

<https://doi.org/10.15388/vu.thesis.1008>

<https://orcid.org/0000-0003-1331-6752>

VILNIUS UNIVERSITY

Gediminas Plakys

# Bacterial Enzymes that Degrade Yeast Cell Wall Polysaccharides

**DOCTORAL DISSERTATION**

Technological Sciences,  
Chemical Engineering (T 005)

VILNIUS 2026

The dissertation was prepared between 2021 and 2025 at the Department of Molecular Microbiology and Biotechnology, Institute of Biochemistry, Life Sciences Center, Vilnius University.

**Academic Supervisor** – Prof. Dr. Rolandas Meškys (Vilnius University, Natural Sciences, Biochemistry, N 004).

**Academic Consultant** – Dr. Edita Mažonienė (Kaunas University of Technology, Technological Sciences, Chemical Engineering, T 005).

This doctoral dissertation will be defended in a public meeting of the Dissertation Defence Panel:

**Chairman** – Dr. Dalius Ratautas (Vilnius University, Technological Sciences, Chemical Engineering, T 005).

**Members:**

Prof. Dr. Renata Gudiukaitė (Vilnius University, Technological Sciences, Chemical Engineering, T 005),

Dr. Inga Matijošytė (Vilnius University, Technological Sciences, Chemical Engineering, T 005),

Prof. Dr. Saulius Serva (Vilnius University, Natural Sciences, Biochemistry, N 004),

Dr. Ilma Tapio (Natural Resources Institute, Finland, Technological Sciences, Chemical Engineering, T 005).

The dissertation shall be defended at a public meeting of the Dissertation Defence Panel at 13:00 on 30th November 2026 in Room R401 of the Vilnius University Life Sciences Centre.

Address: Saulėtekio av. 7, R401, Vilnius, Lithuania

Tel. +37064447646; e-mail: [gediminas.plakys@bchi.stud.vu.lt](mailto:gediminas.plakys@bchi.stud.vu.lt)

<https://doi.org/10.15388/vu.thesis.1008>

<https://orcid.org/0000-0003-1331-6752>

VILNIAUS UNIVERSITETAS

Gediminas Plakys

# Mielių ląstelių sienelių polisacharidus skaidantys bakteriniai fermentai

**DAKTARO DISERTACIJA**

Technologijos mokslai,  
Chemijos inžinerija (T 005)

VILNIUS 2026

Disertacija rengta 2021–2025 metais Vilniaus universiteto Gyvybės mokslų centre, Biochemijos institute, Molekulinės mikrobiologijos ir biotechnologijos skyriuje.

**Mokslinis vadovas** – prof. dr. Rolandas Meškys (Vilniaus universitetas, gamtos mokslai, biochemija, N 004).

**Mokslinė konsultantė** – dr. Edita Mažonienė (Kauno technologijos universitetas, technologijos mokslai, chemijos inžinerija, T 005).

Gynimo taryba:

**Pirmininkas** – dr. Dalius Ratautas (Vilniaus Universitetas, technologijos mokslai, chemijos inžinerija – T005).

**Nariai:**

prof. dr. Renata Gudiukaitė (Vilniaus Universitetas, technologijos mokslai, chemijos inžinerija – T005),

dr. Inga Matijošytė (Vilniaus Universitetas, technologijos mokslai, chemijos inžinerija – T005),

prof. dr. Saulius Serva (Vilniaus universitetas, gamtos mokslai, biochemija – N004),

dr. Ilma Tapio (Gamtos išteklių institutas, Suomija, technologijos mokslai, chemijos inžinerija – T005).

Disertacija ginama viešame Gynimo tarybos posėdyje 2026 m. lapkričio mėn. 30 d. 13:00 val. Vilniaus Universiteto Gyvybės mokslų centro R401 auditorijoje. Adresas: Saulėtekio al. 7, R401, Vilnius, Lietuva, tel. +37064447646; el. paštas [gediminas.plakys@bchi.stud.vu.lt](mailto:gediminas.plakys@bchi.stud.vu.lt)

## CONTENTS

|                                                                     |    |
|---------------------------------------------------------------------|----|
| LIST OF ABBREVIATIONS .....                                         | 7  |
| INTRODUCTION.....                                                   | 8  |
| SCIENTIFIC NOVELTY AND PRACTICAL VALUE .....                        | 11 |
| THESIS STATEMENTS .....                                             | 13 |
| 1. LITERATURE OVERVIEW .....                                        | 14 |
| 1.1 Yeast cell wall.....                                            | 14 |
| 1.1.1 Saccharomyces yeast .....                                     | 14 |
| 1.1.2 Yeast of other genera .....                                   | 19 |
| 1.1.3 YCW – an industrial scale by-product.....                     | 21 |
| 1.2 Structure and applications of $\beta$ -glucans .....            | 25 |
| 1.2.1 Biological activity.....                                      | 25 |
| 1.2.2 Commercial and clinical use.....                              | 30 |
| 1.3 Bioactive and functional oligosaccharides .....                 | 32 |
| 1.3.1 Structure and applications of laminaribiose.....              | 34 |
| 1.3.2 Structure and applications of laminaripentaose .....          | 35 |
| 1.3.3 Structure and applications of gentiobiose .....               | 36 |
| 1.3.4 Structure and applications of branched oligosaccharides ..... | 37 |
| 1.4 Carbohydrate active enzymes (CAZymes) .....                     | 38 |
| 1.5 Enzymatic yeast cell lysis methods.....                         | 41 |
| 1.5.1 Bacterial lytic enzyme mixtures .....                         | 43 |
| 1.5.2 Purified bacterial lytic enzymes.....                         | 45 |
| 2. MATERIALS AND METHODS .....                                      | 48 |
| 2.1 Materials.....                                                  | 48 |
| 2.1.1 Chemicals .....                                               | 48 |
| 2.1.2 Substrates.....                                               | 48 |
| 2.1.3 Bacterial strains.....                                        | 48 |
| 2.1.4 Nucleic acids.....                                            | 49 |
| 2.2 Methods.....                                                    | 49 |
| 2.2.1 Isolation of YCW-degrading bacteria.....                      | 49 |
| 2.2.2 Identification of YCW-degrading bacteria .....                | 49 |
| 2.2.3 Production and collection of CAZymes fractions.....           | 50 |
| 2.2.4 Identification of CAZymes .....                               | 52 |
| 2.2.5 Cloning of target genes .....                                 | 56 |
| 2.2.6 Synthesis of the recombinant proteins .....                   | 58 |
| 2.2.7 Purification of the recombinant proteins.....                 | 59 |
| 2.2.8 Determination of substrate specificity .....                  | 62 |

|        |                                                                            |     |
|--------|----------------------------------------------------------------------------|-----|
| 2.2.9  | Optimisation of enzymatic reaction conditions .....                        | 64  |
| 2.2.10 | TLC analysis of hydrolysis products .....                                  | 65  |
| 2.2.11 | DNS-based enzymatic activity assay .....                                   | 65  |
| 2.2.12 | Quantitative analysis of YCW conversion yield.....                         | 66  |
| 2.2.13 | <i>p</i> NP- $\beta$ -gentiobioside-based enzymatic activity assay.....    | 67  |
| 2.2.14 | Pull-down assay for protein-carbohydrate interaction.....                  | 67  |
| 2.2.15 | Yeast cell lysis .....                                                     | 68  |
| 2.2.16 | Nucleotide, amino acid sequence accession numbers.....                     | 69  |
| 2.2.17 | Computer software, tools and servers.....                                  | 70  |
| 3.     | RESULTS AND DISCUSSION .....                                               | 71  |
| 3.1    | Identification of YCW-degrading CAZymes.....                               | 71  |
| 3.1.1  | Screening for YCW-degrading bacteria.....                                  | 72  |
| 3.1.2  | Proteomic analysis of secretome proteins.....                              | 74  |
| 3.2    | Characterisation of YCW-targeting recombinant GHs .....                    | 80  |
| 3.2.1  | YCW utilisation-ensuring GHs from <i>Rheinheimera</i> .....                | 80  |
| 3.2.2  | Disaccharides-producing GHs from <i>Paenibacillus</i> .....                | 87  |
| 3.2.3  | Yeast-lytic GHs from <i>Cellulosimicrobium</i> .....                       | 108 |
| 3.3    | Application of GHs to convert YCW to oligosaccharides .....                | 110 |
| 3.3.1  | Quantitative evaluation of oligosaccharide yield.....                      | 111 |
| 3.3.2  | <i>p</i> NP- $\beta$ -gentiobioside for residual activity measurement..... | 113 |
| 3.4    | Application of GHs to lyse living yeast cells .....                        | 115 |
| 3.4.1  | Evaluation of yeast cell susceptibility to lysis .....                     | 115 |
| 3.4.2  | Substitution of $\beta$ -ME with glycerol .....                            | 117 |
| 3.4.3  | Lysis of non- <i>Saccharomyces</i> yeast .....                             | 118 |
|        | FINAL REMARKS.....                                                         | 121 |
|        | CONCLUSIONS.....                                                           | 123 |
|        | REFERENCES.....                                                            | 124 |
|        | SANTRAUKA .....                                                            | 146 |
|        | APPENDICES.....                                                            | 169 |
|        | ACKNOWLEDGEMENTS .....                                                     | 191 |
|        | LIST OF PUBLICATIONS.....                                                  | 192 |
|        | CONFERENCE PRESENTATIONS.....                                              | 193 |
|        | CURRICULUM VITAE .....                                                     | 194 |
|        | NOTES.....                                                                 | 196 |

## LIST OF ABBREVIATIONS

|                |                                                          |
|----------------|----------------------------------------------------------|
| AA             | – auxiliary activity                                     |
| $\beta$ -ME    | – $\beta$ -mercaptoethanol                               |
| BSY            | – brewer's spent yeast                                   |
| CE             | – carbohydrate esterase                                  |
| CAZymes        | – carbohydrate-active enzymes                            |
| CBM            | – carbohydrate-binding module                            |
| CF             | – cell factory                                           |
| CWP            | – cell wall proteins                                     |
| DNS            | – 3,5-dinitrosalicylic acid                              |
| DTT            | – dithiotreitol                                          |
| DP             | – degree of polymerisation                               |
| GH             | – glycosyl hydrolase                                     |
| GnOS           | – gentio-oligosaccharides                                |
| GPI            | – glycosylphosphatidylinositol                           |
| MGCP           | – mannan/ $\beta$ -1,6-glucan-containing polysaccharides |
| MOS            | – mannan-oligosaccharides                                |
| M <sub>w</sub> | – molecular weight                                       |
| PI             | – prebiotic index                                        |
| PIR            | – proteins with internal repeats                         |
| PL             | – polysaccharide lyase                                   |
| <i>p</i> NP    | – para-nitrophenol                                       |
| <i>p</i> NPG2  | – para-nitrophenyl- $\beta$ -gentiobioside               |
| TLC            | – thin layer chromatography                              |
| t <sub>R</sub> | – retention time                                         |
| YCW            | – yeast cell wall                                        |

## INTRODUCTION

The history of fermented foods and beverages dates back to around 6,000–7,000 BC, with the yeast *Saccharomyces cerevisiae* being the primary microorganism employed in the production of bread, wine and beer (Legras et al., 2007). Using yeast as a whole cell in the fermentation process has been its primary application. Recently, however, the individual components of yeast cells have attracted increasing attention due to advancements in biotechnology and new market requirements. The intracellular water-soluble components obtained as “yeast extract” have become a source of flavour enhancers and essential amino acids. Meanwhile, the yeast cell wall (YCW) has emerged as a source of fungal  $\beta$ -glucans, which are mostly used in the feed industry and as food supplements. The YCW and  $\beta$ -glucans can be applied either after extraction from spent brewer’s yeast or as spent brewer’s yeast itself, without extraction and purification procedures (Jacob et al., 2019).

In fact, both baker’s and brewer’s yeast can serve as a source of yeast  $\beta$ -glucans and YCW (Freimund et al., 2003; Samuelsen et al., 2014; Varelas et al., 2016). YCW is generally considered to be a low-value by-product accumulated during yeast extract production (Borchani et al., 2014). Spent brewer’s yeast and YCW are inexpensive, nontoxic, and compositionally valuable large-scale by-products (Avramia and Amariei, 2021; Li and Karboune, 2019).

According to the European Polysaccharide Network of Excellence (EPNOE) research roadmap 2040 (Gericke et al., 2023), the use of biomass-derived polysaccharides for food and nutrition is one of the areas to be developed towards a sustainable circular bioeconomy. Therefore, valorisation of YCW into food-grade products is a step forward towards sustainability.

Since the 1980s, the immunomodulatory effects of  $\beta$ -glucans have been investigated in clinical trials, with much focus dedicated to improving cancer treatment therapies (Steimbach et al., 2021). Despite promising results obtained in numerous *in vitro* studies, pre-clinical and clinical trials have not produced significant results (De Marco Castro et al., 2021; Steimbach et al., 2021). No major breakthrough has yet been achieved, as almost all  $\beta$ -glucans on the market exist only as food supplements and not as drugs. The only exception is non-*Saccharomyces*  $\beta$ -glucan, which has been clinically used in Japan since 1977 (Maehara et al., 2012). There are two main reasons why therapeutic applications are expected to be limited: the variability of  $\beta$ -glucan structure and composition when obtained from different sources (Lee et al., 2024), and the differences between simplified *in vitro* systems, animal models,

and human organisms, which result in inconsistent clinical trial results (Markovina et al., 2020; Steimbach et al., 2021).

As an alternative to current applications and breakthroughs lacking in the cancer therapy area, YCW can be utilised as a substrate for the enzymatic conversion of polysaccharides into different classes of bioactive or food-functionality-improving molecules, including oligosaccharides. This would address the issue of inconsistent structural features and bioactivity in  $\beta$ -glucans. YCW has been identified as a suitable source for producing bioactive and functional oligosaccharides such as laminaripentaose, laminaribiose, and gentiobiose. This approach not only serves to valorise YCW but also utilises a cost-efficient industrial source of YCW to produce these oligosaccharides. The most abundant polysaccharides of YCW are  $\beta$ -glucans (Klis et al., 2002) and bacteria are a common source of microbial  $\beta$ -glucanases (Jin et al., 2023).

An important feature of some YCW-degrading enzymes is their ability to disrupt living yeast cells. Currently, mixtures of lytic enzymes, such as *Zymolyase*, *Lyticase*, and *Glusulase* are commercially available (Brettrager et al., 2024). Commercial lytic enzyme mixtures *Zymolyase* and *Lyticase* are sourced from bacteria *Cellulosimicrobium cellulans*. However, common lysis mixtures contain proteases that can have off-target activities that degrade not only YCW mannoproteins, but intracellular target proteins as well (Brettrager et al., 2024). Yeast can serve as a source of proteins: either native (C. Ma et al., 2024) or recombinant (Liu et al., 2012) ones. The side activities of lytic enzyme mixtures limit their applications when extractable proteins need to remain intact. Reducing agents are an alternative to proteases (Zlotnik et al., 1984), as they disrupt the mannoprotein layer of YCW. However, they also affect intracellular proteins. Therefore, conditions in which specific endo-1,3- $\beta$ -glucanases can lyse living yeast cells without the addition of proteases or reducing agents are desirable.

As complete disruption of the YCW by enzymatic hydrolysis requires multiple enzymatic activities of carbohydrate-active enzymes (CAZymes), the YCW itself is an excellent substrate for screening microorganisms that produce YCW polysaccharide-targeting enzymes. Identifying new microorganisms that produce YCW-degrading enzymes and characterising these enzymes has two benefits: it provides additional tools for the protease- and reducing agent-free lysis of yeast cells, and it provides YCW valorisation possibilities.

The main aim of this work is to identify and apply novel YCW polysaccharide-degrading bacterial enzymes to produce higher-added-value products.

The following objectives were set out to achieve the aim of this study:

1. To identify bacteria capable of YCW degradation.
2. To identify the CAZymes responsible for YCW decomposition.
3. To determine the substrate specificity and hydrolysis products of recombinant variants of the target CAZymes.
4. To evaluate the ability of these enzymes to convert YCW into functional and bioactive oligosaccharides.
5. To identify yeast lysis reaction conditions and combinations of CAZymes that can substitute for reducing agents and protease additives.

## SCIENTIFIC NOVELTY AND PRACTICAL VALUE

Firstly, this study explores the possibility of converting YCW industrial by-product into functional and bioactive oligosaccharides via enzymatic hydrolysis. The characterised YCW-degrading enzymes from *Paenibacillus* sp. GKG and *Rheinheimera* sp. GKU bacteria were the first representatives of GH3, GH16, GH30, and GH81 families at the genus level. Among these enzymes, the GH16\_306 from *Rheinheimera* sp. GKU has the most scientific significance due to its interesting domain architecture. It has one catalytic module, annotated as a GH16 family member and two non-catalytic domains, annotated as internal repeat domains. However, structural model analysis and functional experiments revealed that these modules exhibit carbohydrate-binding activity towards  $\beta$ -1,3-glucans. Since it is not similar to the representatives of current carbohydrate-binding module (CBM) families, this module is the potential first representative of a new CBM family. These results expand current knowledge on CBM modules and their affinity to different carbohydrates. Whereas enzymes from *Paenibacillus* sp. GKG showed an exceptional property to produce oligosaccharides in high laminaribiose-to-glucose (PsLam81A enzyme) and gentiobiose-to-glucose (PsGly30A enzyme) ratios. In addition, the GH64 enzyme from *Cellulosimicrobium* sp. GKH produced laminaripentaose as the dominant product.

From a practical application standpoint, CBM modules can be applied as ligands in affinity chromatography to separate oligosaccharides with different glycosidic linkages (Johansson et al., 2006). Based on this example, putative CBMs of the GH16\_306 protein could be explored for the separation of  $\beta$ -1,3- and  $\beta$ -1,6-oligosaccharides. PsLam81A, PsGly30A and GH64 enzymes are useful for converting YCW into oligosaccharides with high conversion yields. The ultimate application of the oligosaccharides explored in this work would be to include them in food products as functional or bioactive ingredients. Laminaribiose would act as a prebiotic (Sanz et al., 2005), gentiobiose would enhance the taste (Xia et al., 2022), whereas laminaripentaose would provide a prebiotic and anti-obesity activity (Y. Xu et al., 2024). Moreover, crude preparations of already commercialised yeast extracts applied as flavour enhancers could be enriched for functional oligosaccharides upon treatment with GHs described in this work. YCW  $\beta$ -glucans could be converted to functional and bioactive oligosaccharides with the application of enzymatic activities described in this work during or after the autolysis step that is applied in the preparation of such extracts (Tomé, 2021).

Secondly, the ability of endo-1,3- $\beta$ -glucanases to induce yeast cell lysis is investigated. The ability of certain endo-1,3- $\beta$ -glucanases to lyse living yeast

cells was first discovered several decades ago. However, proteases or reducing agents have been reported to be necessary for lytic activity. This work reports protease-free and reducing agent-free lysis of living *Saccharomyces cerevisiae* yeast cells, which was previously reported not to occur (Scott and Schekman, 1980).

From a practical standpoint, the ability to lyse yeast cells is important for laboratory and commercial applications. Laboratory-scale applications include YCW disruption for spheroplast generation (Karas et al., 2014) and intracellular protein extraction (Dunn and Wobbe, 1993). DNA-free (Miyajima et al., 2009; Rimek et al., 1999) and RNase-free (Collart and Oliviero, 1993) cell-lysis protocols are also important for nucleic acid amplification and sequencing applications. Commercial applications include extracting recombinant intracellular proteins for use in biopharmaceuticals for therapeutic purposes (Kulagina et al., 2021). Another emerging application is the extraction of native yeast proteins for use in the food industry as a source of amino acids or functional ingredients (Jach et al., 2022; C. Ma et al., 2024). A promising area of application of lytic endo-1,3- $\beta$ -glucanases that has not been explored in this study is the development of antifungal preparations (Bai et al., 2021).

Overall, this study identifies the GH16\_306 protein's internal repeat domain as a putative CBM domain that is not currently assigned to any CBM family. PsLam81A, PsGly30A, and GH64 enzymes, on the other hand, are shown to be suitable tools for YCW valorisation. Lastly, the study outlines the conditions for protease- and  $\beta$ -ME-free lysis of *Saccharomyces cerevisiae* BY4741 yeast cells. These findings provide support for further practical application trials.

## THESIS STATEMENTS

1. *Rheinheimera* sp. GKU, *Paenibacillus* sp. GKG, and *Cellulosmicrobium* sp. GKH bacteria can degrade YCW and use it as a carbon source.
2. *Rheinheimera* sp. GKU, *Paenibacillus* sp. GKG, and *Cellulosmicrobium* sp. GKH secrete CAZymes that convert YCW into water-soluble oligosaccharides and glucose.
3. Three GHs of *Rheinheimera* sp. GKU, namely GH16\_3455, GH16\_306, and GH3\_3456, are responsible for YCW decomposition to glucose.
4. Three characterised enzymes, namely PsLam81A, PsGly30A and GH64, are suitable to convert YCW  $\beta$ -glucans into bioactive and functional products – laminaribiose, gentiobiose, and laminaripentaose, respectively.
5. Synergistic action of GH16 and GH64 enzymes allows lysis of living *Saccharomyces cerevisiae* BY4741 yeast cells without proteases or a reducing agent additive when glycerol is used instead.

# 1. LITERATURE OVERVIEW

## 1.1 Yeast cell wall

The composition, structure and dynamics of the yeast cell wall (YCW) have been extensively studied and comprehensively described. The YCW consists of an inner layer rich in  $\beta$ -glucan and chitin, and an outer layer rich in mannoproteins (Fig. 1.1). The inner layer provides the cell wall with skeletal function and mechanical rigidity. The outer layer interacts with the environment, ensures nutrient uptake and protects the inner layer from enzymatic damage (Gow and Lenardon, 2023). The outer mannoprotein layer protects the inner layer of the YCW from external glucanases and chitinases. Consequently, in addition to glucanases, yeast lytic enzyme mixtures typically require proteases or reducing agents, such as  $\beta$ -mercaptoethanol ( $\beta$ -ME) (Zlotnik et al., 1984). If the inner layer is hydrolysed, osmotically driven cell lysis occurs (Gow and Lenardon, 2023). The composition of YCW may change based on cultivation conditions or consist of different polymers in yeast of different genera.

### 1.1.1 *Saccharomyces* yeast

The cell wall of *Saccharomyces cerevisiae* is mechanically resistant and is mainly composed of polysaccharides and glycoproteins (Avramia and Amariei, 2021). Depending on the culturing conditions, the cell wall of the yeast *S. cerevisiae* makes up between 14% and 25% of the dry weight of the yeast cell (Aguilar-Uscanga and Francois, 2003).

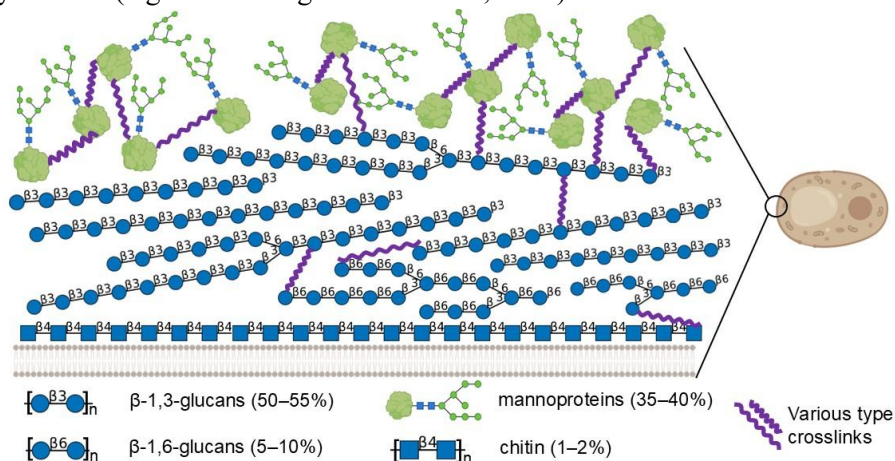


**Figure 1.1.** Schematic structure of YCW. Created according to (Orlean, 2012), using <https://biorender.com/>.

The four most abundant polymers on a dry weight basis of *S. cerevisiae* YCW are  $\beta$ -1,3-glucan (50–55%), mannoproteins (35–40%),  $\beta$ -1,6-glucan (5–10%), and chitin (1–2%) (Klis et al., 2002). The proportion of these polymers depends on yeast growth conditions (Table 1.1). Consequently, by adjusting growth conditions, it is possible to increase the initial amount of the target polysaccharide, e.g.  $\beta$ -1,6-glucan. An increase in the proportion of  $\beta$ -1,6-glucan in the YCW substrate would result in higher yields of higher-value oligosaccharides, e.g., gentiobiose, after treatment of YCW with glycosyl hydrolases (GHs). Additionally, the susceptibility of living yeast cells to the lytic enzyme mixture also depends on growth conditions.

**Table 1.1.** Effects of growth conditions on cell wall mass,  $\beta$ -glucans,  $\beta$ -1,6-glucan and on the sensitivity of living yeast cells to *Zymolyase*. Reprinted from (Aguilar-Uscanga and Francois, 2003).

| Growth conditions | Cell wall dry mass (%)      | Total $\beta$ -glucan /cell mass (%) | $\beta$ -1,6-glucan/total glucan (%) | <i>Zymolyase</i> sensitivity (MLR) ( $\times 10^{-2}$ min) |
|-------------------|-----------------------------|--------------------------------------|--------------------------------------|------------------------------------------------------------|
| YPD               | 24.5 $\pm$ 2.5 $\uparrow$   | 12.7 $\pm$ 0.3 $\uparrow$            | 18 $\pm$ 3 $\uparrow$                | 0.28                                                       |
| YNB               | 21.2 $\pm$ 2.4              | 7.3 $\pm$ 0.4                        | 18 $\pm$ 3                           | 0.32                                                       |
| CF                | 20.4 $\pm$ 2.8              | 7.1 $\pm$ 0.3                        | 15 $\pm$ 2                           | 0.68                                                       |
| Glucose           | 18.3 $\pm$ 2.0              | 6.6 $\pm$ 0.3                        | 14 $\pm$ 3                           | 0.75                                                       |
| Mannose           | 14.2 $\pm$ 1.8              | 6.4 $\pm$ 0.2                        | 14 $\pm$ 2                           | 0.66                                                       |
| Sucrose           | 15.2 $\pm$ 1.8              | 6.3 $\pm$ 0.3                        | 13 $\pm$ 4                           | 0.68                                                       |
| Maltose           | 14.5 $\pm$ 2.0              | 5.1 $\pm$ 0.2                        | 16 $\pm$ 3                           | 0.35                                                       |
| Galactose         | 16.4 $\pm$ 2.0              | 7.8 $\pm$ 0.2                        | 19 $\pm$ 3                           | 0.15                                                       |
| Ethanol           | 10.8 $\pm$ 1.5 $\downarrow$ | 3.9 $\pm$ 0.3 $\downarrow$           | 21 $\pm$ 2                           | 0.03 $\downarrow$                                          |
| pH 3              | 17.9 $\pm$ 3.0              | 6.0 $\pm$ 0.3                        | 15 $\pm$ 2                           | 0.49                                                       |
| pH 4              | 18.9 $\pm$ 2.0              | 8.1 $\pm$ 0.4                        | 17 $\pm$ 3                           | 0.62                                                       |
| pH 5              | 20.5 $\pm$ 2.0              | 6.7 $\pm$ 0.3                        | 14 $\pm$ 2                           | 0.75                                                       |
| pH 6              | 14.1 $\pm$ 1.6              | 5.8 $\pm$ 0.3                        | 12 $\pm$ 2                           | 0.86 $\uparrow$                                            |
| 22 °C             | 12.4 $\pm$ 2.1              | 6.0 $\pm$ 0.3                        | 10 $\pm$ 2 $\downarrow$              | 0.85                                                       |
| 30 °C             | 18.3 $\pm$ 2.6              | 6.9 $\pm$ 0.5                        | 14 $\pm$ 2                           | 0.75                                                       |
| 37 °C             | 15.5 $\pm$ 2.0              | 8.9 $\pm$ 0.5                        | 20 $\pm$ 2                           | 0.59                                                       |

Yeast cells were cultivated in a controlled batch-fermentor, unless otherwise stated, in a cell factory (CF) media. Other media were YPD, YNB. Unless otherwise stated (see column 1), the pH was kept constant at 5.0, temperature at 30 °C and aeration at  $pO_2 \sim 50\%$  saturation in the medium. The values reported are the mean  $\pm$  S.E.M. from four independent experiments, and for each experiment, three samples were taken for cell wall analysis. MLR - maximal lysis rate. Arrows indicate minimum and maximal values in each column, green colour indicates favourable parameters, red indicates undesirable parameters for either oligosaccharide production or lysis of living yeast cells.

The data (Table 1.1) shows that growth in YPD medium ensures the highest YCW proportion and total  $\beta$ -glucan proportion from dry cell mass.

Although synthetic CF media supplemented with ethanol as a carbon source gives the highest proportion of  $\beta$ -1,6-glucan/total glucan (%), the total  $\beta$ -glucan /cell mass (%) proportion is more than 3-fold lower than that of YPD media. Therefore, YPD media would be more favourable in all criteria for oligosaccharide production. Regarding the lysis of living yeast cells, CF media supplemented with ethanol resulted in the highest yeast cell resistance to *Zymolyase* treatment, while the yeast grown in CF medium supplemented with glucose showed the highest sensitivity to *Zymolyase*.

The YCW structure itself is water-insoluble, and multiple steps are required to obtain pure polymers with respective solubilities (Fig. 1.2). Based on their structural features, YCW polysaccharides exhibit distinct properties when isolated as sole polymers.

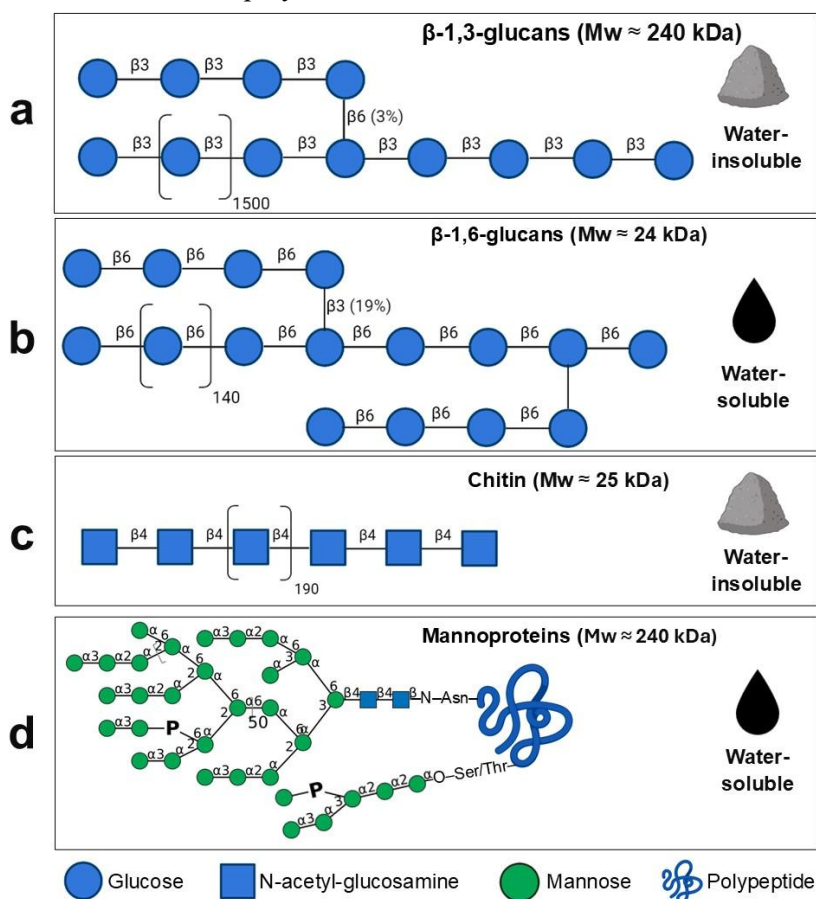


**Figure 1.2.** Characteristics of separate *Saccharomyces* yeast YCW polymers. Molecular weight and solubility correspond to purified components. a –  $\beta$ -1,3-glucans, b –  $\beta$ -1,6-glucans, c – chitin, d – mannoproteins. Polysaccharide structures were visualised according to symbol nomenclature for glycans (SNFG) using the DrawGlycan-SNFG web tool (Cheng et al., 2016). Image created according to (Bastos et al., 2022; Klis et al., 2002; Orlean, 2012).

The degree of polymerisation (DP) and the extent of branching of these polymers vary.  $\beta$ -1,3-glucans have the longest polymeric chains with a DP of  $\geq 1500$ , whereas mannoproteins, chitin and  $\beta$ -1,6-glucans have lower DPs of 200, 190 and 140, respectively (Klis et al., 2002). Yeast  $\beta$ -1,3/1,6-glucan extracted from cell walls is considered water-insoluble (Manners et al., 1973). However, isolated  $\beta$ -glucan polymers have different solubilities:  $\beta$ -1,3-glucan is water-insoluble (Isbell, 1973) (Fig. 1.2a), whereas  $\beta$ -1,6-glucan is water-soluble (Klis et al., 2002) (Fig. 1.2b). The DP and branching degree are the main factors determining the solubility difference between  $\beta$ -1,3-glucans and  $\beta$ -1,6-glucans – low DP and high branching degree ensure water-solubility (Klis et al., 2002).  $\beta$ -1,3-glucan acts as the cell wall's framework, while  $\beta$ -1,6-glucan acts as a linker between its different components (Lipke and Ovalle, 1998). Chitin is water-insoluble (Fig. 1.2c), because of its crystalline structure and intra-chain and inter-chain hydrogen bonding (Orlean, 2012). Mannoprotein polymer is water-soluble (Fig. 1.2d) due to the amphiphilic nature of the protein moiety and highly branched mannan carbohydrate moiety (Qiao et al., 2022).

In YCW, these different polymers are tightly connected via various cross-links. The extent of cross-link variety extends beyond  $\beta$ -1,6-glucans. Multiple treatments are necessary if these cross-links need to be disrupted. In some cases, it may not be possible to remove cross-links without disrupting individual polymer structures. Several different cross-link types connecting YCW polymers are depicted in Figure 1.3. First three crosslinks (A–C) are between polysaccharides, the second kind are between polysaccharides and proteins (D, E), and the last kind is between proteins (F). Firstly, the  $\beta$ -1,6 glucan has  $\beta$ -1,3-braches connected to the reducing end of chitin via  $\beta$ -1,2 or  $\beta$ -1,4- bonds (A) (Kollár et al., 1997). Secondly, the reducing end of a chitin and the nonreducing end of a  $\beta$ -1,3- are linked via  $\beta$ -1,4-linkage (B). The frequency of this cross-link is low – about one chitin- $\beta$ -glucan linkage per 8000 hexoses. However, these rare cross-links have a major impact on the solubility of  $\beta$ -glucan (Kollár et al., 1995). Chitin-cross-linked  $\beta$ -glucan is alkali- and acid-insoluble. Whereas,  $\beta$ -glucan fraction, which does not have such cross-links, e.g. after treatment with chitinase, becomes alkali- and acid-soluble (Kollár et al., 1995). Lastly, reducing ends of  $\beta$ -1,3-glucan can be attached to  $\beta$ -1,6-glucan chains that are attached to  $\beta$ -1,3-glucan (C), but the bond type is unknown (Kollár et al., 1997).

The proteins that are covalently linked to  $\beta$ -glucans are called cell wall proteins (CWP). CWP can have either enzymatic function (e.g., GHs participating in YCW remodelling during YCW polymer synthesis) or structural function (e.g. regions rich in serine and threonine) (Yin et al., 2005).

Initially, glycosylphosphatidylinositol (GPI) anchors CWP to the outer side of the plasma membrane. During the cell wall assembly process, the cleavage of the GPI glycan between the glucosamine residue and the first mannose residue occurs, and it is followed by a transglycosylation reaction that links the reducing end of the GPI glycan remnant to the cell wall  $\beta$ -1,6-glucan (D) (Gonzalez et al., 2009). Cell wall proteins with internal repeats (PIR proteins) are rich in serine and threonine residues. These proteins do not have a GPI anchor, but are cross-linked via the  $\gamma$ -carboxyl group of glutamic acids, arising from specific glutamines to the hydroxyl groups of glucoses of  $\beta$ -1,3-glucan chains (E) (Ecker et al., 2006). PIR proteins can be released using mild alkaline conditions or treatment with  $\beta$ -1,3-glucanase (Mrsa et al., 1997). Proteins that can be released with sulfhydryl reagents indicate that they are directly attached via disulfides or retained behind a network of disulfide-linked proteins (F) (Orlean, 2012). CWP are highly glycosylated – the carbohydrate part accounts for 50–95% of this co-polymer by weight (Lipke and Ovalle, 1998).

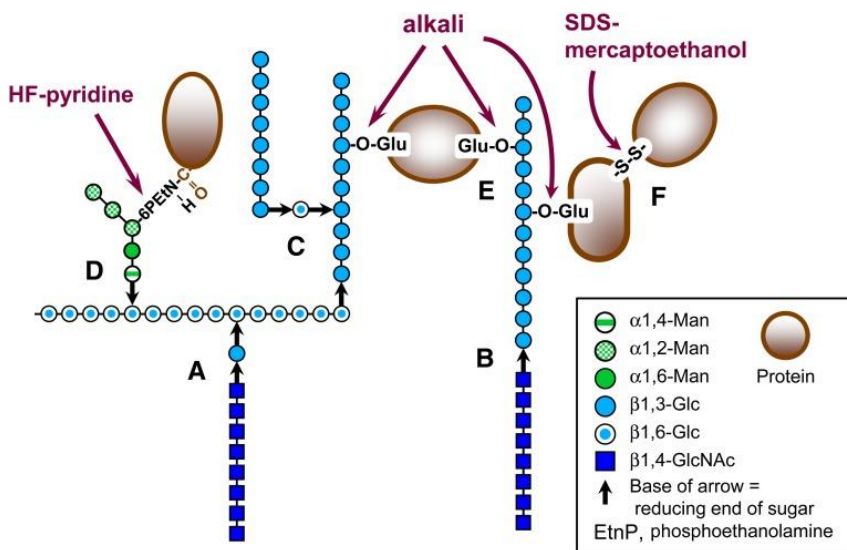


**Figure 1.3.** Examples of cross-links between YCW components. (A) Reducing end of chitin linked to a side-branching  $\beta$ -1,3-Glc on  $\beta$ -1,6-glucan. (B) Reducing end of chitin linked to a non-reducing end of  $\beta$ -1,3-glucan. (C) Reducing end of  $\beta$ -1,3-glucan chain linked to a side-branching  $\beta$ -1,6-Glc on  $\beta$ -1,3-glucan. (D) Reducing end of GPI glycan (possibly the  $\alpha$ 1,4-Man) to internal Glc in  $\beta$ -1,6-glucan (linkage to nonreducing end of  $\beta$ -1,6-glucan is also possible). (E) Ester linkages between  $\beta$ -1,3-Glc and  $\gamma$ -carboxyl groups of glutamates in PIR protein internal repeats. (F) Disulfide bond between CWP. Reprinted from (Orlean, 2012).

These components form a complex water-insoluble YCW structure with various intra-chain and inter-chain bonds. Polysaccharides can be linked via glycosidic bonds, such as  $\beta$ -1,3-,  $\beta$ -1,4-,  $\beta$ -1,6-,  $\alpha$ -1,2,  $\alpha$ -1,4,  $\alpha$ -1,6 or linked

to proteins via amino or hydroxy groups of amino acid residues (Orlean, 2012). This trait makes YCW a broad-spectrum substrate, making it suitable for screening microorganisms that produce various types of GHs. Screening involves observing the appearance of hydrolysis zones on solid agar plates. The appearance of hydrolysis zones can be expected in the presence of GHs such as  $\beta$ -1,3-glycosidases,  $\beta$ -1,6-glycosidases,  $\alpha$ -1,2-,  $\alpha$ -1,4-, and  $\alpha$ -1,6-mannosidases and chitinases. When YCW is treated with such GHs, the DP of the backbone may decrease, as may the length of the branches and/or inter-chain bonds, which makes the substrate soluble. Polysaccharide substrates are used as the sole carbon source in agar plates. For example, Avicel or CMC-Na were used as the carbon source during the screening for cellulolytic bacteria, and hydrolysis zones were inspected after visualisation using Congo red (Ma et al., 2020).

### 1.1.2 Yeast of other genera

The main focus of YCW applications is on *Saccharomyces* and *Candida* yeast (Jofre et al., 2024). Nevertheless, non-*Saccharomyces* yeast is also an emerging object for applications as probiotics or as a source of YCW components such as  $\beta$ -glucans and mannans (Schiavone et al., 2023). Yeast of different genera show similar composition of YCW (Table 1.2).

**Table 1.2.** Cell wall polysaccharides in different yeast genera. Adapted from (Ruiz-Herrera et al., 2006; Schiavone et al., 2023).

| Yeast species                    | Cell wall polysaccharide                |                      |            |                |                |
|----------------------------------|-----------------------------------------|----------------------|------------|----------------|----------------|
|                                  | $\beta$ -1,3- and $\beta$ -1,6- glucans | $\alpha$ -1,3 glucan | Chitin     | Manno-proteins | Galacto-mannan |
| <i>Saccharomyces cerevisiae</i>  | 50–65%                                  | none                 | 1–5%       | 35–40%         | none           |
| <i>Kluyveromyces lactis</i>      | 50–55%                                  | none                 | 1–3%       | 30–40%         | none           |
| <i>Schizosaccharomyces pombe</i> | 54–60%                                  | 28–32%               | in conidia | none           | 9–14%          |
| <i>Debaryomyces hansenii</i>     | 50–60%                                  | nd                   | 2–7%       | 29–35%         | none           |
| <i>Kluyveromyces marxianus</i>   | 50%                                     | nd                   | 1–4%       | 20–25%         | none           |
| <i>Candida albicans</i>          | 58-60%                                  | none                 | 2%         | 38-40%         | none           |

Listed examples contain  $\beta$ -glucans and mannoproteins as the major polymers with minor amounts of chitin. The exception is

*Schizosaccharomyces pombe* yeast as their YCW does not contain mannoproteins, but contain  $\alpha$ -1,3-glucan and galacto-mannan instead. The proportion between  $\beta$ -1,3-glucans and  $\beta$ -1,6-glucans is important if the conversion of  $\beta$ -glucans to disaccharides is considered. The selection of different types of source yeast allows for increasing the yield of target oligosaccharide products by exploiting different glucan proportions and types in the cell walls of different yeasts and fungi. For example, *Candida albicans* yeast contains a high proportion of  $\beta$ -1,6-glucans (43–53%), compared with the YCW of *S. cerevisiae*, which contains 10–15% of  $\beta$ -1,6-glucans (Free, 2013). However, the pathogenicity, toxicity and safety of the source microorganism should be considered. Therefore, *S. cerevisiae* is a more conventional choice for practical applications. *S. cerevisiae* yeast is the main yeast species used in baking (Parapouli et al., 2020) or brewing (Lodolo et al., 2008) industries. The terms baker's or brewer's yeast represent the industry in which these strains are primarily used. Baker's yeast strains are adapted as leavening agents, while brewer's yeast strains are responsible for alcoholic fermentation. Another conventional source could be *Aureobasidium pullulans* yeast. A previous study showed that gentiobiose yield can be two times higher if  $\beta$ -glucan from *A. pullulans* is used as a starting material instead of  $\beta$ -glucan from *S. cerevisiae* (Hirabayashi et al., 2017).

Another trait that differs across yeast genera is the degree of crosslinking between  $\beta$ -1,3/1,6-glucans. *S. cerevisiae* yeast has the lowest crosslinking degree – 7%, followed by *C. albicans* – 15% and *S. pombe* – 75% (Aimanianda et al., 2009). The higher the crosslinking degree, the lower the yield of linear oligosaccharides, such as laminari-oligosaccharides or gentio-oligosaccharides, can be expected. Nevertheless, if  $\beta$ -1,3/1,6-branched oligosaccharides are the target product of  $\beta$ -glucan conversion, then *S. pombe* yeast would be a preferable source.

In fact, even the screening of a most suitable yeast strain can also help to improve the yield of desired product, whether it is a laminaribiose, gentiobiose or 3,6-branched DP3 and DP4 oligosaccharides. According to results reported by Boutros and colleagues, the calculated ratio between 3-linked and 6-linked glucose may vary from 6.1 to 50.2, and the proportion of 3,6-linked glucose may comprise 1.2 to 4.9% based on molar ratio when different strains of *S. cerevisiae* are used (Boutros et al., 2022). However, it is difficult to evaluate from the presented results how much of the  $\beta$ -glucan structure variability was determined by the yeast strain type and how much by the extraction method, since a full comparison of extraction protocols was not available.

Overall, baker's or brewer's yeast *S. cerevisiae* YCW is the most efficient and safe source to produce prebiotic laminari-oligosaccharides (e.g.

laminaribiose), since  $\beta$ -1,3-glucans account for a major part of yeast cell walls' dry weight and have a relatively low proportion of 3,6-branches. Although the contents of  $\beta$ -1,6-glucans are not as high, they offer a possibility to exploit them for the production of  $\beta$ -1,6-disaccharide – gentiobiose.

### 1.1.3 YCW – an industrial scale by-product

Yeast-derived products are commercialised on an industrial scale. Depending on the chemical compounds extracted and their processing strategies, these compounds can be applied in different industrial sectors (Fig. 1.4). Yeasts are rich in high-quality peptides and proteins, B vitamins (B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, B<sub>6</sub>, and folate), minerals such as selenium, zinc, and iron, microbial oils from oleaginous species, and cell wall polysaccharides like  $\beta$ -glucans and mannans (Deng et al., 2026). Upon yeast cell lysis, water-soluble compounds are collected as yeast extract, while the water-insoluble fraction, composed primarily of YCW, remains in the pellet fraction. Differently processed yeast extracts are mostly used in food and biotechnology industries, while water-insoluble YCW polysaccharides are used in feed and medicine (Fig. 1.4) (Deng et al., 2026).

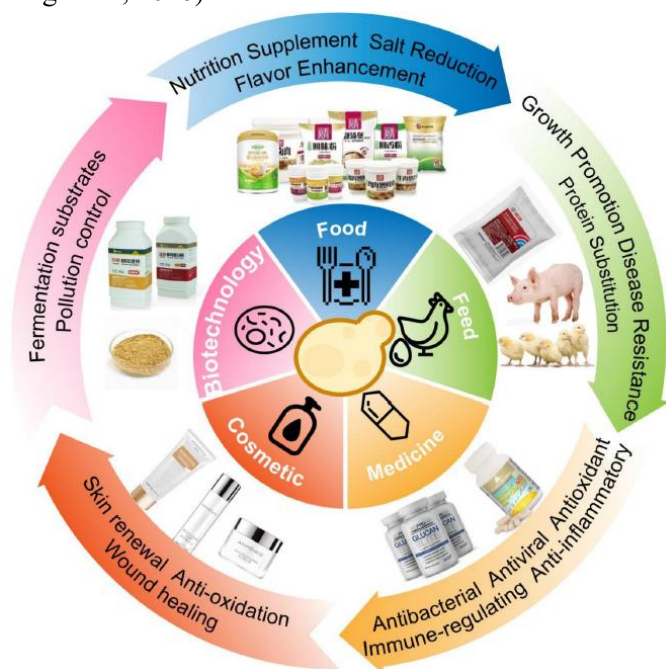


**Figure 1.4.** Applications of yeast-based products in various sectors, sorted by different industrial uses of processed yeast products. Reprinted from (Deng et al., 2026).

The major industrial source of yeast  $\beta$ -glucans in a form of YCW or spent brewer's yeast is brewing industry. Spent brewer's yeast is considered to be a

waste (Gao et al., 2024). Water-soluble intracellular components can be used for yeast extract production (Gao et al., 2024), while water-insoluble YCW is mostly utilised as a source of  $\beta$ -glucans and mannans as dietary supplements (Samuelsen et al., 2014) or livestock feed ingredient (Broadway et al., 2015). A global yearly beer production of 188 million tons was recorded in 2024 (BarthHaas GmbH & Co. KG, 2025). One hundred litres of produced beer generates from 0.5–1.1 kg (Jacob et al., 2019) to 2–4 kg (San Martin et al., 2020) of spent brewer's yeast. This results in a yearly accumulation of 0.9–7.5 million tons of spent brewer's yeast worldwide. Avramia and colleagues used the latter range calculating that the EU, the second largest beer producer, alone accumulates 0.9 million tonnes of spent brewer's yeast (Avramia and Amariei, 2021). As spent brewer's yeast contains 8% of  $\beta$ -glucans, the yearly accumulation of spent brewer's yeast in Europe would be equivalent to 72 000 tons of  $\beta$ -glucan, which could be converted into bioactive and functional oligosaccharides (San Martin et al., 2020). With regard to YCW, the content of YCW is 18% of dry cell weight when glucose is used as a growth substrate (Aguilar-Uscanga and Francois, 2003). This results in 162,000 tons of YCW fraction accumulating yearly from spent brewer's yeast.

Another industrial source of YCWs is the yeast extract industry. Yeast extract usually includes all soluble components of the yeast cell, released after cell lysis and after removal of the insoluble cell wall (Jacob et al., 2019). Brewer's yeast is the major raw material for yeast extract production, but other yeast sources can be used as well, e.g. baker's yeast, *Candida utilis*, *Candida tropicalis*, and *Kluyveromyces marxianus* (Tao et al., 2023). Spent brewer's yeast is primarily used because the biomass is ready for the disruption and extraction process after it is collected from the brewing process. Whereas *Saccharomyces*, *Candida*, and *Kluyveromyces* yeast require a separate biomass growth step. Nevertheless, these yeasts are known to utilise cheap raw materials, e.g., molasses (El-Helow et al., 2015).

The demand for converting by-products, such as spent brewer's yeast and YCW, into value-added products is increasing due to their large-scale accumulation, low price, non-toxicity, and valuable compositional properties (Li and Karboune, 2019). Initially, spent brewers' yeast and YCW attracted attention as sources of  $\beta$ -glucans for use in the feed industry and in foods such as dietary fibres, emulsifying agents and fat replacers. YCW, as a crude fraction or its components,  $\beta$ -glucans and mannoproteins, have the main application area as feed additives for livestock and pets, such as broiler chickens, milk cows, fish, and dogs (Jofre et al., 2024). Additionally, less common broader application areas include bioremediation, drug delivery, and emulsifiers in food (Jofre et al., 2024). Several examples of potential

applications of different YCW compounds are presented in Table 1.3. A broader list with application examples can be found in a review by Baek and colleagues (Baek et al., 2024). In addition,  $\beta$ -glucans can be used as functional food additives to improve emulsion stability, water-holding capacity and as a fat replacer, resulting in enhanced textural characteristics (Avramia and Amariei, 2021). Most of these application areas are based on scientific study results rather than commercial applications.

On a commercial scale, spent brewer's yeast is usually the most common source for YCW, while YCW is a source for  $\beta$ -glucan, mannoprotein or mannan polymers, and either of these products can be used in the livestock feed industry (Rakowska et al., 2017). Purified  $\beta$ -glucans are used in food supplements and investigated in clinical trials as immunomodulators (Thomas et al., 2022).

**Table 1.3.** Potential biotechnological applications of yeast cell wall compounds. Adapted from (Jofre et al., 2024).

| Compound                      | Application                                                | Key Findings                                                                     |
|-------------------------------|------------------------------------------------------------|----------------------------------------------------------------------------------|
| Mannoprotein                  | Wine production <sup>1</sup>                               | Improved the quality and stability of wine by removing proteins and polyphenols. |
|                               | Food industry <sup>2</sup>                                 | Emulsifier and stabiliser agent of French salad dressing.                        |
|                               | Medical <sup>3</sup>                                       | Anti-oxidative and antiaging activities.                                         |
|                               | Veterinary <sup>1</sup>                                    | Modulation of the immune system of elderly dogs.                                 |
| $\beta$ -glucans and chitosan | Medical <sup>4</sup>                                       | Antibacterial activity for wound healing.                                        |
| $\beta$ -glucans              | Medical <sup>3</sup>                                       | Protective effect on the respiratory system against viral infections.            |
|                               | Pharmaceutical <sup>3</sup>                                | Drug delivery.                                                                   |
|                               | Bioremediation <sup>3</sup>                                | The adsorbent of lead ions.                                                      |
| Chitin                        | Agriculture, water treatment, and biomedicine <sup>5</sup> | Hydrogel production.                                                             |
|                               | Materials science <sup>3</sup>                             | Efficient material to extract nanofibril production.                             |
|                               | Water treatment <sup>3</sup>                               | Chitin-nanocrystals scaffold for water purification                              |

Compound sourced from: *Saccharomyces cerevisiae*<sup>1</sup>, *Saccharomyces uvarum*<sup>2</sup>, no data<sup>3</sup>, baker's yeast<sup>4</sup>, *Komagataella pastoris*<sup>5</sup>.

Biomedical and pharmaceutical effects of  $\beta$ -glucans have attracted the most attention recently (Avramia and Amariei, 2021; Bastos et al., 2022; Jofre et al., 2024). The area with the most anticipation is the adjuvants that would be able to improve cancer treatment methods by modulation of the immune system (Steimbach et al., 2021). However, going from *in vitro* studies towards clinical trials, there is still a lot of uncertainty in cancer treatment (Steimbach et al., 2021) and even in simpler conditions, such as upper tract respiratory diseases (De Marco Castro et al., 2021). Therefore, nutritional supplements and feed additives remain the primary application areas of extracted  $\beta$ -glucans or crude preparations of  $\beta$ -glucans – YCW.

However, with increasing attention being dedicated to plant-based meat analogues, edible insects and cultured meat, the demand for yeast  $\beta$ -glucans as feed industry ingredients may decline in the future (Lee et al., 2020). Additionally, using YCW-derived products in the food and pharmaceutical industries rather than the animal feed industry adds value to this source material. Ciobanu and colleagues have concluded that spent brewers' yeast is currently underutilised (Ciobanu et al., 2024). The conclusion was based on observations that, despite the high accumulation capacity of spent brewers' yeast during the brewing process and its high potential benefits, it is disposed of as a by-product. Particularly, YCW potential is addressed for medical applications due to its immunomodulatory activity and for food applications due to its thickening, water-holding, oil-binding, gelifying, and fat-replacing properties (Ciobanu et al., 2024).

According to the European Polysaccharide Network of Excellence (EPNOE) research roadmap 2040 (Gericke et al., 2023), the utilisation of biomass-derived polysaccharides for food and nutrition represents a key area for development towards a sustainable circular bioeconomy. In addition, as outlined by Oliveira and colleagues (Oliveira et al., 2022) the valorisation of spent brewers' yeast as an industrial by-product for the food industry is a strategy that should be employed to achieve the objectives of the circular economy, with the ultimate aim of achieving a carbon-neutral economy by 2050 (Yang et al., 2023). Both highly abundant YCW polymers,  $\beta$ -glucans and mannoproteins, have found certain commercial applications in food supplements (De Marco Castro et al., 2021) and beverage (Lomolino et al., 2024) industries, respectively. Nevertheless,  $\beta$ -glucans are better candidates for the valorisation by conversion to oligosaccharides, in comparison to mannans. This is mainly due to a less diverse and branched glycosidic bond network of  $\beta$ -glucans (Fig. 1.2). Therefore, in this study, YCW  $\beta$ -glucans were selected as the main substrates that should be converted to oligosaccharides.

## 1.2 Structure and applications of $\beta$ -glucans

Polysaccharide laminarin exhibits structural features analogous to those of yeast- $\beta$ -glucans, with both comprising a  $\beta$ -1,3-glucan as a primary structural element, accompanied by  $\beta$ -1,6 branches. Compared to yeast  $\beta$ -glucan from YCW, laminarin is less complex due to lower DP, shorter branches and the absence of intra- or inter-crosslinking. Laminarin is a standard substrate used to characterise  $\beta$ -1,3-glycosidases and  $\beta$ -1,6-glycosidases. This substrate is a water-soluble  $\beta$ -1,3-glucan with  $\beta$ -1,6-side-chain branches, the majority of which are single glucosyl residues. The most common type of laminarin is sourced from *Laminaria digitata* and has a heterogeneous structure, comprising a  $\beta$ -1,3-glucan backbone with a DP 20–30, and a 3:1 ratio of M-chain to G-chain molecules. M-chain molecules are terminated with a non-reducing 1-linked D-mannitol, while G-chain molecules are terminated with a reducing 3-linked glucose residue. On average, one  $\beta$ -1,3 backbone has 1.3 branches per molecule, with a range of zero to four  $\beta$ -1,6 branches (Read et al., 1996). The water-solubility of laminarin is determined by these short, occasional branches. Unbranched laminarin oligosaccharides are only freely water-soluble if their DP is lower than 20. Unbranched laminarin oligosaccharides with a DP higher than 36 (e.g., curdlan) are water-insoluble. An increase in  $\beta$ -1,3-backbone length (DP) or the increase in branch length decreases the solubility of  $\beta$ -1,3-glucan (Legentil et al., 2015). Therefore, yeast  $\beta$ -1,3-glucan with an average DP of around 1,500 and  $\beta$ -1,3 branches linked via a  $\beta$ -1,6 bond is water-insoluble (Legentil et al., 2015; Lipke and Ovalle, 1998).

As discussed earlier (Chapter 1.1), YCW is a complex structure composed of several polymers that are linked by inter- and intra-chain bonds. Therefore, like  $\beta$ -glucans, YCW is water-insoluble. Various physical, chemical and enzymatic methods are employed to solubilise and purify specific components, for example,  $\beta$ -glucans from YCW or yeast biomass. Ever since the discovery of  $\beta$ -glucans' biological activity, major efforts have been dedicated to investigating their mechanism of action and finding therapeutic applications.

### 1.2.1 Biological activity

The main current applications of yeast  $\beta$ -glucans are food supplements, and YCW are used as feed supplements. These application areas are mostly based on the immunomodulatory activity of yeast  $\beta$ -glucans. Almost all YCW polysaccharides ( $\beta$ -1,3-glucan,  $\beta$ -1,6-glucan, mannan), and YCW as a crude

preparation, are reported to have biological activity. The animal feed supplemented with YCW is used as an alternative to antibiotics, as a growth promoter, and as a prophylactic measure against infectious diseases induced by *Clostridium perfringens*, *Salmonella*, *Escherichia coli*, and *Listeria monocytogenes* (Santovito et al., 2018). Zymosan is a purified polysaccharide fraction of YCW (Holan et al., 1980). The anti-tumour properties of  $\beta$ -1,3-glucan in mice were demonstrated by depleting zymosan of  $\beta$ -1,3-glucan upon treatment with  $\beta$ -1,3-glucanase (Lee et al., 2021). In this same study,  $\beta$ -1,6-glucan was demonstrated to be the main immunoregulator in zymosan-derived mannan/ $\beta$ -1,6-glucan-containing polysaccharides (MGCP) fraction. Purified  $\beta$ -glucans are available as commercial products, such as *Wellmune* and *Yestimun* (Fig. 1.5).

Although yeast  $\beta$ -1,3/1,6-glucans have demonstrated immunomodulatory properties, human evidence is weaker than evidence obtained during pre-clinical studies, and much uncertainty still exists (De Marco Castro et al., 2021). One potential explanation for this difference could be attributed to the highly dynamic structural properties of yeast  $\beta$ -glucans, which were extracted during separate experiments (Fig. 1.5). Yeast  $\beta$ -glucans are extracted using various physical, chemical and enzymatic methods (Varelas et al., 2016).

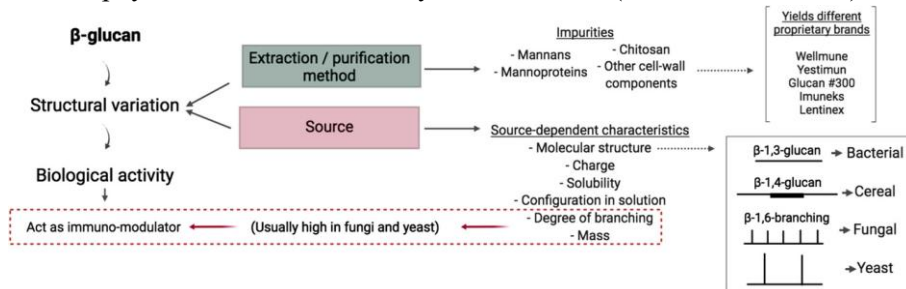


**Figure 1.5.**  $\beta$ -Glucan structure–activity relationship. The variability in  $\beta$ -glucan structure is due to differences in the source, as well as the extraction and/or purification methods used. This likely explains the divergent functionalities that exist among  $\beta$ -glucans. Differences arise depending on the source, such as in the nature of molecular linkages and the degree of branching. There is also variability in mass, charge, solubility and configuration in solution (single helix, triple helix or random coil), as well as in impurity levels and content. These variabilities will result in different interactions with the host. It has been demonstrated that only glucans which are highly purified and possess a high degree of branching along the  $\beta$ -1,3-glucan backbone, in conjunction with a high molecular weight, are capable of exerting immunomodulatory properties. Reprinted from (De Marco Castro et al., 2021).

Each of these methods has distinct characteristics that impact the structure of  $\beta$ -glucans. Chemical hydrolysis methods require extreme pH values, physical methods expose  $\beta$ -glucans to high shear or high temperature and enzymatic methods require reaction termination at a certain time point. This is because the  $\beta$ -glycosidases used are the major enzymes responsible for both

the efficient extraction and the degradation of  $\beta$ -glucans. In addition, the source strain of yeast and the processing methods used are key factors influencing the purity profile, branching, and linkage patterns of isolated yeast  $\beta$ -glucan. Out of five investigated commercial  $\beta$ -glucan samples several differences were observed – one sample had high content of glycogen, other had unusually high percentage of terminal residues, while another one had lower branching degree and shorter branches (Boutros et al., 2022). For instance, the stimulation of cytokines can be induced by the  $\beta$ -1,3-glucan with a triple-helix configuration (Falch et al., 2000). Nonetheless, in order to sustain the triple-helix configuration, it is necessary that the extraction process should follow low temperature and certain pH requirements (Novák et al., 2012). The limited solubility, structure and purity of  $\beta$ -1,3/1,6-glucans products can result in a number of practical issues, including batch-to-batch inconsistency, contrary immunomodulatory effect between products of different vendors, leading to limited clinical usage (Xiao et al., 2022).

The  $\beta$ -glucan content and molecular weight ( $M_w$ ) profile between the samples extracted from cultured yeast and brewer's spent yeast (BSY) are different. Lee and colleagues have determined that BSY ( $201.80 \pm 5.41 \mu\text{g/mg}$ ) contains a higher content of alkali-extractable  $\beta$ -glucans compared to cultured *S. cerevisiae* ( $87.89 \pm 2.42 \mu\text{g/mg}$ ) or *Saccharomyces bouldarii* ( $56.21 \pm 2.70 \mu\text{g/mg}$ ) species yeast when the autolysis residue fractions were analysed (Lee et al., 2024). However, the BSY yeast  $\beta$ -glucan profile was shifted towards lower  $M_w$ . The most abundant fraction of  $\beta$ -glucan in BSY was 19.3 kDa in  $M_w$ , while *S. cerevisiae* – 452.5 kDa and *S. bouldarii* – 405.3 kDa. As pure  $\beta$ -1,3-glucans have a  $M_w$  of 240 kDa and  $\beta$ -1,6-glucans – 24 kDa (Fig. 1.2), the  $M_w$  of 19.3 kDa from BSY hints at an extensive hydrolysis during the autolysis/fermentation process. The authors hypothesised that the lower  $M_w$  may ensure better water-solubility and higher bioactivity (Lee et al., 2024). However, it is unclear whether lower  $M_w$   $\beta$ -glucans possess better immunomodulatory activity compared to particulate  $\beta$ -glucans. As an earlier review conducted by Bastos and others listed several studies that showed higher immunomodulatory activity of higher  $M_w$  particulate  $\beta$ -glucans in comparison to low  $M_w$   $\beta$ -glucans (Bastos et al., 2022).

Investigations into immunomodulatory activity have received the majority of attention. The highest expectations have been set for cancer therapy. At the research and pre-clinical trial levels, therapeutic effects on colorectal, pancreatic, and gastric cancers showed promising efficacy (Wang et al., 2024). However, despite ongoing studies and clinical trials,  $\beta$ -glucans are still considered compounds with promising potential rather than an efficient therapeutic tool (Noorbakhsh Varnosfaderani et al., 2024). In a significant

number of reviews,  $\beta$ -glucans derived from different sources (bacterial, cereal, fungal, yeast, algae) are all discussed as a more or less distinctive class of compounds showing various health benefits (Ameri Shah Reza et al., 2025; Noorbakhsh Varnosfaderani et al., 2024; Wang et al., 2024). Among these, due to  $\beta$ -1,3/1,6-branched glucan structure, yeast and fungal  $\beta$ -glucans are the main ones associated with the immunomodulatory activity (Ameri Shah Reza et al., 2025; Noorbakhsh Varnosfaderani et al., 2024). A study by Driscoll showed that when  $\beta$ -glucans of various origins are combined with monoclonal antibody therapy, yeast  $\beta$ -glucan exhibits stronger anti-tumour activity compared to fungal  $\beta$ -glucan (Driscoll et al., 2009).

Depending on DP, branch type, and branching degree,  $\beta$ -glucan polysaccharides and oligosaccharides exert immunomodulatory effects via distinct receptors. The overview of the relationship between  $\beta$ -glucan structure and affinity towards different receptors is presented in Figure 1.6. Long water-insoluble linear  $\beta$ -1,3-glucan oligosaccharides and  $\beta$ -1,6-glucans with  $\beta$ -1,3-branches stimulate the immune system by binding the dectin-1 receptor. However, short water-soluble linear glucan oligosaccharides suppress the immune system via the dectin-1 receptors. On the other hand, soluble linear  $\beta$ -1,3-glucan oligosaccharides and branched  $\beta$ -1,3/1,6-glucans bind to CR3 receptors (Bastos et al., 2022). Other YCW polysaccharides can also activate the immune system via distinct receptors (Fig. 1.6), but they are beyond the scope of this study and are not reviewed.

Dectin-1 receptors are present on immune cells from the myeloid (macrophages, monocytes, dendritic cells, and neutrophils) or lymphoid ( $\gamma\delta$  T cells) lineages (Mata-Martínez et al., 2022). CR3 receptors are also abundant on similar types of cells: myeloid (neutrophil granulocytes, monocytes, macrophages, and activated lymphocytes) or lymphoid (natural killer cells) (Fernández et al., 2022).

Dectin-1 receptors are reported to be responsible for various immunological defence mechanisms, such as phagocytosis, release of inflammatory cytokines, production of reactive oxygen species and inflammasome complex activation (Elder et al., 2017). CR-3 receptors participate in leukocyte migration and adhesion, the phagocytosis of pathogens, and the induction of both inflammatory and tolerogenic responses (Lamers et al., 2021). The immune response triggered by the interaction of  $\beta$ -glucans with the dectin-1 and CR3 receptors is initiated via pathogen-associated molecular patterns (PAMPs). These pathways are naturally present in humans and are dedicated to protection against bacteria and fungi. Yeast  $\beta$ -glucans, having conserved structural features, activate the first line of defence known as pattern-triggered immunity (PTI) (Zhou et al., 2024).

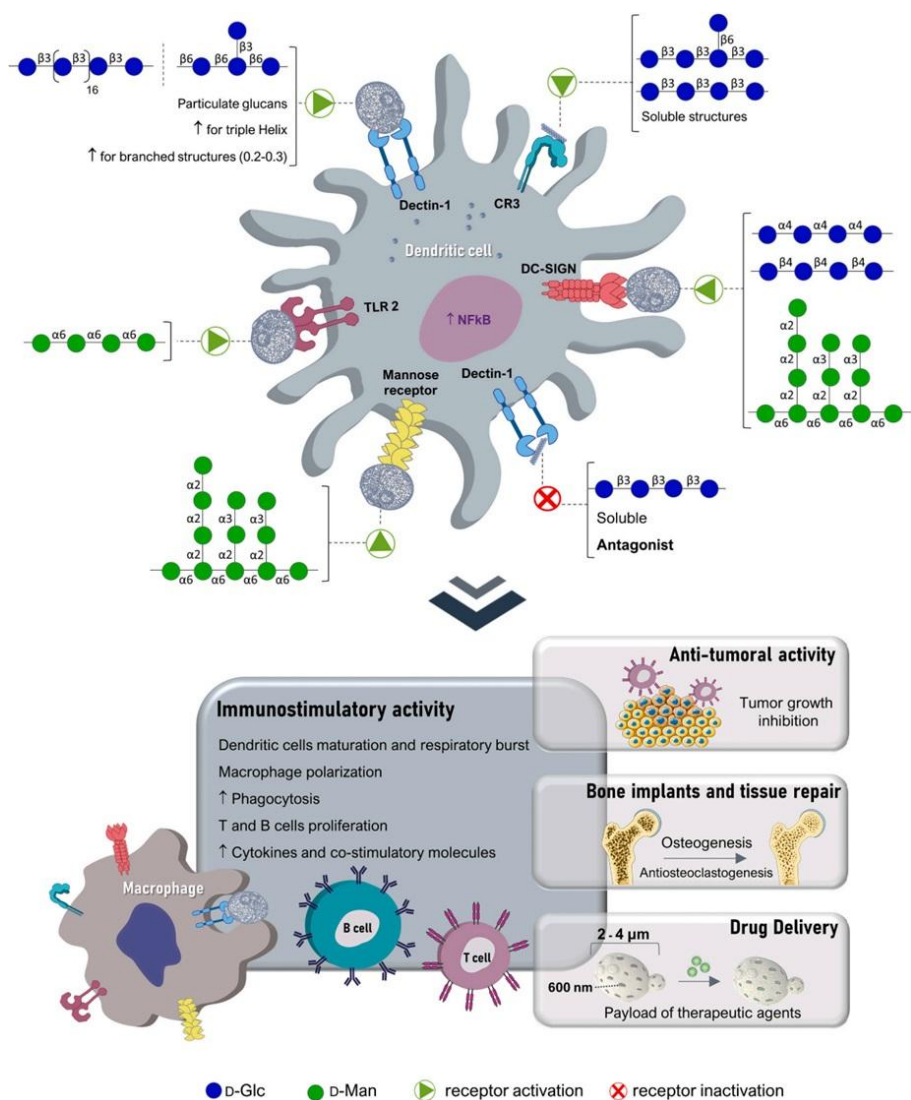


**Figure 1.6.** Illustration of immune system modulation by yeast glucan and mannan structures, through mediation of immune system cells (e.g. dendritic cells and macrophages) surface receptors (Dectin-1, CR3 — Complement Receptor 3; DC-SIG — Dendritic cell-specific ICAM-3-grabbing nonintegrin; Mannose receptor; TLR — Toll like receptor). Schematic overview of immunostimulatory response (signaling, cells activation and proliferation) and related biomedical applications (antitumoral, bone implant and regeneration field, drug delivery systems). Reprinted from (Bastos et al., 2022).

Water-insoluble particulate  $\beta$ -glucans are more bioactive. Nevertheless, they are more difficult to handle and process in laboratory conditions (Bastos et al., 2022). In addition to major structural characteristics, such as DP and branching degree (De Marco Castro et al., 2021), basic handling steps (e.g. drying) are important for bioactivity as well. For water-insoluble  $\beta$ -glucan,

spray-drying ensured 2-fold higher bioactivity, compared to the same preparation of  $\beta$ -glucan that was dried by lyophilisation or solvent exchange methods (Hromádková et al., 2003).

It is obvious that the characteristics of  $\beta$ -glucans isolated from different sources may vary greatly. This feature limits current applications as a therapeutic agent since  $\beta$ -glucans exhibit a structure-dependent, inconsistent immunomodulatory effect (De Marco Castro et al., 2021) or low added value of yeast CW when used as feed supplements instead of food applications. Therefore, the further enzymatic hydrolysis for conversion to oligosaccharides appears to be a lower-risk approach than using intact molecules to exert immunostimulatory activity. To sum up, despite extensive research on the application of yeast  $\beta$ -glucans as a source of high-value, immunostimulatory properties in supplements, many obstacles remain. However, despite existing obstacles in cancer therapy, some applications of  $\beta$ -glucan have been found to be commercially relevant.

### 1.2.2 Commercial and clinical use

Since the 1980s, research on the immunomodulatory effects of  $\beta$ -glucans has been conducted in clinical trials, with a particular focus on enhancing cancer therapies (Steimbach et al., 2021). Despite encouraging results from numerous *in vitro* studies, the pre-clinical and clinical trials have not yielded significant enough results for  $\beta$ -glucans to become a conventional drug or adjuvant in cancer treatment (De Marco Castro et al., 2021; Steimbach et al., 2021). As no significant breakthrough has been achieved to date, most  $\beta$ -glucans available on the market are marketed as food supplements and not drugs. The exception to this is non-*Saccharomyces*-derived  $\beta$ -glucans, which have been licensed as drugs in Japan since 1983 (Vetvicka et al., 2019). Another source claims that clinical usage started in 1977, and since 1989, it has been used in combination with chemotherapy (Maehara et al., 2012). These  $\beta$ -glucans are derived from the Shiitake mushroom *Lentinula edodes* (lentinan) and from *Coriolus versicolor* (polysaccharide-K). It is important to note that these  $\beta$ -glucans are structurally different to *Saccharomyces*-derived  $\beta$ -glucans. Lentinan has a linear  $\beta$ -1,3-glucan backbone with single  $\beta$ -1,6-glucose branches every fifth glucose residue and a  $M_w$  of 300–1000 kDa; it is water-soluble and forms triple-helices in water solutions (Zhang et al., 2011). Meanwhile, polysaccharide-K has a  $\beta$ -1,4-glucan backbone with  $\beta$ -1,3- and  $\beta$ -1,6- side branches and a  $M_w$  of 100 kDa (Dou et al., 2019). It has protein bound to  $\beta$ -1,6-branches and protein accounts for 18–38% of its composition (Maehara et al., 2012). However, even after over 40 years of clinical usage,

the difficulties in its extraction, purification, chain conformation, and the relationship between structure and biological activities have remained obstacles to its development as a therapeutic drug (Zhang et al., 2011).

Nowadays, commercially world-wide available food supplements such as “Wellmune”, “Yestimun”, “Glucan#300” show immunostimulatory effects – daily consumption shortens the time needed to recover from the symptoms of a cold or flu and increases the levels of lysozyme, immunoglobulins, interleukins, B- and T-cells in the blood or saliva (De Marco Castro et al., 2021). However, the clinical implementation of  $\beta$ -glucan therapy for the treatment of inflammation, cancer, and cardiometabolic diseases is still facing various challenges. The diversity of  $\beta$ -glucan structure is identified as one of the primary reasons limiting the elucidation of its mechanism of action and the establishment of the structure-function relationship (Z. Xu et al., 2024). The stimulation of the immune system, the enhancement of macrophage activity, and the increased production of cytokines are extensively demonstrated benefits of yeast  $\beta$ -glucans. These properties are being applied in the development of vaccine adjuvants, immunotherapeutic drugs, and wound-healing formulations. Nevertheless, conventional pharmaceutical use is still underway, and all these different potential application areas remain promising until solutions to current limitations are discovered (Mundada and Mahajan, 2025).

Another point indicating limited development of  $\beta$ -glucans as conventional immunomodulators is the inconsistent or inconclusive results of clinical trials. Up-to-date performed clinical trials lack direct proof of immunomodulatory activity, e.g. only three out of 106 analysed clinical trials claims could be considered clinically relevant, as others reported effects on various biomarkers and surrogate outcomes (Markovina et al., 2020). Moreover, the small groups that usually participate in such trials are another drawback of up-to-date studies. Therefore, larger group sizes are suggested for future studies (Markovina et al., 2020; Steimbach et al., 2021). In some cases, when  $\beta$ -glucans were used in combination with chemotherapy, patients who were treated with  $\beta$ -glucan experienced more nausea compared to those who received chemotherapy only. In addition, the decrease in the number of registered clinical trials shows a loss of interest in this topic (Markovina et al., 2020). Based on these observations and the ongoing investigations over several decades, it seems that a significant breakthrough is needed to determine the mechanism of action in the human organism, or to unify the structural properties across different experiments, in order for the current situation to change.

In the meantime, as an alternative to the current applications, yeast  $\beta$ -glucans or YCW can be used as a source of another class of bioactive or food-functionality-improving molecules, such as disaccharides or oligosaccharides. The use of enzymatic hydrolysis to produce low DP oligosaccharides as the final hydrolysis product, rather than as intermediate products with high DP, overcomes the issue of final product degradation. Unintended hydrolysis of  $\beta$ -glucan is encountered during  $\beta$ -glucan extraction from YCW using enzymatic methods. YCW is a suitable source to produce laminaribiose, laminaripentaose, and gentiobiose, not only in the context of YCW valorisation, but also as a cost-efficient industrial source with the main purpose of producing laminaribiose, laminaripentaose and gentiobiose.

### 1.3 Bioactive and functional oligosaccharides

Several different disaccharides and oligosaccharides can be generated as final bioconversion products upon enzymatic hydrolysis of YCW. The application potential depends on several factors, of which yield and demonstrated benefits are the most important. The yield is mainly determined by the structure of YCW – the proportion of substrate polysaccharide to be hydrolysed to smaller oligosaccharides. Another important trait is crosslinking degree, which determines how much of these polysaccharides are accessible to CAZymes and whether the hydrolysis products are uniform. Based on the structure of YCW polysaccharides, several different types of oligosaccharides can be obtained after treatment of YCW with different specificity CAZymes. Laminari-oligosaccharides can be obtained from the most abundant  $\beta$ -1,3-glucan (50–55%), mannan-oligosaccharides (MOS) from mannoproteins (35–40%), gentio-oligosaccharides from  $\beta$ -1,6-glucan (5–10%), and chitin-oligosaccharides from chitin (1–2%) (Klis et al., 2002).

Laminari-oligosaccharides, particularly laminaribiose and laminaripentaose, and gentio-oligosaccharides, particularly gentiobiose, are the major focus of this study. This focus is based on the relatively high abundance of  $\beta$ -1,3-glucans and  $\beta$ -1,6-glucans in YCW and their low-branching degree (Fig. 1.2). In comparison, MOS have a similar abundance to  $\beta$ -glucans in YCW, but their branching pattern is more complex, and they contain an additional protein moiety (Fig. 1.2). This makes production of low-molecular-weight oligosaccharides a very complex task, as different multiple glycosidic bonds ( $\alpha$ -1,2,  $\alpha$ -1,3,  $\alpha$ -1,6) are present in densely branched structures. Based on scientific studies, the benefits of mannans and MOS include improved gut health and immune function. In addition, they show antioxidative, anticancer, antihyperlipidemic, and vaccine-enhancing

activities (Baek et al., 2024). Given the difficult MOS extraction strategy and the existing benefits, the application of mannans rather than the production of specific MOS seems more relevant. Chitin hydrolysis products, monomeric N-acetyl glucosamine and dimeric chitobiose, are used as osteoarthritis therapeutics, dietary supplements, and cosmetic ingredients (Rani et al., 2020). However, due to the very low chitin proportion in YCW, these products are less relevant than  $\beta$ -glucan-derived oligosaccharides.

One of the main areas of oligosaccharide applications is as functional food additives, where they are used for their health benefits or for improving food attractiveness. A study conducted by Sanz et al. compared the prebiotic index (PI) score of disaccharides consisting of different monosaccharides linked via different glycosidic bonds (Sanz et al., 2005). Among the tested disaccharides (Fig. 1.7), several that can be obtained after hydrolysis of polysaccharides in YCW were investigated as well. 6 $\alpha$ -mannobiose showed the highest prebiotic potential, with laminaribiose being the second-best YCW-derived oligosaccharide. However, laminaribiose was less effective than the conventional prebiotic supplement, fructo-oligosaccharides.

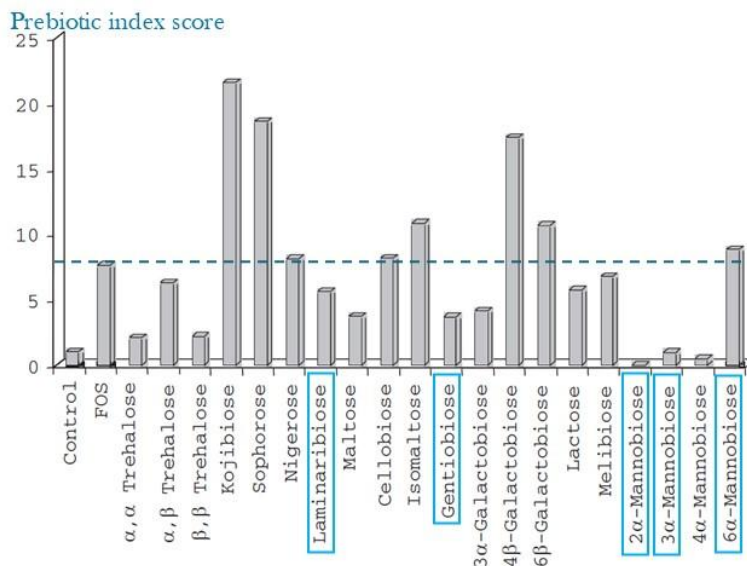


**Figure 1.7.** Fermentation selectivity of disaccharides as a function of structure. Dissaccharides were used as the sole carbon source in microscale non-pH-controlled faecal fermentations. Control fermentations had no added carbohydrate. Blue boxes represent YCW-derived oligosaccharides. Adapted from (Paeschke and Aimutis, 2010; Sanz et al., 2005).

In addition to linear oligosaccharides with a single type of glycosidic linkage, mixed glycosidic linkage oligosaccharides can also be obtained. The diversity is determined by multiple cross-links present between YCW polysaccharides (Fig. 1.3) (Orlean, 2012). However, due to low abundance,

the characterisation and investigation of these minor components is difficult. The most abundant mixed glycosidic linkage oligosaccharides are expected to be  $\beta$ -1,3/1,6-branched oligosaccharides. These oligosaccharides might be promising immunostimulants, as soluble  $\beta$ -1,3/ $\beta$ -1,6-structures are known to possess these properties (Fig. 1.6) (Bastos et al., 2022). The main benefit that could be obtained from using such oligosaccharides would be the structural consistency of these oligosaccharides. If these structures exhibit immunostimulatory activity comparable to particulate  $\beta$ -glucans, this could help overcome the main challenge of applying  $\beta$ -glucan products as conventional therapeutics or adjuvants. The first outcome could be a simplified investigation of the structure-function relationship and the mechanism of action of yeast  $\beta$ -glucans' biological activity (De Marco Castro et al., 2021; Mundada and Mahajan, 2025; Z. Xu et al., 2024).

A significant proportion of primary polysaccharides in YCW, a relatively simple crosslinking pattern between  $\beta$ -glucan chains, and multiple application areas, make these laminaribiose, laminaripentaose and gentiobiose oligosaccharides the main objects selected for investigation in this study.  $\beta$ -1,3/ $\beta$ -1,6-Branched oligosaccharides are a promising immunostimulant. Due to the more difficult extraction and characterisation, these oligosaccharides are not the primary focus of this study.

### 1.3.1 Structure and applications of laminaribiose

Laminaribiose is a  $\beta$ -1,3-disaccharide (Fig. 1.8) with prebiotic properties. Compared to gentiobiose (PI score = 3.70), laminaribiose (PI score = 5.60) has a higher prebiotic potential due to its higher PI score. However, neither laminaribiose nor gentiobiose has a PI score equivalent to that of the commercial prebiotic product fructooligosaccharides (PI score = 7.64) (Sanz et al., 2005). Another application of laminaribiose is in agriculture, where it is used to improve seed germination (Jamois et al., 2008). The chemical synthesis of laminaribiose is complicated, resulting in a high production cost of 3,200 EUR/g (Abi et al., 2019). Alternative synthesis methods are based on enzymatic reactions. GHs can be used to produce laminaribiose from various  $\beta$ -1,3-glucans, such as laminarin (Kumagai and Ojima, 2010), pachyman (McGrath and Wilson, 2006), and curdlan (Kurakake et al., 2013). The cell walls of baker's yeast are also reported to yield laminaribiose as a hydrolysis product following treatment with endo- $\beta$ -1,3-glucanase (Rombouts and Phaff, 1976a).

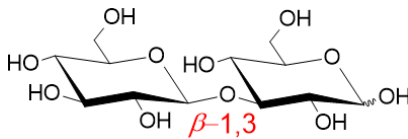


**Figure 1.8.** Laminaribiose depicted in chair conformation.

Currently, the focus is mainly on multienzymatic synthesis reactions. Several strategies for the synthesis of laminaribiose using various sources and multistep enzymatic treatments have been described to date. For example, a tetra-enzymatic process was designed for the synthesis of laminaribiose using glucose as the starting material (Niu et al., 2020); a bienzymatic synthesis using glucose and sucrose as the primary substrates (Müller et al., 2017); a tri-enzymatic system using sucrose (Kitaoka et al., 1993); a tetra-enzymatic system using cellulose (Du et al., 2022); and a tetra-enzymatic system using maltodextrins and glucose (Sun et al., 2019).

However, these processes are multienzymatic, and disaccharide side-products are produced alongside laminaribiose. Therefore, the main advantage of using GHs to hydrolyse polysaccharides to laminaribiose rather than synthesising it from monosaccharides or disaccharides using  $\beta$ -glucosidases or sugar phosphorylases is the lower process cost. Lower processing cost could be expected as fewer different enzymes are needed and side-products with the same DP are not formed, which can later complicate the purification process.

### 1.3.2 Structure and applications of laminaripentaose

Laminaripentaose is a  $\beta$ -1,3-pentasaccharide (Fig. 1.9) with health-promoting properties. Laminaripentaose has limited studies available on its application areas. Recently, a study demonstrated the therapeutic effect of laminaripentaose in the treatment of obesity and obesity-induced cognition impairment (Y. Xu et al., 2024). Mice fed with a high-fat diet for 12 weeks and treated with laminaripentaose (500 mg/kg) reduced weight gain by 32%, in comparison to no laminaripentaose treatment. In addition, laminaripentaose increased gut beneficial bacteria: *Bifidobacterium* increased by 422%, and *Butyricimonas* increased by 94%. This was accompanied by an increase in faecal short-chain fatty acids. Moreover, various other health benefits were observed: reduced systemic low-grade inflammation and intestinal barrier injury, improved memory functions, reduced hippocampal insulin resistance and neuronal injury.

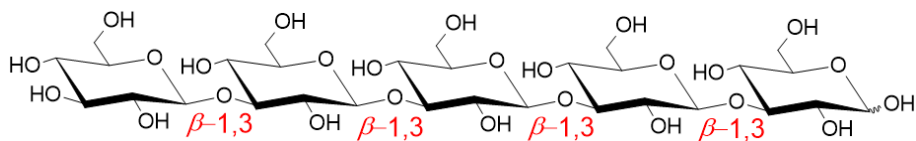


**Figure 1.9.** Laminaripentaose depicted in chair conformation.

The ability to produce laminaripentaose is attributed to the GH64 family endo- $\beta$ -1,3-glucanases (Drula et al., 2022). Previously characterised enzymes were able to use different  $\beta$ -1,3-glucans as substrates, releasing laminaripentaose as a hydrolysis product. Curdlan, laminarin, and yeast  $\beta$ -glucan substrates were hydrolysed to release laminaripentaose when appropriate enzymes were used.

SpGlu64A from *Streptomyces pratensis* was the most efficient on curdlan; the efficiency of laminarin hydrolysis was more than 400-fold lower, and no activity on yeast  $\beta$ -glucan was detected (J. Ma et al., 2024). Whereas, KfGH64 from *Kribbella flavida* had the highest activity on laminarin, followed by curdlan (Kumagai and Ojima, 2010). Interestingly, MoGluB from *Magnaporthe oryzae* was more efficient on yeast  $\beta$ -glucan than on laminarin (Wang et al., 2021). The purity of laminaripentaose can be adjusted by selecting different  $\beta$ -1,3-glucan substrates. The GluY from *Cellulosimicrobium funkei* produced the lowest proportion of oligosaccharide by-products with  $M_w$  higher than laminaripentaose, when curdlan was used as a substrate, in comparison to laminarin or pachyman (Bai et al., 2021).

Overall, recently reported health benefits of laminaripentaose increase its application potential. To date, many studies have focused on the exploration and the characterisation of laminaripentaose-producing enzymes. Not all these enzymes are capable of efficiently producing laminaripentaose from yeast  $\beta$ -glucan. Therefore, the combination of an efficient enzyme and a cost-effective substrate, such as YCW, could represent a promising strategy for synthesising an oligosaccharide with various health benefits.

### 1.3.3 Structure and applications of gentiobiose

Gentiobiose is a  $\beta$ -1,6-disaccharide (Fig. 1.10) with two main applications: as a prebiotic and as a flavour enhancer. Firstly, prebiotic effects have been demonstrated, as gentiobiose can modulate the gut microbiota by resisting digestion by the common oral bacterium *Streptococcus mutans* and mammalian intestinal enzymes (Hodoniczky et al., 2012). In addition, gentiobiose promotes the growth of *Lactobacillus acidophilus* and *Bifidobacterium animalis* (van Zanten et al., 2012) and has a PI score equal to 3.70 (Sanz et al., 2005). Secondly, gentiobiose has a refreshing, bitter taste

similar to chocolate, cocoa, coffee, and beer, and can reduce the astringency of fruits and vegetables. Consequently, gentiobiose and gentio-oligosaccharides (GnOS) are used as flavour enhancers in a variety of foods, such as chocolate, ice cream, jam and coffee (Xia et al., 2022).

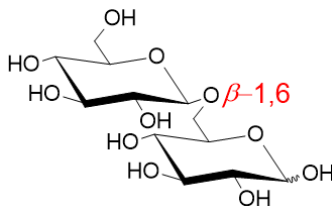


**Figure 1.10.** Gentiobiose depicted in chair conformation.

In some cases, gentiobiose is blended with sweet-tasting sugars, such as glucose and isomaltose, to improve the taste of foods such as soft drinks, dairy drinks, chocolate paste, jam, confectionery, spices and sauces (Aizawa et al., 2017). Gentiobiose is responsible for the bitter taste of GnOS products, as gentiobiose is five times more bitter than gentiotriose (Fujimoto et al., 2009), and gentiobiose is the main ingredient in commercial GnOS products Gentose#45 and Gentose#80 (Xia et al., 2022). In general, gentiobiose and GnOS are not freely available in nature. Only a small amount of free gentiobiose (approximately 2 mg/kg) has been detected in the locule of a ripe tomato (Dumville and Fry, 2003).

Gentiobiose can be prepared by chemical synthesis or enzymatic methods. The main disadvantages of chemical synthesis (Zhu and Kong, 2001) are multiple reactions required: protecting, activating and deprotecting, and low yields. Trans-glycosylation reactions produce non-specific disaccharides (Da Silva et al., 2017; He et al., 2013; Xia et al., 2022), oligosaccharides with higher DPs (Fujimoto et al., 2009; Wang et al., 2018) or mixed-type glycosidic linkage oligosaccharides (Seidle and Huber, 2005) in addition to the target disaccharide gentiobiose. In contrast, treatment of  $\beta$ -1,6-glucans with endo-1,6- $\beta$ -glucanases (Lu et al., 2023; Temple et al., 2017) allows the production of glucose and gentiobiose as the main reaction products, with glucose being easily removed using microbiological techniques (Moreno et al., 2014).

#### 1.3.4 Structure and applications of branched oligosaccharides

In addition to linear oligosaccharides, mixed glycosidic linkage oligosaccharides can be generated from YCW as well. Based on the accessibility to the specific GH enzymes,  $\beta$ -1,3/1,6-glucans are easier to obtain in comparison to  $\alpha$ -1,2/1,3/1,6-mannans (Fig. 1.2). Due to a less complex structure,  $\beta$ -1,3/1,6-oligosaccharides can be an alternative

immunomodulator to  $\beta$ -1,3/1,6-glucans. The main benefit would be a more consistent, more uniform and more easily monitored structure compared to  $\beta$ -1,3/1,6-glucans. The complexity and inconsistency of yeast  $\beta$ -glucan structure are considered the main limiting factors in further clinical applications (De Marco Castro et al., 2021; Z. Xu et al., 2024).

Previous studies report data on the immunomodulatory properties not only on high molecular weight particulate  $\beta$ -glucans, but for lower  $M_w$  water-soluble structures as well (Bastos et al., 2022). Soluble linear  $\beta$ -1,3-glucans and branched  $\beta$ -1,3/1,6-glucans can act as activators of CR3 receptor of dendritic cells (Fig. 1.6). In addition,  $\alpha$ -1,6-linear mannans can act as Toll-like receptor 2 (TLR-2) receptors, while branched  $\alpha$ -1,2/1,3/1,6-mannans can activate Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) and mannose receptors (Fig. 1.6). Another study investigating the immunomodulatory properties of zymosan and MGCP showed that  $\beta$ -1,6-glucan with single branches of  $\beta$ -1,3-glucose can act as an immunoregulator, while  $\beta$ -1,6-glucan with single branches of  $\alpha$ -1,2-mannose cannot (Lee et al., 2021). Aimanianda and others have shown that  $\beta$ -1,3/ $\beta$ -1,6-mixed linkage oligosaccharides with DP>4 are produced after treatment of yeast  $\beta$ -1,6-glucan with endo- $\beta$ -1,6-glucanase (Aimanianda et al., 2009). A  $\beta$ -1,3/1,6-branched oligosaccharide mixture obtained from the fungus *Alternaria alternata* with DP 8–17 showed 1000 times higher elicitor activity on tobacco BY-2 cells, in comparison to laminarin, having DP 20–30 with  $\beta$ -1,3-backbone and  $\beta$ -1,6-single glucose branches (Shinya et al., 2006).

To conclude, depending on the GH enzyme used to hydrolyse YCW polysaccharides, different oligosaccharides with distinct application fields can be produced. Laminaribiose can be used as a prebiotic, gentiobiose as a taste enhancer, laminaripentaose as an anti-obesity therapeutic agent, and branched oligosaccharides as immunostimulators. To achieve the desired hydrolysis products, a suitable enzyme must be selected from the vast diversity of available ones.

#### 1.4 Carbohydrate active enzymes (CAZymes)

The enzymes that build, modify, and break down carbohydrates are known as carbohydrate-active enzymes (CAZymes). These enzymes are curated in the CAZy database, where classification and functional information for each family is available (Drula et al., 2022). These enzymes are classified into families in the CAZy database (Drula et al., 2022) according to their amino acid sequences (Henrissat, 1991). Based on the type of activity towards carbohydrates, these enzymes are classified into five major families: glycoside

hydrolases (GHs), glycosyltransferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), auxiliary activities (AAs) and carbohydrate-binding modules (CBMs). GHs are enzymes that break down complex carbohydrates into oligosaccharides, sugars, or monosaccharides. These enzymes are of major interest due to their ability to produce oligosaccharides from polysaccharides as substrates. Additionally, CBMs are relevant when encoded alongside catalytic GH modules, as CBMs can enhance hydrolytic activity by improving enzyme binding to the polysaccharide substrate. The principal structure of GHs is presented in Figure 1.11. GHs have at least one GH catalytic module and none or multiple CBM modules. The type of GH module determines substrate specificity, while CBMs enhance hydrolysis efficiency. Among GHs, endo-1,3- $\beta$ -glucanases and endo-1,6- $\beta$ -glucanases are important for the hydrolysis of yeast  $\beta$ -1,3/1,6-glucan into functional and bioactive oligosaccharides.

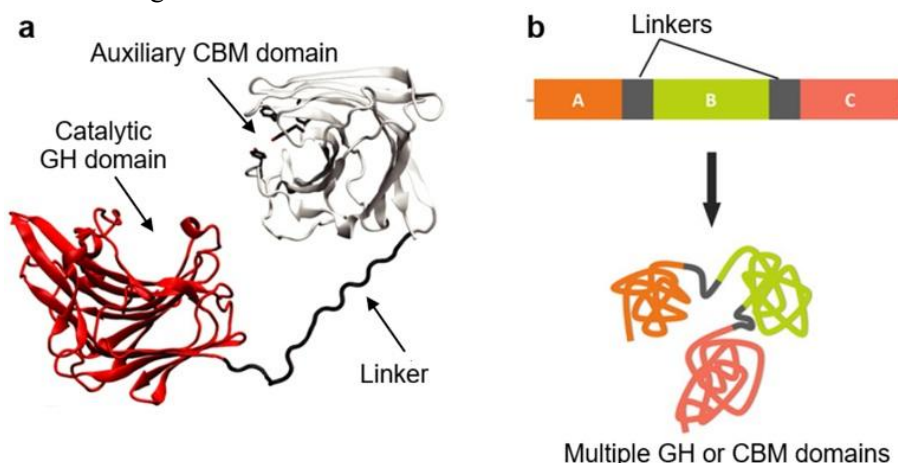


**Figure 1.11.** A representative structure of GH proteins consisting of catalytic and auxiliary modules covalently linked via amino acid linkers. a – GH protein with a single GH module (red) and single CBM module (grey). b – GH protein with multiple either GH or CBM domains. Adapted from (Pradeep and Edison, 2022).

So far, a vast diversity of GHs has been discovered. Those GHs that catalyse the hydrolysis of  $\beta$ -1,3-D-glycosidic and, in some cases,  $\beta$ -1,4-D-glycosidic bonds are subdivided into three classes. The first class comprises exo- $\beta$ -1,3-glucanases (EC 3.2.1.58), which cleave D-glucose from the non-reducing end of a glucan molecule. The second class comprises endo-1,3- $\beta$ -glucanases (EC 3.2.1.39), which require at least two  $\beta$ -1,3-bound glucose residues adjacent to the digested bond. The third class comprises endo-1,3(4)- $\beta$ -glucanases hydrolysing both  $\beta$ -1,3 and  $\beta$ -1,4 bonds, provided that the glucose residue whose reducing group is involved in the linkage to be hydrolysed is itself substituted at C-3 (Caseiro et al., 2022).

Endo-1,3- $\beta$ -glucanases (EC 3.2.1.39) are found in ten families: GH5, GH16, GH17, GH55, GH64, GH81, GH128, GH152, GH157 and GH158. Most bacterial endo-1,3- $\beta$ -glucanases belong to family GH16, whereas most fungal endo-1,3- $\beta$ -glucanases belong to family GH81 (Caseiro et al., 2022). GH16, GH64, and GH81 family members are explored in this study.

Endo-1,6- $\beta$ -glucanases (EC 3.2.1.75) belong to the GH5 and GH30 families and degrade a variety of  $\beta$ -1,6-glucans to oligosaccharides and glucose. Within the GH30 family, the enzymes with  $\beta$ -1,6-glucosidase (EC 3.2.1.-) and endo-1,6- $\beta$ -glucanase (EC 3.2.1.75) activities are divided into GH30\_1 and GH30\_3 subfamilies. GH30\_3 family member is explored in this study.

$\beta$ -glucosidases ( $\beta$ -D-glucoside glucohydrolases, EC 3.2.1.21) belong to the GH1, GH2, GH5, GH16, GH30, and GH39 families (Drula et al., 2022).  $\beta$ -glucosidases (EC 3.2.1.21) catalyse the hydrolysis of terminal, non-reducing  $\beta$ -D-glucosyl residues with the release of  $\beta$ -D-glucose. The GH3 family comprises mostly  $\beta$ -glucosidases. (Zhang et al., 2025). The GH3 family member is explored in this study. However, since one of the objectives of this study is the production of oligosaccharides rather than their breakdown into glucose, this  $\beta$ -glucosidase enzyme is less important in this study.

Advances in next-generation sequencing technologies have enabled a continuous increase in the number of gene sequences deposited in the CAZy database. Meanwhile, the number of CAZymes that have been biochemically or structurally characterised is growing much more slowly. Biochemical characterisation of enzymes identified during the sequencing of microbial genomes is important because newly discovered enzymes can offer advanced properties for industrial applications. Examples of industrially relevant traits that these enzymes can have include cold-adaptivity and high thermal stability (Chen et al., 2020; Yan et al., 2018).

The combination of  $\beta$ -glucans and  $\beta$ -1,3-glucanases serves applications across multiple fields (Fig. 1.12). Endo-1,3- $\beta$ -glucanases have a wide range of biotechnological applications, including the conversion of lignocellulosic biomass into sugars, as clarification agents in winemaking, as feed additives, and controlling pathogenic fungi in various crops (Caseiro et al., 2022). The main application area of endo-1,6- $\beta$ -glucanases is the synthesis of gentiobiose and gentio-oligosaccharides (Temple et al., 2017).  $\beta$ -Glucosidases can be used to synthesise oligosaccharides. However, alongside target products, side products with different glycosidic linkages (Seidle and Huber, 2005) or DPs (Fujimoto et al., 2009) are produced.

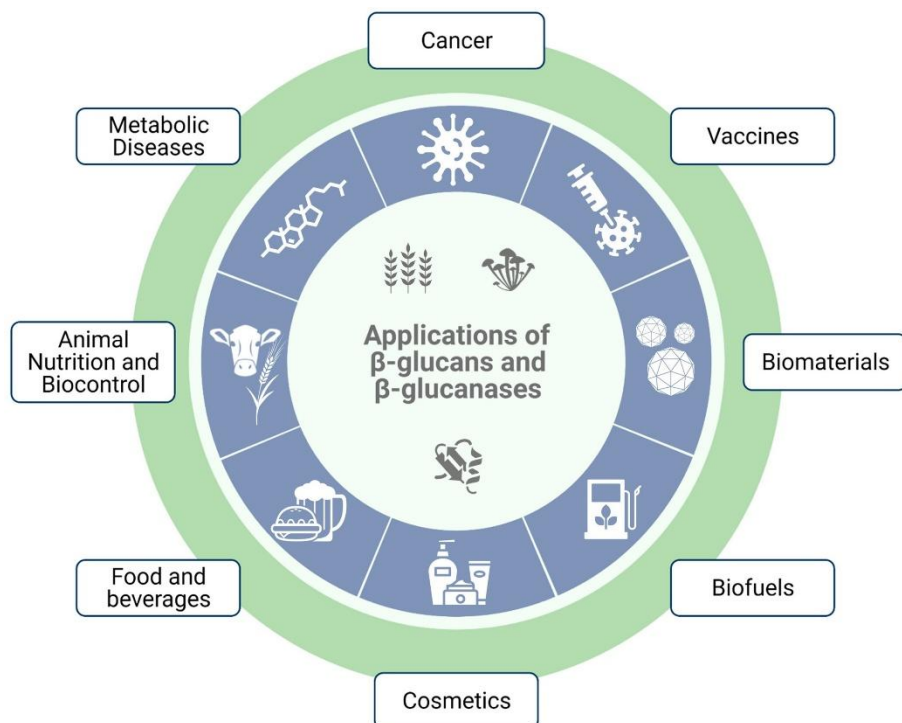


**Figure 1.12.** The applications of  $\beta$ -glucans and  $\beta$ -1,3-glucanases. Due to their unique physicochemical and bioactive properties,  $\beta$ -glucans and their processing enzymes are attractive molecules with multiple applications, ranging from cancer treatment to brewing. Reprinted from (Caseiro et al., 2022).

Two different strategies can be used to search for the CAZymes for the required applications. Firstly, enzyme sequences deposited into the CAZyme database can be used. These sequences are often supplemented with existing functional characterisation data from previous studies that can be used for further exploration. Secondly, enzymes with targeted activity can be discovered by isolating strains with desired functionalities and then by mapping these functionalities to particular enzymes, once genomic (Park et al., 2018), transcriptomic (Yang et al., 2025) or proteomic (Contato et al., 2024) data is retrieved. An online web server dbCAN3 allows the extraction of a list of CAZymes using genome or proteome sequences as an input (Zheng et al., 2023). Both strategies were applied in this study to achieve two distinct goals: the conversion of yeast  $\beta$ -glucans into functional, bioactive oligosaccharides and the application of GHs to the lysis of living yeast cells.

### 1.5 Enzymatic yeast cell lysis methods

Yeast cell lysis involves the disruption of the cell wall and plasma membrane, releasing intracellular components. Various methods for lysing

yeast cells are available. Each method has its own advantages and disadvantages. The selection of the most appropriate method is primarily based on the cell type to be disrupted and the particular intracellular component to be extracted (Ciobanu et al., 2024). The additional selection criteria can include lysis efficiency, extraction yield of intracellular components, application scale, method accessibility, and its effect on the intracellular components.

The scope of lysis methods that would be suitable for the lysis of brewer's or baker's yeast is wide. It includes non-mechanical methods sub-classified as biological (autolysis and enzymatic lysis), physical (thermolysis), and chemical (organic solvent treatment, osmotic shock). Another class is mechanical methods, such as ultrasonication, bead milling, and high-pressure homogenisation (Ciobanu et al., 2024). Avramia and colleagues have reviewed multiple studies aiming to extract yeast  $\beta$ -glucans from yeast (Avramia and Amariei, 2021). This review showed that the variety of applicable methods is large (Fig. 1.13). Both non-mechanical and mechanical methods are used, in some cases in various combinations of two methods.

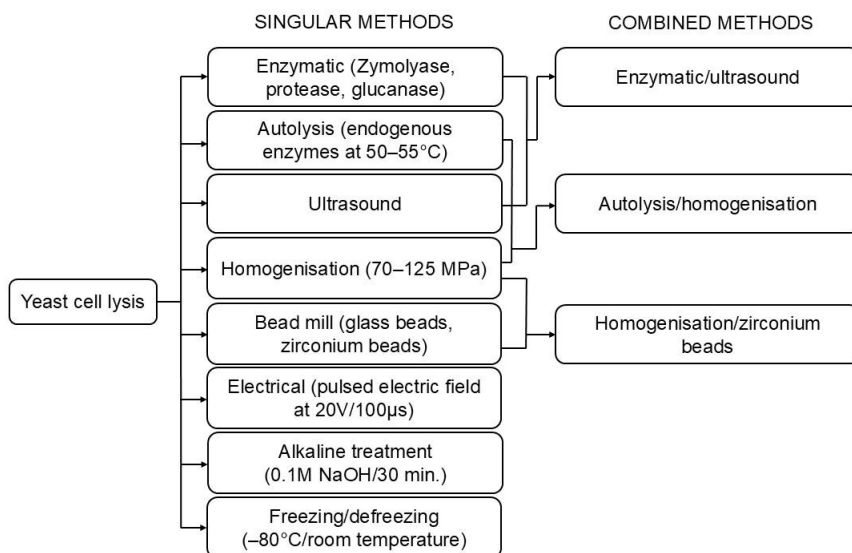


**Figure 1.13.** Different cell lysis methods for obtaining  $\beta$ -glucans. Adapted from (Avramia and Amariei, 2021).

Based on the numerous examples described in scientific papers, non-mechanical methods seem to be more preferred in research. However, mechanical methods, such as high-pressure homogenisation, are more easily scalable (Ciobanu et al., 2024). For YCW extraction, selecting the best cell lysis method is not straightforward. On the one hand, autolysis and enzymatic

lysis are cheap and readily accessible methods, but they share the main drawback of uncontrollable lysis of YCW components. Enzymatic lysis leads to the solubilisation of  $\beta$ -glucans, which retain immunostimulatory properties (Xin et al., 2022). However, insoluble yeast  $\beta$ -glucans are considered more potent immunostimulants (De Marco Castro et al., 2021). This ambiguity leads back to the previously discussed (Chapter 1.2.2) main issue of  $\beta$ -glucan application – the inconsistency of  $\beta$ -glucan structure when currently available yeast cell lysis and  $\beta$ -glucan extraction methods are used. The solubilisation effect shows that  $\beta$ -glucans are not only released from other YCW components but also have their structure altered, making them water-soluble rather than water-insoluble (Fig. 1.2). On the other hand, mechanical lysis methods do not degrade YCW polymers; however, if the aim of application is therapeutic, then the crude preparation of YCW may not show immunomodulatory activity, as appropriate cell lysis and extraction are required to access particular YCW polysaccharides (Günel-Köroğlu et al., 2025). In such cases, an additional isolation step for  $\beta$ -glucans, e.g., alkaline extraction, is required. Nevertheless, when YCW is used as a substrate for screening of yeast polysaccharides-degrading enzymes, the more intact the YCW structure is, the more suitable the substrate is.

The ability of certain endo-1,3- $\beta$ -glucanases to lyse living yeast cells was discovered several decades ago. During the investigation of lytic enzyme mixtures, activities of endo-1,3- $\beta$ -glucanases, proteases,  $\beta$ -1,6-glucanases, mannanases and chitinases targeting every polymer of *Saccharomyces* YCW were detected (Ferrer, 2006). Two main enzyme classes – proteases and endo-1,3- $\beta$ -glucanases – were identified as key to lytic ability (Scott and Schekman, 1980). Proteases were found to disrupt the external mannoprotein layer. Disruption of the external mannoprotein layer provides access for glucanases to target  $\beta$ -glucans, which ensure the mechanical strength of YCW. Enzymatic yeast cell lysis enzyme mixtures relying on endo-1,3- $\beta$ -glucanase and protease activities are commercially available.

### 1.5.1 Bacterial lytic enzyme mixtures

Enzymes that can lyse yeast cells can be either endogenous or exogenous. Endogenous  $\beta$ -glucanases primarily function to remodel the YCW (Lesage and Bussey, 2006), while endogenous proteases maintain protein turnover (Jones, 1984). Furthermore, these enzymes participate in autolysis, which occurs at the end of the cell's life cycle. Artificial autolysis can also be initiated to extract  $\beta$ -glucans or produce yeast extract. However, this requires incubation at elevated temperatures of 55 °C for 48 hours (Takaloo et al.,

2020). In this case, alongside autolysis, the degradation and hydrolysis of multiple cellular components occur simultaneously (Fig. 1.14). Organelles such as the YCW, cell membrane, nucleus and vacuole are degraded, while intracellular proteins are hydrolysed. This long, low-specificity process is not favoured when intracellular compounds need to remain intact.

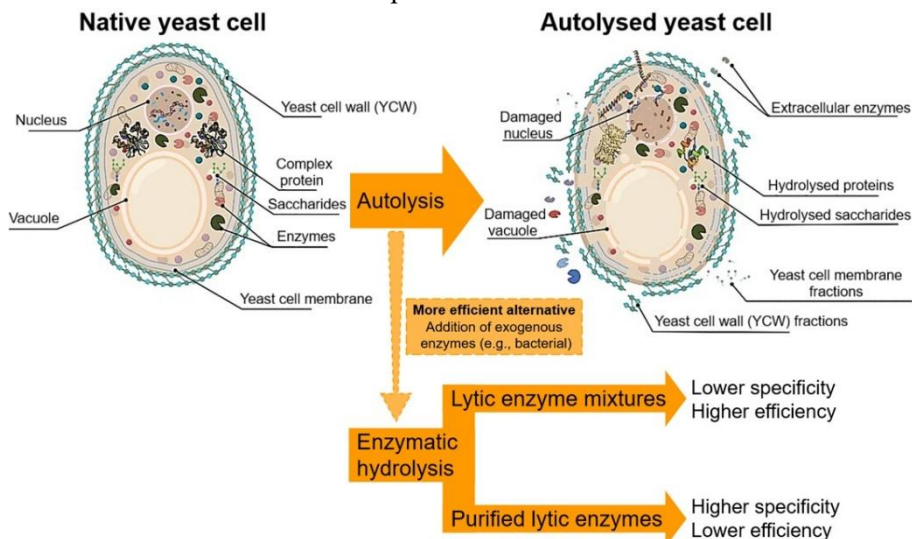


**Figure 1.14.** Yeast cell's modifications during autolysis and autolysis comparison with enzymatic hydrolysis. Adapted from (Constantin et al., 2024).

Alternatively to autolysis, enzymatic hydrolysis induced by added exogenous enzymes is more efficient (Fig. 1.14). Such hydrolysis usually requires a lower temperature (25–35°C) and a shorter period (2 hours) than autolysis (Kitamura et al., 1972; Nacalai Tesque, Inc., 2025). There are two types of enzymatic hydrolysis that can be applied: one uses lytic enzyme mixtures obtained from secretomes, and the other uses purified lytic enzymes. The main differences are efficiency and specificity. Purified lytic enzymes enable targeted lysis of specific YCW polymers, however, with lower lysis efficiency. Lytic enzyme mixtures, on the other hand, have higher lysis efficiency due to targeting multiple YCW polymers. The main disadvantage of lytic enzyme mixtures is their lower specificity, meaning that they may also target intracellular compounds (Salazar et al., 2006). To date, lytic enzyme mixtures have shown greater success than purified lytic enzymes in commercial applications. The selection of yeast lytic enzymes is limited because not every GH that can hydrolyse yeast  $\beta$ -glucan or YCW can lyse living yeast cells (Ferrer, 2006).

*Cellulosimicrobium cellulans* and *Paenibacillus* sp. bacteria are already known producers of  $\beta$ -glucanases with multiple enzymes characterised is

different studies (Pradeep, 2022). On the other hand, *Rheinheimera* sp. bacteria are not known as typical producers of  $\beta$ -glucanases. Nevertheless, among these bacteria, only *C. cellulans* is reported to have yeast cell lysis ability. Currently, mixtures of lytic enzymes such as *Zymolyase*, *Lyticase*, or *Glusulase* are commercially available (Brettrager et al., 2024). The actinomycete *C. cellulans* (also known as *Cellulomonas cellulans*, *Oerskovia xanthineolytica* and *Arthrobacter luteus*) is the main source of yeast lytic enzymes (Ferrer, 2006). *Zymolyase* and *Lyticase* are lytic enzyme mixtures derived from *C. cellulans* (Ferrer, 2006), while *Glusulase* is sourced from snail intestinal juice (Marshall and Grand, 1975). According to the manufacturer's product information (Nacalai Tesque, Inc., 2025) and previous studies (Brettrager et al., 2024; Kitamura et al., 1972), *Zymolyase-20T* (Nacalai Tesque, Kyoto, Japan) contains 20,000 U/g of  $\beta$ -1,3-glucan laminaripentaohydrolase which is responsible for the lytic activity. It also contains approximately  $1.5 \times 10^6$  units/g of  $\beta$ -1,3-glucanase, approximately  $1.0 \times 10^4$  units/g of protease and approximately  $1.0 \times 10^6$  units/g of mannanase.

However, common lysis mixtures contain proteases with off-target activities that degrade, in addition to YCW mannoproteins, intracellular target proteins as well (Brettrager et al., 2024). Proteinase K is also sometimes used to accelerate yeast cell lysis (Hamouda et al., 2023). These side activities limit the applications of lytic enzyme mixtures when it is necessary to keep the extractable proteins intact. Reducing agents are an alternative to proteases (Zlotnik et al., 1984), but they also affect intracellular proteins. Nucleases are another undesirable contaminant in yeast lysis mixtures (Scott and Schekman, 1980). Therefore, conditions in which pure endo-1,3- $\beta$ -glucanases can lyse living yeast cells without the presence of proteases or reducing agents are desirable.

### 1.5.2 Purified bacterial lytic enzymes

The main goal of using purified lytic enzymes is to prevent off-target enzymatic activities of non-specific lysis (Deng et al., 2026). Either purified proteases or endo-1,3- $\beta$ -glucanases can be used. Proteases are a less common choice, suitable when hydrolysis of intracellular yeast proteins is of no concern and bioactive or intact proteins are not the target product. In such cases, proteases, e.g. papain, can be used as an autolysis-enhancing additive or yeast lysis-inducing enzyme (Deng et al., 2026). However, if functional or intact proteins need to be extracted from yeast (Dunn and Wobbe, 1993; Kulagina et al., 2021), the use of proteases is not suitable, and endo-1,3- $\beta$ -glucanases are a more suitable option.

Evaluation of purified endo-1,3- $\beta$ -glucanases revealed that they can possess lytic activity in the absence of proteases. However, reducing agents, e.g.  $\beta$ -ME and DTT, must be used in such instances. These reducing agents are used in high concentrations – for example, 30 mM DTT (Dunn and Wobbe, 1993); 20 mM  $\beta$ -ME (Karas et al., 2014); and 100 mM  $\beta$ -ME (Hamouda et al., 2023; Kitamura and Yamamoto, 1981).

According to the CAZy database (Drula et al., 2022), endo-1,3- $\beta$ -glucanases are classified within the following families: GH5, GH16, GH17, GH50, GH55, GH64, GH81, GH128, GH152, GH157 and GH158. However, only the endo-1,3- $\beta$ -glucanases in the GH16 (Salazar et al., 2006) and GH64 (Shen et al., 1991) families can lyse living yeast cells. Recombinant endo-1,3- $\beta$ -glucanase from *Oerskovia xanthineolytica*, which belongs to the GH64 family, has been commercially available with some periods of discontinuation (Carvalho et al., 2021). Over the years, it has been demonstrated that lytic endo-1,3- $\beta$ -glucanases belong to the GH16 or GH64 families. However, using these enzymes requires high concentrations of DTT or  $\beta$ -ME. Previous studies have reported on the use of GH16 or GH64 lytic endo-1,3- $\beta$ -glucanases, but have not compared their efficiencies or tested combinations across a range of reducing agent concentrations, or in the absence of reducing agents.

*Cellulosimicrobium* genus bacteria-encoded GH16 and GH64 family yeast lytic glucanases were discovered and characterised several decades ago. However, previous studies focused on either one or the other of these enzymes rather than on their combination or on comparing their lytic activity. This work focuses on comparing the lytic activities of GH16 and GH64 lytic glucanases, as well as *Zymolyase-20T*. It also screens for conditions that would enable the lysis of living yeast cells without the use of protease or a reducing agent. Additionally, the minimum  $\beta$ -ME concentration required for yeast lysis of 16 strains representing eight different genera is determined by screening a range of reducing agent concentrations (0.5–50 mM).

To sum up, a great deal of attention has been devoted to investigating YCW polysaccharides to date. The immunomodulatory properties of  $\beta$ -glucans for use in cancer therapy are of major interest. However, regardless of extensive experimental data collected on  $\beta$ -glucan structure-bioactivity relation over several decades, this knowledge is insufficient for conventional therapeutic use. The best-to-date solution for yeast  $\beta$ -glucan application is the food supplement status, which still produces inconsistent results. On the other hand, enzymatic yeast cell lysis based on the degradation of YCW polymers is another topic that has attracted attention. Current solutions offer either low-efficiency lysis under protease-free and  $\beta$ -ME-free conditions or efficient lysis with proteases and  $\beta$ -ME. Despite previous research, new approaches are

possible: YCW-derived oligosaccharides have a simple structure and distinct health-promoting properties, while combinations of pure lytic enzymes may surpass the efficiency of single-enzyme use. The aim of this work is to explore alternatives to current solutions: to convert  $\beta$ -glucans into bioactive and functional oligosaccharides, and to make the enzymatic yeast cell lysis method less prone to degradation and native structure alteration of intracellular yeast proteins. New enzymes can address the current issues and expand the scope of higher-added-value products that can be produced using yeast or YCW.

## 2. MATERIALS AND METHODS

### 2.1 Materials

#### 2.1.1 Chemicals

Chemicals and solvents were purchased from Calbiochem (San Diego, CA, USA), Carl-Roth (Karlsruhe, Germany), Chem-Lab NV (Zedelgem, Belgium), ChemPur (Piekary Slaskie, Poland), Fluka (Buchs, Switzerland), Formedium (Hunstanton, UK), Honeywell (Seelze, Germany), Lach-Ner (Neratovice, Czech Republic), Merck (Darmstadt, Germany), PENTA (Prague, Czech Republic), Polysciences (Warrington, PA, USA), Sigma-Aldrich (St. Louis, MO, USA), and Thermo Fisher Scientific (Vilnius, Lithuania).

#### 2.1.2 Substrates

Laminari-oligosaccharides, curdlan, pachyman, barley  $\beta$ -glucan, yeast  $\beta$ -glucan, and  $\beta$ -1,2-glucan were purchased from Megazyme (Wicklow, Ireland). Laminarin, carboxymethyl cellulose, cellobiose and microcrystalline cellulose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gentiobiose and pustulan were purchased from Biosynth-Carbosynth (Bratislava, Slovakia).

The YCW substrate was prepared as described previously (Plakys et al., 2022). Briefly, yeast cells were mechanically disrupted, then YCW was separated from soluble cell components, washed with water and lyophilised. *p*NP-gentiobioside was synthesised by Gintaras Urbelis as described previously (Plakys et al., 2024).

#### 2.1.3 Bacterial strains

*Escherichia coli* DH5 $\alpha$  (Fermentas, Vilnius, Lithuania) was used for plasmid amplification, and *E. coli* BL21 (DE3) (Novagen, Darmstadt, Germany), *E. coli* LOBSTR (Kerafast, Boston, MA, USA), *E. coli* soluBL21 cells (Amsbio, Milton, UK), *E. coli* SHuffle T7 (New England Biolabs, Ipswich, MA, USA), *E. coli* HMS174(DE3) (Novagen, Darmstadt, Germany), and *E. coli* KRX (Promega, Vienna, Austria) were used for synthesis of recombinant proteins.

## 2.1.4 Nucleic acids

Genes encoding GH3\_3456, GH16\_306, and GH16\_3455 glycosidases were cloned from *Rheinheimera* sp. GKU. PsLam81A and PsGly30A glycosidases coding genes were cloned from *Paenibacillus* sp. GKG. Both bacteria were isolated during this work. Genes were cloned using specifically designed primers (Appendix 1). DNA primers were synthesised by Metabion International AG (Germany) and Azenta (Germany). Genome sequence of *Rheinheimera* sp. GKU was determined by Dr. Aleksandras Kononov using Oxford Nanopore long-read sequencing.

Synthetic gene sequences encoding the GH16 and GH64 enzymes were sourced from *Cellulosimicrobium cellulans* LLG109 and *Cellulosimicrobium cellulans* ATCC 21606, respectively. These strains were isolated during previous studies (Ferrer, 2006). Clonal genes were purchased in pET-24(+) plasmids from Twist Bioscience (San Francisco, CA, USA).

## 2.2 Methods

### 2.2.1 Isolation of YCW-degrading bacteria

Soil and water samples were collected in Lithuania. The samples were enriched for YCW-degrading microorganisms by adding YCW to a final concentration of 10 mg/mL and incubated at 22 °C for 120 hours. The enriched samples were diluted 25-fold using 0.9% NaCl, then 50 µL aliquots were plated onto a solid mineral medium containing a water-insoluble YCW substrate as a carbon source. The solid mineral medium contained 5 g/L NaCl, 1 g/L NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 1 g/L K<sub>2</sub>HPO<sub>4</sub>, 2 g/L MgSO<sub>4</sub> × 7H<sub>2</sub>O, 15 g/L agar, 3 g/L lyophilized YCW and 6 mg/L clotrimazole, at pH 7.2. The plates were then incubated at 30 °C for 72 hours. After that, the plates were visually inspected, and colonies that produced hydrolysis zones were isolated by repeated streaking.

### 2.2.2 Identification of YCW-degrading bacteria

Chromosomal DNA was extracted from the isolated colonies as described previously (Woo et al., 1992). The 16S rRNA gene was amplified by PCR using chromosomal DNA as a template, Phusion Plus PCR Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania) and primers Woo1 and Woo2 (Appendix 1) (Weisburg et al., 1991). The PCR products were purified using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Vilnius, Lithuania). The purified PCR products were cloned into the pJET1.2/blunt cloning vector

using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Vilnius, Lithuania). *E. coli* strain DH5 $\alpha$  cells were transformed with the ligation mixtures and spread onto LB agar plates supplemented with 50  $\mu$ g/mL ampicillin. The pDNA was extracted and purified using the ZR Plasmid Miniprep kit (Zymo Research, Irvine, CA, USA) and sequenced using pJET1.2 sequencing primers (Appendix 1) by the Sanger method (Macrogen Europe, Amsterdam, the Netherlands or Genewiz Germany GmbH, Leipzig, Germany).

### 2.2.3 Production and collection of CAZymes fractions

#### 2.2.3.1 *Rheinheimera* sp. GKU CAZymes fractions

Isolated culture of *Rheinheimera* sp. GKU was grown on solid LB medium at 30 °C for 48 hours. Grown biomass was collected into a sterile 1.5 mL microcentrifuge tube and suspended in 0.9% NaCl solution. Suspension was transferred to the 100 mL of liquid mineral medium containing 5 g/L NaCl, 1 g/L NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 1 g/L K<sub>2</sub>HPO<sub>4</sub>, 2 g/L MgSO<sub>4</sub>, 0.02 g/L CaCl<sub>2</sub>, 0.01 g/L MnSO<sub>4</sub>, 5 mg/L FeSO<sub>4</sub>, 1 mM HCl, pH 7.2 and 5 g/L of glucose or 5 g/L of lyophilised YCW. The amount of suspension necessary was calculated to yield an initial OD<sub>600nm</sub> of 0.1. The cultures were cultivated at 30 °C, 180 rpm for 48 hours. The biomass and supernatant were separated by centrifugation at 7,200 $\times$  g for 15 min at 4 °C and collected for secretome, periplasmic and intracellular protein analysis.

Culture medium was used for secreted protein analysis. Proteins were precipitated by autoclaving 100 mL of supernatant at 1.5 atm for 30 min at 105 °C. The pellet was collected by centrifugation at 7,200 $\times$  g for 15 min at 4 °C, supernatant was discarded. Pellets were suspended in 1 mL of denaturing buffer (8 M urea, 100 mM ammonium bicarbonate, pH 8.0). The resulting suspension was mixed for 20 min at 22 °C using the tube rotator Multi RS-60 (Biosan, Riga, Latvia). The resulting suspension was centrifuged at 21,300 $\times$  g for 15 min at 20 °C. Two aliquots of 500  $\mu$ L were separated and used as technical replicates (n=2).

Biomass was used to extract periplasmic and intracellular proteins. The biomass was resuspended in 25 mM Tris-HCl, 500 mM NaCl, pH 8.0 buffer to yield 15 % (w/w) pellet concentration.

Two aliquots of 500  $\mu$ L were separated as technical replicates (n=2) for intracellular protein extraction using ultrasonic homogeniser Sonopuls HD 2070 (BANDELIN electronic GmbH & Co. KG, Berlin, Germany). The resulting suspension was centrifuged at 21,300 $\times$  g for 15 min at 20 °C. Supernatants were collected, and pellets were discarded.

Two aliquots of 500  $\mu$ L suspension were separated as technical replicates (n=2) for periplasmic protein heat and EDTA extraction. The suspension was centrifuged at 21,300 $\times$  g for 15 min at 20  $^{\circ}$ C, supernatant was discarded. Pellet was resuspended in 500  $\mu$ L of 25 mM Tris-HCl, 500 mM NaCl, 10 mM EDTA, pH 8.0 buffer. Extraction was performed by incubating the pellets at 60  $^{\circ}$ C for 2 hours in thermoblock TS-100 (Biosan, Riga, Latvia). All resulting samples were subjected to tryptic digestion as described in Chapter 2.2.4.1.

#### 2.2.3.2 *Paenibacillus* sp. GKG CAZymes fraction

The liquid mineral medium used to produce extracellular enzymes contained 5 g/L NaCl, 1 g/L  $\text{NH}_4\text{H}_2\text{PO}_4$ , 1 g/L  $\text{K}_2\text{HPO}_4$ , 2 g/L  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$  and 3 g/L lyophilised YCW, the pH was adjusted to 7.2. A single colony from a solid mineral medium was inoculated into 20 mL of the liquid mineral medium and incubated at 30  $^{\circ}$ C, 180 rpm for 24 hours. Then, 10 mL of the inoculum were transferred to 200 mL of liquid mineral medium and incubated at 30  $^{\circ}$ C 180 rpm for 48 hours.

The culture medium was centrifuged at 7,200 $\times$  g for 20 min at 4  $^{\circ}$ C. The supernatant was collected, and the pellet was discarded. The supernatant was concentrated 20-fold using an Amicon Stirred Ultrafiltration Cell 8200 (Millipore, Burlington, USA), which was equipped with Omega 76 mm 30K membrane discs (Pall Corporation, New York, NY, USA). The concentrated supernatant was dialysed in a 14K dialysis membrane tubing (Carl-Roth, Karlsruhe, Germany) at 4  $^{\circ}$ C against 10 mM Tris-HCl, pH 7.0, for 18 hours. Afterwards, the dialysed sample was subjected to a tryptic digestion as described in Chapter 2.2.4.1.

#### 2.2.3.3 *Cellulosimicrobium* sp. GKH CAZymes fractions

Bacteria were cultivated on both solid and liquid media to produce secretome proteins. The solid mineral medium consisted of 5 g/L NaCl, 1 g/L  $\text{NH}_4\text{H}_2\text{PO}_4$ , 1 g/L  $\text{K}_2\text{HPO}_4$ , 2 g/L  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ , 15 g/L agar, 3 g/L lyophilised YCW, pH 7.2. In this case, lyophilised YCW were added before autoclaving. A purified colony was streaked on solid mineral medium and incubated at 30  $^{\circ}$ C for 72 hours. The hydrolysis zones formed, were used to extract the secreted proteins.

A single colony of bacteria was transferred to 100 mL of liquid mineral medium containing 5 g/L NaCl, 1 g/L  $\text{NH}_4\text{H}_2\text{PO}_4$ , 1 g/L  $\text{K}_2\text{HPO}_4$ , 2 g/L  $\text{MgSO}_4$ , 0.02 g/L  $\text{CaCl}_2$ , 0.01 g/L  $\text{MnSO}_4$ , 5 mg/L  $\text{FeSO}_4$ , 1 mM HCl, and 5 g/L of YCW, pH 7.2. After incubation at 30  $^{\circ}$ C, 180 rpm for 48 h, the culture medium was collected by centrifugation at 8,000 $\times$  g at 20  $^{\circ}$ C for 20 minutes. The collected supernatant was subjected to autoclaving. The resulting

precipitate was collected by centrifugation at  $8,000\times g$  at  $20\text{ }^{\circ}\text{C}$  for 20 min and was used to analyse secreted protein fraction. All resulting samples were subjected to tryptic digestion as described in Chapter 2.2.4.1.

## 2.2.4 Identification of CAZymes

### 2.2.4.1 FASP digestion

Filter-assisted sample preparation (FASP) of protein samples was performed using Nanosep®, MWCO 10 kDa ultrafiltration centrifugal units (Pall Corporation, New York, NY, USA) according to a modified FASP method described earlier (Erde et al., 2014). All centrifugations were performed at  $20\text{ }^{\circ}\text{C}$ ,  $13,500\times g$ , until remaining buffer volume on the centrifugal unit was  $<10\text{ }\mu\text{L}$ . All buffer exchanges were performed on centrifugal units, which were then shaken in a Thermomixer C (Eppendorf, Hamburg, Germany) at  $20\text{ }^{\circ}\text{C}$  and 900 rpm for 30 seconds.

The initial steps up to the protein-reducing step differed depending on the previous steps in protein fraction collection (Chapter 2.2.3). *Rheinheimera* sp. GKU protein fractions were prepared in the following manner. 500  $\mu\text{L}$  of periplasmic protein and intracellular protein fractions were loaded on centrifugal units and concentrated to a volume of  $<30\text{ }\mu\text{L}$ . 210  $\mu\text{L}$  of denaturing buffer was added. The resulting mixture was incubated in a Thermomixer C at  $20\text{ }^{\circ}\text{C}$  for 15 min, then concentrated to a volume of  $<10\text{ }\mu\text{L}$ . 500  $\mu\text{L}$  of secretome protein fraction was loaded on a filter unit and concentrated to a volume of  $<10\text{ }\mu\text{L}$ . 100  $\mu\text{L}$  reducing buffer (8 M urea, 100 mM ammonium bicarbonate, 10 mM DTT, pH 8.0) was loaded on centrifugal units with samples concentrated to a volume of  $<10\text{ }\mu\text{L}$ , and the following procedures were the same for all prepared samples.

500  $\mu\text{L}$  of *Paenibacillus* sp. GKG concentrated and dialysed secretome sample was loaded on centrifugal units and concentrated to a volume  $<30\text{ }\mu\text{L}$ . 210  $\mu\text{L}$  of denaturing buffer was added. The resulting mixture was incubated in a Thermomixer C at  $20\text{ }^{\circ}\text{C}$  for 15 min and then concentrated to a volume of  $<10\text{ }\mu\text{L}$ . 100  $\mu\text{L}$  reducing buffer was loaded on centrifugal units, and the following procedures were the same for all prepared samples.

The secreted protein fraction from the liquid culture medium of *Cellulosimicrobium* sp. GKH supplemented with YCW was collected by thermal precipitation. The precipitated proteins collected after thermal treatment of 100 mL of culture medium were dissolved in 1000  $\mu\text{L}$  of denaturing buffer. The samples were then clarified by centrifugation at  $20,000\times g$ ,  $20\text{ }^{\circ}\text{C}$  for 20 minutes. 500  $\mu\text{L}$  of supernatant was loaded onto the centrifugal unit and concentrated to a volume  $<10\text{ }\mu\text{L}$ . The secreted protein

fraction from the solid culture medium was extracted using deionised water. Solid culture medium pieces (0.5 g) were excised and incubated with 500  $\mu$ L of deionised water at 20 °C for 2 hours. The sample was then centrifuged at 20,000 $\times$ g for 30 minutes at 20 °C, and the supernatant was collected. This procedure was repeated once, and the resulting supernatants were pooled together and concentrated to a volume of <30  $\mu$ L using a centrifugal unit. Then, 210  $\mu$ L of denaturing buffer was added. The resulting mixture was incubated in a Thermomixer C at 20 °C for 15 min, then concentrated to a volume of <10  $\mu$ L. 100  $\mu$ L of reducing buffer was loaded on centrifugal units, concentrated to a volume of <10  $\mu$ L, and the following procedures were the same for all prepared samples.

The resulting mixtures of all samples in the reducing buffer were loaded onto centrifugal units and incubated in a thermomixer at 37 °C, 600 rpm for 60 minutes. After incubation, 200  $\mu$ L of denaturing buffer was added to the centrifugal units, and the flow-through was discarded after centrifugation. 100  $\mu$ L of alkylation buffer (50 mM iodoacetamide in 8 M urea, 100 mM ammonium bicarbonate) was added, and centrifugal units were incubated at 37 °C, 600 rpm for 60 minutes. 200 mM DTT solution was added to yield a final concentration of 50 mM DTT to deactivate residual iodoacetamide. Alkylation reagents were removed by centrifugation, flow-through was discarded. Centrifugal units were washed once with 200  $\mu$ L of the denaturing buffer, and the flow-through was discarded. Two additional buffer exchanges (200  $\mu$ L) were performed with the FASP digestion buffer (50 mM ammonium bicarbonate, pH 8). Proteins were digested overnight in 100  $\mu$ L FASP digestion buffer containing 0.02  $\mu$ g/mL of trypsin (Pierce MS-grade; Thermo Fisher Scientific, Vilnius, Lithuania). Digestion reaction mixtures were incubated in a 37 °C water bath Sub36 (Grant Instruments, Shepreth, UK) for 16 hours. After digestion, the flow-through was collected into new 1.5 mL centrifugal tubes, the centrifugal filter units were rinsed twice with 50  $\mu$ L of FASP digestion buffer, and the flow-through was collected. The solvent from the recovered peptide fraction was evaporated using a vacuum dryer Speed Vac SC110 (Thermo Savant, Waltham, MA, USA).

Dried peptides were redissolved in 0.1% formic acid, and a Hi3 PhosB standard peptide mixture (Waters Corporation, Milford, MA, USA) was added to give a final concentration of 0.1 or 0.2 pmol/ $\mu$ L. The prepared samples were analysed by LC-MS/MS.

#### 2.2.4.2 Data-independent LC-MS/MS

Liquid chromatography (LC) separation of trypsin-cleaved *Paenibacillus* secretome peptides was performed with the ACQUITY UPLC I-Class System

(Waters Corporation, Milford, MA, USA). The peptides were separated on a reversed-phase analytical column ACQUITY UPLC Peptide BEH C18 Column 300 Å, 1.7 µm, 2.1 mm × 150 mm (Waters Corporation, Milford, MA, USA) at a flow rate of 40 µL/minute. The isocratic gradient of 5% solvent B was set until 2.5 min, then a linear gradient from 5% to 35% solvent B was set until 50 min, followed by a linear gradient from 35 to 85% solvent B until 51 min, then held at 85% until 53 min, followed by a linear gradient from 85 to 5% solvent B until 53.5 min, followed by The isocratic gradient of 5% solvent B until 60 min (solvent A: 0.1% formic acid, solvent B: 100% acetonitrile and 0.1% formic acid). The analytical column temperature was set to 40 °C.

The LC was coupled online through an ESI ionisation source to a Synapt G2 mass spectrometer (Waters Corporation, Milford, MA, USA). Data acquired in positive ionisation mode using MassLynx version 4.2 software (Waters Corporation, Milford, MA, USA). LC-MS data collected in data-independent acquisition (DIA) mode MS<sup>E</sup>. The source/TOF conditions were set as follows: resolution mode, capillary voltage 2.6 kV, sampling cone voltage 40 V, extraction cone voltage 4 V, source temperature 120 °C, desolvation gas flow 800 L/h at 450 °C. During spectral data acquisition, the trap collision energy of the mass spectrometer was ramped from 18 to 35 eV for high-energy scans in MS<sup>E</sup> mode; for low-energy scans, collision energy was disabled. The mass range was set to 50–2000 Da with a scan time set to 0.5 s. A reference compound (2 ng/µL in 50% acetonitrile, 0.1% formic acid) leucine enkephalin (Waters Corporation, Milford, MA, USA) was co-infused continuously at a 7 µL/min flow rate and scanned every 45 s as a reference for accurate mass measurements (reference mass: m/z 556.2771).

The raw data files were processed for peptide and protein identification using ProteinLynx Global SERVER (PLGS) version 3.0.3 (Waters Corporation, Milford, MA, USA). The following parameters were used to generate peak lists: low and elevated energy thresholds, 135 and 20 counts, respectively; reference mass correction window, 0.25 Da at 556.2771 Da/e. Processed data analysed using the following parameters: trypsin was selected as the primary digest reagent, one missed cleavage was permitted, and carbamidomethylation of cysteines was set as a fixed modification. Deamidation of asparagine and glutamine, and oxidation of methionine were set as variable modifications. A reference database with 1,671,348 protein sequences, retrieved from UniProt using the *Paenibacillus* genus keyword, was used. The minimum identification criteria were two fragment ions per peptide, five fragment ions per protein, and at least one peptide per protein. The false discovery rate (FDR) was set to 2%.

### 2.2.4.3 Data-dependent LC-MS/MS

Digested peptides of *Rheinheimera* and *Cellulosimicrobium* were analysed using a data-dependent LC-MS/MS method. LC separation of trypsin-digested peptides was performed using Dionex Ultimate3000 RSLCnano (Dionex Softron GmbH, Germering, Germany). Peptides were concentrated on an Acclaim PepMap 100 5  $\mu\text{m}$ , 100  $\mu\text{m} \times 2 \text{ cm}$  (Thermo Fisher Scientific, Vilnius, Lithuania) trap column equilibrated with 2% acetonitrile with 0.1% trifluoroacetic acid at a 5  $\mu\text{L}/\text{min}$  flow rate. Afterwards, peptides were separated on an Acclaim PepMap RSLC 3  $\mu\text{m}$ , 75  $\mu\text{m} \times 15 \text{ cm}$  (Thermo Fisher Scientific, Vilnius, Lithuania) analytical column at a flow rate of 0.3  $\mu\text{L}/\text{min}$ . The isocratic elution 4% solvent B was set until 10 min, then a linear gradient from 4% to 45% solvent B was set until 70 min, followed by a linear gradient from 45 to 99% solvent B until 75 min, held at 99% until 85 min, from 99% to 4% solvent B until 86 min, isocratic 4% solvent B until 96 min (solvent A: 0.1% formic acid, solvent B: 80% acetonitrile and 0.1% formic acid). The column compartment temperature was set to 30  $^{\circ}\text{C}$ .

The LC system was coupled online to an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), which was equipped with a Nanospray Flex ionisation source. Prior to analysis, the instrument was calibrated using a Pierce LTQ ESI positive ion calibration solution (Thermo Fisher Scientific, Rockford, IL, USA). Data acquired using Chromeleon version 6.8 software (Dionex Corporation, Sunnyvale, CA, USA) and XCalibur version 2.1 software (Thermo Fisher Scientific, Bremen, Germany). LC-MS/MS data collected in data-dependent acquisition (DDA) mode for the six most intense ions over 85 minutes. Dynamic exclusion was set to 120 seconds, and the mass range for full-scan was set to 275–2000 Da. The Orbitrap detector resolution was 30,000, and the automatic gain control (AGC) was set to  $2 \times 10^5$  ions with a maximum injection time of 10 ms. A normalised collision energy of 35.0 was used for collision-induced dissociation (CID) fragmentation, and charge-state rejection of +1 was enabled. MS/MS spectra were acquired using an ion trap detector. The source conditions were set as follows: positive polarity, capillary voltage 1.77 kV, capillary temperature 200  $^{\circ}\text{C}$ . Peptide and protein identification was performed by processing Thermo.RAW files using PEAKS Studio version 8.5 software (Bioinformatics Solution, Waterloo, Canada).

The amino acid sequences of the *Cellulosimicrobium cellulans* reference proteome (UniProt ID: UP000319068) were validated using the PEAKS Studio tool, and 3,607 sequences were used to create a custom database. Translated amino acid sequences of *Rheinheimera* sp. GKU sequenced

genome (3,785 sequences) and rabbit glycogen phosphorylase (UniProt ID: P00489) were used to create a custom database. Mass error tolerances of 10 ppm and 0.5 Da were used for parent and fragment ions, respectively. Carbamidomethylation of cysteine was set as a fixed modification, whereas deamidation of asparagine and glutamine, oxidation of methionine and carbamylation of lysine or N-terminal were set as variable modifications. Trypsin was selected as the digestion enzyme, and non-specific cleavage was allowed at both ends of the peptide. The thresholds of  $-10\lg P > 15$  and  $-10\lg P > 20$  were selected for peptide and protein identification, respectively.

### 2.2.5 Cloning of target genes

Putative secreted protein lists for *Paenibacillus* sp. GKG, *Cellulosimicrobium* sp. GKH, and *Rheinheimera* sp. GKU were obtained after proteomic analysis (Chapter 2.2.4). For *Paenibacillus* sp. GKG, the resulting protein list was manually curated from UniProt database annotations to select only relevant enzymes. Lists of relevant CAZymes for *Cellulosimicrobium* sp. GKH and *Rheinheimera* sp. GKU were compiled using the identified protein list and the CAZyme selection dbCAN3 web tool (Zheng et al., 2023). Further, regardless of the bacterial protein fraction analysed, the CAZyme list was narrowed using CAZy database records as decision criteria. The selection criteria were YCW-inducibility, enzymatic activities typical of the putative glycosyl hydrolase family (endo-1,3- $\beta$ -glucanase and endo-1,6- $\beta$ -glucanase activities were preferred), and the existing number of representatives of *Paenibacillus*, *Rheinheimera*, or *Cellulosimicrobium* in the corresponding glycosyl hydrolase families.

Three *Rheinheimera* sp. GKU protein (GH3\_3456, GH16\_306, and GH16\_3455) encoding genes were cloned. Additionally, truncated versions of the GH16\_306 protein, GH16\_306\_CBM\_CBM, GH16\_306\_CBM\_1, and GH16\_306\_CBM\_2 (Fig. 3.5), were cloned. Genes coding proteins of interest (GH3\_3456, GH16\_306, GH16\_3455) were amplified from GKU genomic DNA using specific expression vector-compatible (pLATE31) primers (Appendix 1) and Phusion Plus PCR Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania). Obtained fragments were extracted from the gel using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Vilnius, Lithuania) and were cloned into pLATE31 (C-terminal His-tag). Cloning procedures were performed according to the manufacturer's recommendations (Thermo Fisher Scientific, Vilnius, Lithuania). Plasmids corresponding to full-length proteins were constructed – pLATE31\_GH3\_3456, pLATE31\_GH16\_306, pLATE31\_GH16\_3455.

Truncated variant of GH16\_306 harbouring C-terminal non-catalytic domains (GH16\_306\_CBM\_CBM protein) was cloned by amplifying the plasmid pLATE31\_GH16\_306 using primer pairs GH16\_306\_del3 and GH16\_306\_del4, respectively. pLATE31\_GH16\_306\_CBM\_CBM plasmid was constructed. To create plasmids containing one of the two “internal\_repeat\_1” domains (GH16\_306\_CBM\_1 and GH16\_306\_CBM\_2 proteins), primer pairs GH16\_306\_del2 and GH16\_306\_del5N or GH16\_306\_del4 and GH16\_306\_del6N (Appendix 1) were used to amplify the required fragments from the plasmid pLATE31\_GH16\_306\_CBM\_CBM. As a result, pLATE31\_GH16\_306\_CBM\_1 and pLATE31\_GH16\_306\_CBM\_2 plasmids were constructed. In all aforementioned cases, the obtained fragments were circularised using T4 DNA ligase. The selection and propagation of clones harbouring constructed plasmids was performed in *E. coli* DH5 $\alpha$ . Dr. Rūta Stanislauskienė carried out cloning of *Rheinheimera* sp. GKU proteins encoding genes.

Two *Paenibacillus* sp. GKG protein, PsGly30A and PsLam81A, encoding genes were cloned. Additionally, a truncated version PsLam81A $\Delta$ CBM56 of the PsLam81A protein was cloned as well. The gene sequence variants coding PsGly30A (full-length enzyme sequence with C-terminal 6 $\times$ His tag), PsLam81A (full-length enzyme sequence with C-terminal 6 $\times$ His tag) and PsLam81A $\Delta$ CBM56 (the truncated enzyme sequence lacking a CBM56 domain with a C-terminal 6 $\times$ His tag) were cloned from *Paenibacillus* sp. GKG genomic DNA using the Phusion Plus PCR Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania) and designed primers PsGly30A, PsLam81A and PsLam81A $\Delta$ CBM56 (Appendix 1). The PCR products were stained with ROTI Load DNA stain 1 SYBR Green (Carl-Roth, Karlsruhe, Germany) and ran in a 1% agarose gel electrophoresis. Bands corresponding to target gene sizes were excised from the gel, and the plasmid DNA was extracted from the gel slices using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Vilnius, Lithuania). The purified PCR products were cloned into a pLATE31 cloning vector using an aLICator LIC Cloning and Expression Kit 3 (C-terminal His-tag) (Thermo Fisher Scientific, Vilnius, Lithuania). *E. coli* strain DH5 $\alpha$  cells were transformed with the reaction mixtures and spread on LB agar plates supplemented with 75  $\mu$ g/mL ampicillin. pDNA was extracted and purified using a ZR Plasmid Miniprep kit (Zymo Research, Irvine, CA, USA). pDNA was sequenced using LIC sequencing primers (Appendix 1) by the Sanger method (Macrogen Europe, Amsterdam, The Netherlands or Genewiz Germany GmbH, Leipzig, Germany). Two additional sequencing primers, PsLam81A-mid (Appendix 1), were designed for the native PsLam81A enzyme-coding gene to obtain whole-gene sequence coverage.

Two *Cellulosimicrobium* protein, GH16 and GH64, encoding sequences were obtained as synthetic genes in pET24\_GH16 and pET24\_GH64 plasmids from Twist Bioscience (San Francisco, CA, USA)

## 2.2.6 Synthesis of the recombinant proteins

*Rheinheimera* sp. GKU proteins were synthesised using several different *E. coli* strains. The selected plasmids were transformed into *E. coli* protein expression strains: pLATE31\_GH16\_306 to KRX, pLATE31\_GH3\_3456 and pLATE31\_GH16\_3455 to SHuffle T7, while pLATE31\_GH16\_306\_CBM\_CBM, pLATE31\_GH16\_306\_CBM\_1, and pLATE31\_GH16\_306\_CBM\_2 to HMS174(DE3). Transformed cells were cultured in 50 mL LB medium supplemented with 100 µg/mL of ampicillin at 37 °C, 180 rpm until the optical density OD<sub>600nm</sub> reached 0.4–0.6, then IPTG was added to a final concentration of 0.5 mM. The bacteria were then grown overnight at 20 °C, 180 rpm for 19 h, except those transformed with pLATE31\_GH16\_3455, pLATE31\_GH16\_306\_CBM\_CBM, pLATE31\_GH16\_306\_CBM\_1 or pLATE31\_GH16\_306\_CBM\_2 plasmids, which were grown at 30 °C, 180 rpm for 19 hours. Subsequently, the cells were harvested by centrifugation at 3,200× g for 10 min at 4°C. Dr. Rūta Stanislauskienė carried out a synthesis of *Rheinheimera* sp. GKU proteins.

*Paenibacillus* sp. GKG proteins were synthesised using two different *E. coli* strains. The recombinant pLATE31\_PsLam81A and pLATE31\_PsLam81AΔCBM56 plasmids were transformed into *E. coli* strain BL21(DE3) cells. The transformed bacteria were cultured in 50 mL LB medium supplemented with 75 µg/mL ampicillin at 30 °C, 180 rpm until an OD<sub>600nm</sub> of 0.6–0.8 was reached. Expression of recombinant protein was induced with 0.5 mM IPTG by incubating at 20 °C, 180 rpm for 24 hours. The bacterial cells were harvested by centrifugation at 7,200× g for 30 min at 4 °C. *E. coli* strain LOBSTR cells were transformed with the recombinant pLATE31\_PsGly30A plasmid. The starter culture of the transformed bacteria was grown at 37 °C, 180 rpm for 4 hours in 20 mL of LB medium supplemented with 75 µg/mL ampicillin. Then 10 mL of the starter culture was transferred to 200 mL of LB medium supplemented with 75 µg/mL ampicillin and incubated at 30 °C, 180 rpm until it reached OD<sub>600nm</sub> 0.6–0.8. Expression of the recombinant protein was induced with 0.2 mM IPTG by incubation at 30 °C, 180 rpm for 22 hours. The bacterial cells were harvested by centrifugation at 3,200× g for 10 min at 4°C.

*Cellulosimicrobium* proteins were synthesised using two *E. coli* strains and plasmids harbouring synthetic genes. *E. coli* strain BL21(DE3) cells were

transformed with the pET24\_GH16 plasmid, and *E. coli* strain soluBL21 cells were transformed with the pET24\_GH64 plasmid. The starter culture of the transformed bacteria was grown at 37 °C, 180 rpm for 4 hours in 20 mL of LB medium supplemented with 75 µg/mL ampicillin. 10 mL of this culture were then transferred to 200 mL of LB medium containing 75 µg/mL ampicillin and grown at 30 °C, 180 rpm until an OD<sub>600nm</sub> of 0.6–0.8 was reached. Expression of the recombinant protein was induced with 0.2 mM IPTG for the *E. coli* strain BL21(DE3) and 0.5 mM IPTG for the *E. coli* soluBL21 strain. Recombinant protein synthesis was carried out at 30 °C, 180 rpm for 22 hours. The biomass was harvested by centrifugation at 3,200× g for 10 min at 4°C.

### 2.2.7 Purification of the recombinant proteins

The harvested cells were concentrated tenfold by resuspending the biomass in mobile phase A to a volume equal to one-tenth of that of the initial culture medium. The cells were then disrupted by sonication and centrifuged at 30,100× g for 30 minutes at 4 °C. The supernatants were loaded onto a 1 mL HiTrap Chelating HP column (GE Healthcare, Uppsala, Sweden).

GH3\_3456 protein was purified using a 20 mM Tris-HCl, 300 mM NaCl, 15 mM imidazole, 1 mM PMSF, pH 8.0 buffer as lysis buffer and mobile phase A, while 20 mM Tris-HCl, 300 mM NaCl, 500 mM imidazole, 1 mM PMSF, pH 8.0 buffer was used as mobile phase B. The column was pre-equilibrated with ten column volumes of mobile phase A. Cell-free extract was loaded onto the column, and the column was then washed with five column volumes of mobile phase A. GH3\_3456 protein eluted at 28 mM imidazole during multi-step elution. After purification, the GH3\_3456 protein was dialysed against a 20 mM Tris-HCl, pH 8.0 buffer.

GH16\_306 protein was purified under denaturing conditions. Initially, 50 mM Tris-HCl, 500 mM NaCl, 10% glycerol, 1 mM PMSF, pH 8.0, was used as the lysis buffer and mobile phase A; 50 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, 10% glycerol, 1 mM PMSF, pH 8.0 buffer was used as a mobile phase B. Based on the 3,5-dinitrosalicylic acid (DNS) assay, the majority of enzymatic activity remained in flow-through fractions. Subsequently, these fractions were supplemented with urea to yield a final concentration of 6 M urea. Mobile phases A and B were supplemented with 6 M urea as well. The column was pre-equilibrated with ten column volumes of mobile phase A. A sample was loaded onto the column and then the column was washed with three column volumes of mobile phase A, then washed with five column volumes of mobile phase containing 28 mM imidazole. GH16\_306 protein eluted at 28–500 mM imidazole gradient. After

purification, the GH16\_306 protein was dialysed against 50 mM Tris-HCl, 10% glycerol, pH 8.0 buffer.

The truncated variant GH16\_306\_CBM\_CBM of the GH16\_306 protein was purified using a 20 mM Tris-HCl, 300 mM NaCl, pH 8.0 buffer as lysis buffer and mobile phase A, while 20 mM Tris-HCl, 300 mM NaCl, 500 mM imidazole, pH 8.0 buffer was used as mobile phase B. The column was pre-equilibrated with ten column volumes of mobile phase A. Cell-free extract was loaded onto the column, and the column was then washed with five column volumes of mobile phase A. GH16\_306\_CBM\_CBM protein eluted at 28 mM imidazole during multi-step elution. After purification, the GH16\_306\_CBM\_CBM protein was dialysed against a 20 mM Tris-HCl, pH 8.0 buffer.

Truncated variants GH16\_306\_CBM\_1 and GH16\_306\_CBM\_2 of the GH16\_306 protein were purified using a 20 mM Tris-HCl, 300 mM NaCl, pH 8.0 buffer as lysis buffer. Purification was performed using HisPur™ Ni-NTA Spin Columns, 0.2 mL (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations. After purification, the GH16\_306\_CBM\_1 and GH16\_306\_CBM\_2 proteins were desalted using Zeba™ Spin Desalting Columns, 7K MWCO (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's recommendations. Dr. Rūta Stanislauskienė carried out the purification of the GH16\_306\_CBM\_1 and GH16\_306\_CBM\_2 proteins.

GH16\_3455 protein was purified using a 20 mM Tris-HCl, 300 mM NaCl, 1 mM PMSF, 10% glycerol, pH 8.0 buffer as lysis buffer and mobile phase A, while 20 mM Tris-HCl, 300 mM NaCl, 500 mM imidazole, 1 mM PMSF, 10% glycerol, pH 8.0 buffer was used as mobile phase B. The column was pre-equilibrated with ten column volumes of mobile phase A. Cell-free extract was loaded onto the column, and the column was then washed with five column volumes of mobile phase A. GH16\_3455 protein eluted at 28–500 mM imidazole gradient. After purification, the GH16\_3455 protein was dialysed against a 20 mM Tris-HCl, pH 8.0 buffer.

For PsLam81A and PsLam81AΔCBM56 proteins, the column was pre-equilibrated with ten column volumes of mobile phase A consisting of 1 × PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>), pH 7.0. Cell-free protein extracts were loaded onto the column. Then the column was washed with five column volumes of 28 mM imidazole in 1 × PBS, pH 7.0. The recombinant protein was eluted with 400 mM imidazole in 1 × PBS, pH 7.0. The collected recombinant protein fraction was desalted using a 5 mL Sephadex G25 column (GE Healthcare, Uppsala, Sweden) with 1 × PBS, pH 7.0.

For the PsGly30A protein, the column was pre-equilibrated with ten column volumes of 1 × PBS, pH 7.0, containing 15 mM imidazole, supernatant was supplemented with 15 mM imidazole as well. The column was washed with five column volumes of 1× PBS, pH 7.0 containing 15 mM imidazole. The recombinant protein was eluted by gradient elution from 15 to 250 mM imidazole in 1× PBS, pH 7.0, with a gradient length of 12 column volumes. The collected recombinant protein fraction was desalted on a 2×5 mL Sephadex G25 column (GE Healthcare, Uppsala, Sweden) using 50 mM MES, pH 6.0 buffer. The desalted fractions were pooled together and loaded onto 1 mL HiTrap SP XL column (GE Healthcare, Uppsala, Sweden) that had been preequilibrated with ten column volumes of 50mM MES, pH 6.0. The column was washed with five column volumes of 50 mM MES, pH 6.0. The recombinant protein was eluted with 500 mM NaCl in 50 mM MES, pH 6.0. The collected recombinant protein fraction was desalted on a 2×5 mL Sephadex G25 column (GE Healthcare, Uppsala, Sweden) using 1× PBS, pH 7.0 buffer.

After synthesis of GH16 and GH64 enzymes from *Cellulosimicrobium* in 200 mL of culture medium, bacterial cells were harvested by centrifugation at 3,200× g for 10 min at 4°C. The harvested cells were resuspended in 20 mL of a buffer containing 50 mM Tris-HCl, 500 mM NaCl, 15 mM imidazole, 10% glycerol, pH 8.0. Cells were disrupted by sonication and then centrifuged at 30,100× g for 30 min at 4°C. The supernatant was loaded onto a 1 mL HiTrap Chelating HP column (GE Healthcare, Uppsala, Sweden) that had been pre-equilibrated with ten column volumes of 50 mM Tris-HCl, 500 mM NaCl, 15 mM imidazole, 10% glycerol, pH 8.0. The column was then washed with five column volumes of 50 mM Tris-HCl, 500 mM NaCl, 15 mM imidazole, 10% glycerol, pH 8.0. Multi-step elution was then employed, with weakly bound proteins being eluted by raising the imidazole concentration to 28 mM. The target proteins were eluted by further increasing the imidazole concentration to 500 mM. The collected recombinant protein fractions were dialysed against a buffer containing 20 mM Tris-HCl, 10% glycerol, pH 8.0.

The molecular weight of the recombinant proteins was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Prestained Protein Ladder (10–170 kDa) (Thermo Fisher Scientific, Vilnius, Lithuania) or PageRuler™ Plus Prestained (11-250 kDa) (Fermentas, Vilnius, Lithuania) and 12% separating and 4% stacking gels. Protein bands were visualised using Coomassie Brilliant Blue G-250 (Fluka, Buchs, Switzerland) staining. The protein concentration was estimated using the Pierce Coomassie (Bradford) Protein Assay Kit (Thermo Fisher, Rockford, IL, USA).

## 2.2.8 Determination of substrate specificity

### 2.2.8.1 Substrate specificity of PsLam81A

The substrate specificity of PsLam81A and PsLam81A $\Delta$ CBM56 enzymes was determined using various polysaccharides: laminarin, yeast  $\beta$ -glucan, curdlan, pachyman and barley  $\beta$ -glucan (Appendix 2). Reaction mixtures (100  $\mu$ L) contained 25  $\mu$ g/mL of the enzyme and 0.9% (w/v) of the polysaccharide substrate in a 50 mM sodium phosphate buffer at pH 7.0. The mixtures were incubated at 40  $^{\circ}$ C, 450 rpm for 18 hours. In control reactions, the enzymes were absent.

The modes of action of PsLam81A and PsLam81A $\Delta$ CBM56 were determined using laminari-oligosaccharides (laminaribiose, laminaritriose, laminaritetraose, laminaripentaose and laminarihexaose (Appendix 2) as substrates. Reaction mixtures (50  $\mu$ L) contained 25  $\mu$ g/mL of the enzyme and 0.9% (w/v) laminari-oligosaccharide substrate in 50 mM sodium phosphate buffer at pH 7.0, and were incubated at 40  $^{\circ}$ C for 2 hours. In control reactions, the enzymes were absent. The hydrolysis reaction was terminated by transferring the samples to 95  $^{\circ}$ C for 10 minutes. No temperature or chemical pre-treatment was applied to the laminari-oligosaccharides or polysaccharides prior to the hydrolysis reaction. The substrates were dissolved or suspended in the appropriate buffers by mixing at 1000 rpm at 22 $^{\circ}$ C for 30 minutes. Activity units were determined as described in Method 2.2.11.

### 2.2.8.2 Substrate specificity of PsGly30A

The mode of action of PsGly30A was determined using gentiobiose, pustulan and laminarin as substrates (Appendix 2). Reaction mixtures containing 25  $\mu$ g/mL PsGly30A and 0.9% (w/v) of the substrates in 50 mM sodium citrate buffer at pH 5.5 were incubated at 40  $^{\circ}$ C with shaking at 450 rpm for 18 hours. Control reactions were performed in the absence of PsGly30A.

The substrate specificity and activity of PsGly30A were determined using various polysaccharides, including pustulan, yeast  $\beta$ -glucan, YCW, laminarin, curdlan, barley  $\beta$ -glucan, microcrystalline cellulose, carboxymethyl cellulose, and  $\beta$ -1,2-glucan. The reaction mixtures (100  $\mu$ L) contained either 0.5  $\mu$ g/mL of PsGly30A for pustulan and yeast  $\beta$ -glucan, or 2.5  $\mu$ g/mL of PsGly30A for YCW and all the other polysaccharides. All reaction mixtures contained 0.9% (w/v) of the polysaccharide substrate in 50 mM sodium citrate buffer at pH 5.5, and were incubated at 50  $^{\circ}$ C, 450 rpm for 15 minutes. Control reactions were performed in the absence of PsGly30A. Hydrothermally untreated substrates were dissolved or suspended in 50 mM sodium citrate buffer, pH

5.5 by mixing at 22 °C, 1000 rpm for 30 minutes. The hydrolysis reaction was terminated by transferring the samples to 95 °C for 10 minutes. Polysaccharides were hydrothermally treated prior to the hydrolysis reaction in order to assess the effect of pretreatment on hydrolysis efficiency. The hydrothermally treated substrates were dissolved or suspended in 50 mM sodium citrate buffer at pH 5.5, then incubated in a heated thermostat at 100 °C for 30 min with occasional vortexing. PsGly30A activity units were determined as described in Method 2.2.11.

The substrate specificity and specific activity of PsGly30A for *p*NP-glycosides were assessed by determining the amount of *p*NP released during hydrolysis reactions. Reaction mixtures (100  $\mu$ L) contained 4 mM of one of the pro-chromogenic substrates: *p*NP- $\alpha$ -glucoside, *p*NP- $\beta$ -glucoside, *p*NP- $\beta$ -gentiobioside (*p*NPG2) (Appendix 2) in 50 mM sodium citrate buffer pH 5.5 and 25  $\mu$ g/mL PsGly30A. Mixtures were prepared directly into microplate wells, vortexed and incubated at 37°C for 30 min in a microplate reader. After 30 min, the absorbance was measured at 405 nm. PsGly30A was not present in the control reactions. One unit of endo-1,6- $\beta$ -glucanase activity is determined as the amount of PsGly30A required to release 1  $\mu$ mol of *p*NP from the substrate per minute. Measurements were performed at least in triplicate and expressed as mean  $\pm$  standard deviation (SD).

#### 2.2.8.3 Substrate specificity of GH3\_3456, GH16\_306, and GH16\_3455

Reaction mixtures (50  $\mu$ L) contained 40  $\mu$ L of carbohydrate substrate in 50 mM sodium phosphate buffer, pH 6.5, at 5–20 mg/mL concentrations, and 10  $\mu$ L of purified GH3\_3456, GH16\_306, or GH16\_3455 enzymes at 0.5 mg/mL concentrations. For the synergistic action of the three enzymes, each enzyme was used at a concentration of 0.16 mg/mL. Reaction mixtures were incubated at 30 °C, 500 rpm for 18 h in a thermomixer. Control reactions contained 10  $\mu$ L of 20 mM Tris-HCl, 10% glycerol, pH 8.0, instead of enzymes.

No temperature or chemical pre-treatment was applied to carbohydrate substrates. The substrates were dissolved or suspended in 50 mM sodium phosphate buffer, pH 6.5, by mixing at 22 °C, 1000 rpm for 15 minutes. The hydrolysis reaction was terminated by transferring the samples to 95 °C for 10 minutes. YCW substrate was used at 20 mg/mL concentration; laminarin, yeast  $\beta$ -glucan, curdlan, pustulan, pachyman, barley  $\beta$ -glucan, microcrystalline cellulose, carboxymethyl cellulose, and  $\beta$ -1,2-glucan were used at 10 mg/mL concentration; laminaribiose, laminaritetraose, gentiobiose, and cellobiose were used at 5 mg/mL concentration. The profiles of hydrolysis

products were evaluated by thin-layer chromatography (TLC) (Method 2.2.10).

#### 2.2.8.4 Substrate specificity of GH16, GH64

The endo- $\beta$ -1,3-glucanase enzymatic activity of GH16 and GH64, as well as the commercial preparation *Zymolyase-20T* (Nacalai Tesque, Kyoto, Japan), further denoted as *Zymolyase*, was confirmed using laminarin and yeast  $\beta$ -glucan as substrates. Reaction mixtures (100  $\mu$ L) contained either 50  $\mu$ g/mL of GH16 or GH64 enzymes or 500  $\mu$ g/mL of *Zymolyase* and 0.9% (w/v) of polysaccharide substrate in a 50 mM sodium phosphate buffer, pH 6.5. The reaction mixtures were incubated at 30 °C, 450 rpm for 18 hours. During control reactions, the enzymes were absent. No temperature or chemical pre-treatment was applied to the polysaccharides prior to hydrolysis. The substrates were dissolved or suspended in the buffer by mixing at 22 °C, 1000 rpm for 30 minutes. The hydrolysis reaction was terminated after 18 hours by transferring the samples to 95 °C for 10 minutes.

#### 2.2.9 Optimisation of enzymatic reaction conditions

##### 2.2.9.1 Enzymatic reaction conditions for PsLam81A

The enzymatic activity of the recombinant PsLam81A enzyme was determined using laminarin as the substrate. The optimal pH was determined by measuring the activity at pH 4.0–10.0, at 40 °C, 450 rpm for 15 minutes. The reaction mixture (100  $\mu$ L) contained 10  $\mu$ g/mL of the enzyme and 0.9% (w/v) laminarin in 50 mM of various buffers: sodium citrate buffer (pH 3.0–6.5), sodium phosphate buffer (pH 6.5–8.0), Tris-HCl buffer (pH 8.0–9.0) and glycine-NaOH buffer (pH 9.0–10.0).

The effect of temperature on PsLam81A and PsLam81A $\Delta$ CBM56 enzymes' activities was determined in a reaction mixture (100  $\mu$ L) containing 5  $\mu$ g/mL of the enzyme and 0.9% (w/v) laminarin in 50 mM sodium phosphate buffer at pH 7.0, at different temperatures ranging from 10 °C to 80 °C, 450 rpm for 15 minutes.

The effect of temperature on the stability of PsLam81A was determined by incubating the enzyme in 1  $\times$  PBS pH 7.0 at temperatures ranging from 4 °C to 60 °C for 15 or 60 minutes. After incubation, the activity was determined in a reaction mixture (100  $\mu$ L) containing 10  $\mu$ g/mL of the enzyme and 0.9% (w/v) laminarin in 50 mM sodium phosphate buffer at pH 7.0, which was then incubated at 40 °C for 15 minutes. Activity units were determined as described in Method 2.2.11.

### 2.2.9.2 Enzymatic reaction conditions for PsGly30A

The enzymatic activity of the recombinant PsGly30A was determined using pustulan as the substrate. The optimum pH was determined by measuring the activity at pH values ranging from 4.0 to 10.0 at 50 °C, 450 rpm for 15 minutes. The reaction mixture (100 µL) contained 0.5 µg/mL of the enzyme and 0.9% pustulan (w/v) in various buffers: sodium citrate buffer (pH 3.0–6.5), MES buffer (pH 5.5–6.5), sodium phosphate buffer (pH 6.5–8.0), Tris-HCl buffer (pH 8.0–9.0), BICINE buffer (pH 8.0–9.0) and glycine-NaOH buffer (pH 9.0–10.0).

The effect of temperature on PsGly30A activity was determined in a reaction mixture containing 0.5 µg/mL of enzyme and 0.9% (w/v) pustulan in 50 mM sodium citrate buffer at pH 5.5, incubated at temperatures ranging from 4 °C to 80 °C, 450 rpm for 15 minutes.

The effect of temperature on PsGly30A stability was determined by incubating the enzyme in 1× PBS (pH 7.0) at temperatures ranging from 4 °C to 60 °C, 450 rpm for 15 or 60 minutes. After incubation, enzyme activity was determined in a reaction mixture (100 µL) containing 0.5 µg/mL enzyme and 0.9% pustulan (w/v) in 50 mM sodium citrate buffer (pH 5.5), incubated at 30 °C, 450 rpm for 15 minutes. Activity units were determined as described in Method 2.2.11.

### 2.2.10 TLC analysis of hydrolysis products

Samples collected at different times during the hydrolysis reaction were immediately boiled for 10 min, then analysed by thin-layer chromatography (TLC). TLC was performed as described (Qin et al., 2016), with slight modifications. Briefly, the samples were spotted on TLC Aluminium Sheets coated with silica gel matrix and developed in a mixture of butan-1-ol/acetic acid/water (2:1:1, v/v). The TLC results were visualised using *p*-anisaldehyde. The developed silica plates were dipped into a dye solution (*p*-anisaldehyde/ 99.8% acetic acid / 96% ethanol / 96% sulfuric acid 0.54:0.54:50:1.9 v/v/v/v) for a few seconds. They were then heated above the heating plate for a few minutes until the spots corresponding to the hydrolysis products appeared.

### 2.2.11 DNS-based enzymatic activity assay

The activity of recombinant GH enzymes was determined using the 3,5-dinitrosalicylic acid (DNS) method, by measuring the amount of reducing sugars released during the hydrolysis of polysaccharides, as previously described (Wood et al., 2012) with modifications. The DNS reagent contained

10 g/L DNS, 300 g/L sodium potassium tartrate, and 16 g/L NaOH. The hydrolysis reaction was terminated by transferring the tubes immediately to a boiling water bath for 5 minutes. After the hydrolysis reaction, the sample was centrifuged at  $16,000\times g$  for 2 min, and then the sample was mixed with the DNS reagent at a sample-to-DNS reagent ratio (1:5, v/v). The mixture was then heated for 5 min in a boiling water bath, cooled for 5 min in an ice bath and left at 22 °C for 5 min to allow for the sample temperature to equilibrate. Then, 100  $\mu$ L of the reaction mixture was transferred to a round-bottom microplate, and the absorbance at 540 nm was measured. One unit of endo-1,3- $\beta$ -glucanase or endo-1,6- $\beta$ -glucanase activity is defined as the amount of enzyme required to produce the amount of reducing sugar equivalent to 1  $\mu$ mol of glucose per minute of reaction time. Measurements were performed at least in triplicate, and the results are expressed as the mean  $\pm$  standard deviation (SD).

#### 2.2.12 Quantitative analysis of YCW conversion yield

Reaction mixtures containing 25  $\mu$ g/mL PsGly30A and 0.9% (w/v) of the substrates (YCW, yeast  $\beta$ -glucan, and pustulan) in 50 mM MES buffer at pH 5.5 were incubated at 40 °C with shaking at 450 rpm for 18 hours. Control reactions were performed in the absence of PsGly30A. For investigation of PsGly30A enzyme hydrolysis reaction products, heat-inactivated hydrolysates were analysed using a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an Aminex HPX-87 H (300  $\times$  7.8 mm) column (Bio-Rad, Hercules, CA, USA) and a RefractoMax 521 refractive index (RI) detector (ERC Inc., Saitama, Japan). HPLC analysis was performed at 60 °C using aqueous 5 mM H<sub>2</sub>SO<sub>4</sub> as the mobile phase at a flow rate of 0.4 mL/min. The injection volume was set to 20  $\mu$ L. Glucose and gentiobiose were used as standards. Prior to HPLC analysis, the supernatants were filtered through 0.2  $\mu$ m GHP membrane 13 mm diameter syringe filters (Pall Corporation, New York, NY, USA).

YCW for quantitative measurements was prepared by autoclaving 20 mg/mL of YCW in a 50 mM sodium phosphate buffer, pH 7.0. The resulting suspension (180  $\mu$ L) was mixed with 20  $\mu$ L of enzyme solution (0.3 mg/mL of PsGly30A or 1 mg/mL of GH64, or 0.8 mg/mL of PsLam81A). Enzymes were absent in control reactions. Mixtures were incubated at 40 °C, 450 rpm for 18 hours. For quantitative evaluation of laminaribiose, laminaripentaose, and gentiobiose concentrations in YCW hydrolysates, a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) was equipped with two consequently connected Aminex HPX-

42A (300 mm × 7.8 mm) columns (Bio-Rad, Hercules, CA, USA) and a de-ashing cartridge. The HPLC system was coupled with a RID G1362A detector (Agilent Technologies, Santa Clara, CA, USA). HPLC analysis was performed at 55°C using *MilliQ* water as the mobile phase at a flow rate of 0.4 mL/min. The injection volume was set to 5 µL. Laminaribiose, laminaripentaose, and gentiobiose were used as standards to construct calibration curves. Prior to HPLC analysis, the supernatants were filtered through 0.2 µm GHP membrane 13 mm diameter syringe filters (Pall Corporation, New York, NY, USA). In addition, to obtain quantitative conversion yields, the  $\beta$ -glucan content in the YCW sample was determined using a  $\beta$ -glucan assay kit (Yeast and Mushroom) (Megazyme, Wicklow, Ireland). The content of dissolved solids (DS) was determined using a moisture analyser MX-50 (A&D, Tokyo, Japan). All experiments described in Chapter 2.2.12, except for hydrolysate preparations, were carried out by Jurgita Kailiuvienė.

#### 2.2.13 *p*NP- $\beta$ -gentiobioside-based enzymatic activity assay

For the residual activity measurements, the YCW hydrolysate contained 9 mg/mL of autoclaved YCW (1 atm, 121 °C, 30 min) and 25 µg/mL PsGly30A. In the control reaction, PsGly30A was not present. The reaction conditions for *p*NP- $\beta$ -gentiobioside (*p*NPG2) hydrolysis were adjusted to minimise substrate consumption and maximise colourimetric signal intensity. Reaction mixtures (40 µL) were prepared by mixing 20 µL of 8 mM *p*NPG2 in 50 mM sodium citrate buffer (pH 5.5) with 20 µL of YCW hydrolysate collected at 0–24 h time points and containing 25 µg/mL PsGly30A. For the negative control experiment, the YCW hydrolysate containing 25 µg/mL PsGly30A was heated at 80 °C for 10 minutes. For the positive control experiment, 25 µg/mL of PsGly30A in 50 mM sodium citrate buffer at pH 5.5 was used. The reaction mixtures were prepared directly in a 384-well microplate, shaken, and incubated at 37 °C for 30 minutes. After 30 minutes, 4.4 µL of 1:1 v/v 1.0 M NaOH and 1.0 M glycine-NaOH were added. After shaking, absorbance at 405 nm was measured.

#### 2.2.14 Pull-down assay for protein-carbohydrate interaction

The protein-carbohydrate interaction was evaluated using a method described earlier (Hao et al., 2024), with modifications. 90 µL of a 10 mg/mL water-insoluble polysaccharide (yeast  $\beta$ -glucan, curdlan, pachyman, and pustulan) suspension in 50 mM sodium phosphate buffer (pH 6.5) was mixed

with 10  $\mu\text{L}$  of a 0.85 mg/mL GH16\_306\_CBM\_CBM, GH16\_306\_CBM1 or GH16\_306\_CBM\_2 protein solution. The mixture was incubated at 22 °C for 2 hours. Centrifugal tubes were placed on a rocker shaker and were left to gently swing at 40 rpm. After incubation, samples were centrifuged at 16,000 $\times g$  for 10 minutes. An aliquot of supernatant (20  $\mu\text{L}$ ) was transferred to a new tube without disturbing the pellet. Supernatants were analysed by SDS-PAGE. Dr. Rūta Stanislauskienė carried out pull-down assays with GH16\_306\_CBM\_1 and GH16\_306\_CBM\_2 proteins.

In order to check protein-carbohydrate interaction with water-soluble sugars, oligosaccharides or polysaccharides, pull-down assay inhibition was performed. 45  $\mu\text{L}$  of a 20 mg/mL yeast  $\beta$ -glucan suspension in 50 mM sodium phosphate buffer (pH 6.5), 45  $\mu\text{L}$  of a 20 mg/mL water-soluble carbohydrate (glucose, laminaribiose, laminaritetraose, laminarihexaose, laminarin, or gentiobiose) were mixed with 10  $\mu\text{L}$  of a 0.85 mg/mL GH16\_306\_CBM\_CBM protein solution. The mixture was incubated at 22 °C for 2 hours. Centrifugal tubes were placed on a rocker shaker and were left to gently swing at 40 rpm. After incubation, samples were centrifuged at 16,000 $\times g$  for 10 minutes. An aliquot of supernatant (20  $\mu\text{L}$ ) was transferred to a new tube without disturbing the pellet. Supernatants were analysed by SDS-PAGE.

## 2.2.15 Yeast cell lysis

A single colony of *S. cerevisiae* BY4741 strain (*MATa*, *his3 $\Delta$ 1*, *leu2 $\Delta$ 0*, *met15 $\Delta$ 0*, *ura3 $\Delta$ 0*) yeast or yeast of other genera (Appendix 17) grown on a solid YPD medium plate was transferred to 5 mL of liquid YPD medium and incubated at 30 °C (*S. cerevisiae*) or 25 °C (other species), 180 rpm for 18 hours. The OD<sub>600nm</sub> of the inoculum was measured, and an appropriate volume was transferred to 100 mL of YPD medium to achieve an OD<sub>600nm</sub> of 0.1. The cultures were then incubated for 4–7 hours 30 °C (*S. cerevisiae*) or 25 °C (other species), 180 rpm for 18 hours, until an OD<sub>600nm</sub> of 1.0 was reached.

The biomass was collected using 50 mL centrifugal tubes by centrifugation at 3,000 $\times g$  and 4 °C for five minutes. The collected biomass was then washed twice with 100 mL of sterile deionised water under the same centrifugation conditions. During collection and washing, the biomass was stored in an ice bath. Lysis mixtures were prepared as described further, depending on the lysis-enhancing additive ( $\beta$ -ME or glycerol).

For measurements using a  $\beta$ -ME concentration of 0.5–50 mM, the yeast biomass was suspended in sterile deionised water to yield an OD<sub>600nm</sub> of approximately 6.0. Firstly, pre-mixed yeast suspensions were prepared. For the control reactions, a yeast lysis suspension was prepared by mixing 550  $\mu\text{L}$

of yeast suspension with 110  $\mu$ L of 500 mM sodium phosphate buffer, pH 7.5. For the lysis reactions, 550  $\mu$ L of yeast suspension, 110  $\mu$ L of 500 mM sodium phosphate buffer, and 110  $\mu$ L of 5–500 mM  $\beta$ -ME stock solution were mixed. Control and lysis reaction mixtures were prepared directly in microplate wells by pipetting the necessary amounts of components. Control reactions without  $\beta$ -ME consisted of 60  $\mu$ L of pre-mixed yeast lysis suspension, and 0, 10, or 20  $\mu$ L aliquots of the enzymatic preparation. Deionised water was then added to make up the total volume to 100  $\mu$ L. Lysis reactions containing  $\beta$ -ME consisted of 70  $\mu$ L of pre-mixed yeast lysis suspension supplemented with the necessary amount of  $\beta$ -ME, and 0, 10, or 20  $\mu$ L of enzymatic preparation. Deionised water was added to give a final volume of 100  $\mu$ L.

For measurements involving osmotic shock with 5% glycerol, the yeast biomass was suspended in a buffer containing 71.4 mM sodium phosphate, 4.3% glycerol (w/v), pH 7.5, yielding an OD<sub>600nm</sub> of approximately 4.3. Lysis reaction mixtures were prepared directly in microplate wells by adding necessary amounts of solvents. Each reaction consisted of 70  $\mu$ L of yeast lysis suspension, 0, 10, or 20  $\mu$ L aliquots of enzymatic preparation, 0, 10, or 20  $\mu$ L aliquots of enzyme buffer (20 mM Tris-HCl, 10% glycerol (w/v), pH 8), and 0 or 10  $\mu$ L of deionised water, resulting in a total volume of 100  $\mu$ L. These volumes were adjusted to achieve a final glycerol concentration of 5% in all lysis mixtures, depending on the enzymatic preparation used, as each preparation contained different amounts of glycerol. Control reactions did not contain enzymes.

Four different enzymatic preparations were used for the lysis reactions – 10 mg/mL of *Zymolyase* in a 50 mM sodium phosphate buffer, pH 7.5; 1 mg/mL of GH16 enzyme in 20 mM Tris-HCl 10% glycerol (w/v), pH 8, 1 mg/mL of GH64 enzyme in 20 mM Tris-HCl 10% glycerol (w/v), pH 8, a combination of 1 mg/mL of GH16 and GH64 enzymes.

The OD<sub>600nm</sub> was measured using flat-bottomed, clear 96-well microplates. Measurements were taken at one-minute intervals for 60 minutes. Continuous shaking was implemented in between OD<sub>600nm</sub> readings. Depending on the yeast strain, the initial OD<sub>600nm</sub> values range from 0.5 to 0.8 for a 100  $\mu$ L volume of lysis mixture. Dr. Ramunė Stanevičienė conducted a screening of different strains at various concentrations of  $\beta$ -ME and 5% glycerol.

#### 2.2.16 Nucleotide, amino acid sequence accession numbers

The *Paenibacillus* sp. GKG family GH81 endo- $\beta$ -1,3-glucanase (PsLam81A) gene coding sequence has been deposited in GenBank under accession no. OP093985. The nucleotide sequence coding GH30 endo- $\beta$ -1,3-

glucanase (PsGly30A) has been deposited in the GenBank database under the accession number OR933702. GH3\_3456, GH16\_306, and GH16\_3455 protein sequences from *Rheinheimera*, as well as GH16 and GH64 protein sequences from *Cellulosimicrobium* are presented in the Appendix 10.

The *Paenibacillus* sp. GKG 16S rRNA gene sequence was deposited in GenBank under accession no. OP081028.C. 16S rRNA gene sequences of *Rheinheimera* sp. GKG and *Cellulosimicrobium* sp. GKH are presented in Appendix 4.

The LC-MS proteomics data of *Paenibacillus* sp. GKG secreted protein fraction has been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD036565. Proteomic analysis data of *Rheinheimera* sp. GKG secreted, periplasmic and intercellular protein fractions, as well as *Cellulosimicrobium* sp. GKH secreted protein fractions are presented in Appendix 5, and Appendix 6, respectively.

#### 2.2.17 Computer software, tools and servers

For DIA mode, LC-MS/MS data acquired using MassLynx v4.2 (Waters Corporation, Milford, MA, USA), and analysis was performed using ProteinLynx Global Server v3.0.3 (Waters Corporation, Milford, MA, USA) software. For DDA mode, LC-MS/MS data recorded using Chromeleon v6.8 (Dionex Corporation, Sunnyvale, CA, USA) and XCalibur v2.1 software (Thermo Fisher Scientific, Bremen, Germany), while data analysis was performed using PEAKS Studio v8.5 software (Bioinformatics Solution, Waterloo, Canada).

Phylogenetic analysis was performed using MEGA11 software (Tamura et al., 2021). For the identification of putative CAZymes, proteomics data refined with the dbCAN3 web tool (Zheng et al., 2023). Protein sequence similarities were determined using the ClustalW web tool (<https://www.ebi.ac.uk/jdispatcher/msa/clustalo>), and protein domain structures were determined using the SMART server (<http://smart.embl-heidelberg.de/>) (Letunic et al., 2021) or InterPro web tool (<https://www.ebi.ac.uk/interpro/>).

The figures were created using Sigma Plot v15.0, ChemSketch v2025.1.0, MS Office v2062 build 16.0., and webtools BioRender, and DrawGlycan-SNFG. The language was edited using DeepL Write and Grammarly tools. The references were formatted using Zotero v8.0.4.

### 3. RESULTS AND DISCUSSION

This work explores solutions to two existing problems. The first is the low added value of current applications of the YCW fraction (Chapter 1.2.1). The second is the low specificity of enzymatic yeast lysis mixtures, which target not only YCW polymers but also intracellular compounds (Chapter 1.5.1). The work focuses on two objects: YCW and bacterial enzymes that can hydrolyse YCW polysaccharides. The YCW serves as a substrate, while bacterial enzymes act as tools to hydrolyse the YCW polysaccharide, yielding higher-added-value products. The importance of YCW as an industrial by-product is discussed in Chapter 1.1.3. Whereas the bacterial enzymes capable of hydrolysing YCW polysaccharides are reviewed in Chapters 1.4 and 1.5.

In the context of the first problem, YCW is being investigated as a potential substrate for producing higher-value oligosaccharides through treatment with bacterial enzymes. Regarding the second problem, bacterial enzymes are being explored for their ability to hydrolyse YCW with higher specificity than lytic enzyme mixtures, but to the extent that they would cause lysis of living yeast cells without the use of proteases or reducing-agent additives.

The results are presented in the following order. Firstly, the YCW-degrading bacteria isolated for their ability to utilise YCW as a carbon source and to solubilise the water-insoluble YCW substrate are presented. Secondly, the enzymes responsible for YCW hydrolysis identified using LC-MS/MS proteomics are presented. Thirdly, the synthesis and investigation of these enzymes' properties are described. Finally, the potential applications of selected GHs in converting YCW into higher-value oligosaccharides and lysing living yeast cells are demonstrated.

#### 3.1 Identification of YCW-degrading CAZymes

The objective of the study is to convert YCW into functional and bioactive oligosaccharides and to lyse living yeast cells by an enzymatic hydrolysis. To achieve this objective, it is necessary to employ enzymes that are capable of efficiently hydrolysing YCW polysaccharides. Such enzymes can access a major part of glycosidic bonds in the YCW  $\beta$ -glucan network, ensuring an optimal yield when converting YCW to oligosaccharides, and can also lyse living yeast cells. As outlined in Chapter 1.5.1, the former reason is of particular significance, given that only a limited number of characterised YCW-degrading GHs possess the capability to lyse living yeast cells. Various soil and water samples were collected from the environment and screened for YCW-degrading bacteria using YCW as a carbon source. Two strategies

commonly used for novel enzyme discovery were evaluated during preliminary trials: the construction of metagenomic libraries (Prayogo et al., 2020) and the proteomic analysis of secretome fractions from YCW-degrading bacteria (Wakarchuk et al., 2016). The latter strategy was found to be more effective and was therefore employed in this study. This approach led to the selection of *Paenibacillus* sp. GKG, *Rheinheimera* sp. GKH and *Cellulosimicrobium* sp. GKH as the most promising YCW-degrading bacteria.

Additionally, literature analysis revealed that *Cellulosimicrobium* bacteria have been the subject of numerous studies on yeast lytic enzyme mixtures. The secretomes of these bacteria are used to produce yeast lytic enzyme mixtures, such as *Zymolyase* (Brettrager et al., 2024). Based on previous reports, we have decided to focus on the activities of the sole endo-1,3- $\beta$ -glucanases in *Cellulosimicrobium* sp. GKH rather than on the application of crude secretome fractions.

### 3.1.1 Screening for YCW-degrading bacteria

Three different bacteria, *Rheinheimera* sp. GKH, *Paenibacillus* sp. GKG, and *Cellulosimicrobium* sp. GKH were isolated from soil, pond water, and YCW samples. Initial selection of colonies was based on the formation of a hydrolysis zone around a single colony or several colonies. The strains were then purified by repeated streaking and monitoring the ability to produce clear hydrolysis zones when cultivated on a solid mineral medium supplemented with YCWs as a carbon source (Fig. 3.1, 3.2, 3.3). The presence of a hydrolysis zone indicated that YCW hydrolysis was taking place as water-insoluble YCW was converted to water-soluble YCW polymers.

The bacterium, which was later named *Rheinheimera* sp. GKH was isolated from a soil sample based on its ability to produce hydrolysis zones on an agar plate containing insoluble YCW substrate (Fig. 3.1). The isolate was identified using 16S rRNA gene sequence analysis, which showed the highest identity (98%) with *Rheinheimera pacifica* strain SJOD-R-25 16S rRNA gene sequence (GenBank accession no. MK955341).

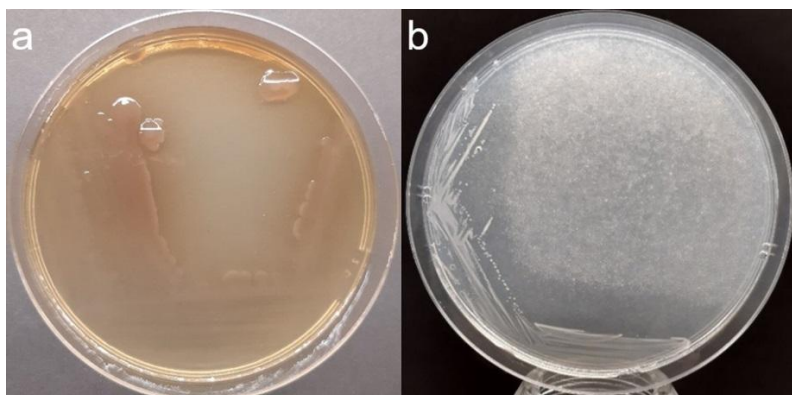


**Figure 3.1.** Isolated *Rheinheimeria* sp. GKU bacterium. a – solid LB medium, b – solid mineral medium containing water-insoluble YCW substrate as a carbon source. A clear hydrolysis zone around colonies shows the presence of extracellular YCW-degrading CAZymes.

The bacterium, which was later named *Paenibacillus* sp. GKG was isolated from a pond water sample based on its ability to produce hydrolysis zones on an agar plate containing an insoluble YCW substrate (Fig. 3.2). The isolate was identified using 16S rRNA gene sequence analysis, which showed the highest identity (99%) with *Paenibacillus* sp. B01 16S rRNA gene sequence (GenBank accession no. CP045802).

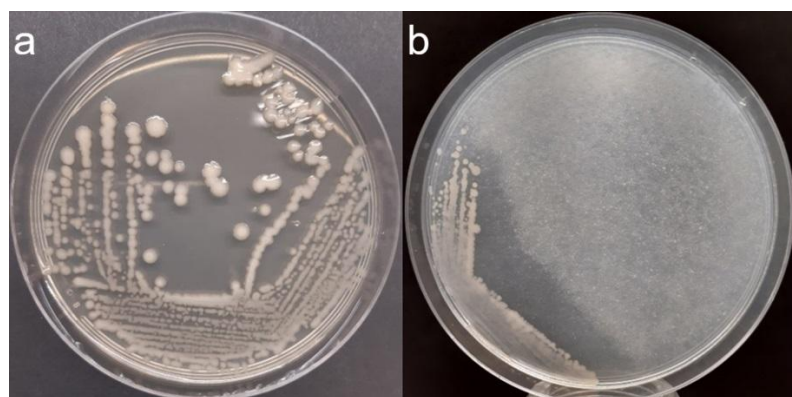


**Figure 3.2.** Isolated *Paenibacillus* sp. GKG bacterium. a – solid LB medium, b – solid mineral medium containing water-insoluble YCW substrate as a carbon source. A clear hydrolysis zone around colonies shows the presence of extracellular YCW-degrading CAZymes.

Another bacterium capable of YCW hydrolysis was isolated from a non-sterile YCW sample (Fig. 3.3). We used YCW as both a carbon source in the medium and as a source of bacteria capable of degrading the YCW substrate. We concluded that YCW was a source of bacteria when we observed that, when the substrate was added after autoclaving, the hydrolysis zones producing bacteria grew throughout the entire depth of the solid medium, not just on its surface. However, when YCW was added before autoclaving, no

colonies formed. A similar example of a yeast-lytic bacterium coexisting with yeast was described earlier, when an *Arthrobacter* strain was isolated from brewery sewage samples (Kitamura et al., 1972). Yeast-lytic enzymes are typically secreted by different strains of bacteria that, in the literature, can be named by either of the following synonyms: *Cellulosimicrobium*, *Arthrobacter* or *Oerskovia* (Ferrer, 2006).

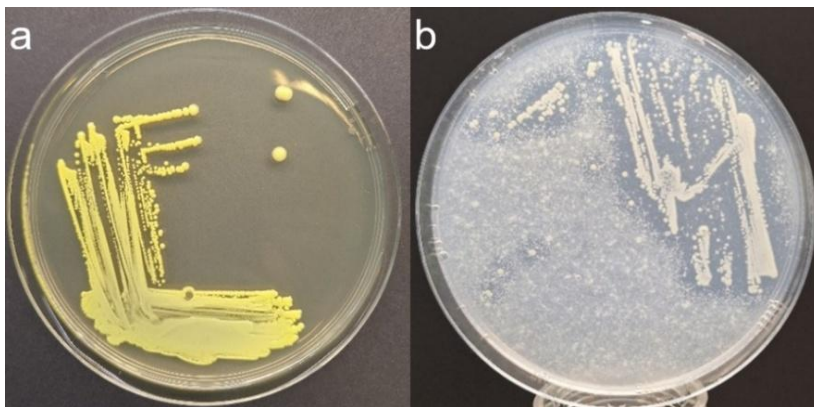


**Figure 3.3.** Isolated *Cellulosimicrobium* sp. GKH bacterium. a – solid LB medium, b – solid mineral medium containing water-insoluble YCW substrate as a carbon source. A clear hydrolysis zone around colonies shows the presence of extracellular YCW-degrading CAZymes.

Analysis of the 16S rRNA gene sequence revealed that the isolated bacterium, subsequently named *Cellulosimicrobium* sp. GKH is similar to representatives of the *Cellulosimicrobium* genus. The isolate exhibited the greatest degree of identity (100.00%) with several *Cellulosimicrobium* strains, including *Cellulosimicrobium cellulans* strain MC29 (GenBank accession no. ON629814.1), and *Cellulosimicrobium funkei* strain NFZ10 (accession no. MW391761.1).

### 3.1.2 Proteomic analysis of secretome proteins

To identify the YCW-degrading enzymes, all three isolated bacteria were cultivated in a liquid mineral medium supplemented with YCW. The resulting culture medium was collected, concentrated, and analysed by LC-MS/MS proteomics to identify the extracellular hydrolytic enzymes. Slightly different experimental designs and approaches were used for each bacterium.

The analysis of *Paenibacillus* sp. GKG proteins, responsible for YCW degradation, was limited to the secretome protein fraction only. The secretome protein fraction was obtained after concentration and dialysis of the culture medium. Samples were collected only using YCW as a carbon source. In addition to the secretome of *Rheinheimera* sp. GKU, intracellular, and

periplasmic protein fractions were collected using heat-induced precipitation. Protein fractions were analysed by comparing differences between samples collected under two conditions: one obtained when glucose was used as a carbon source, the other when YCW was used as a carbon source. By analysing the differences between these two conditions, YCW-induced proteins were identified. The analysis of *Cellulosimicrobium* sp. GKH was limited to the secretome fraction only. However, two secretome samples were collected. The first fraction was collected by heat-induced precipitation, and the second was extracted from a solid mineral medium supplemented with YCW, resulting in clear hydrolysis zones. Samples were collected only using YCW as a carbon source. Lastly, *Paenibacillus* sp. GKG LC-MS/MS bottom-up proteomic analysis was performed using data-independent acquisition, while *Rheinheimera* sp. GKU and *Cellulosimicrobium* sp. GKH bottom-up proteomics analysis was performed using data-dependent LC-MS/MS data acquisition.

After proteomic analysis, putative protein lists were obtained for each analysed protein fraction. The identified protein lists contained up to several hundred proteins. These lists were narrowed down to a sum of seven most promising enzymes based on several criteria. These criteria included YCW-inducibility, enzymatic activities typical of the putative glycosyl hydrolase family, and the existing number of representatives of *Paenibacillus*, *Rheinheimera*, or *Cellulosimicrobium* in the corresponding glycosyl hydrolase families. The selection process for the most relevant enzymes encoded by each bacterium is described in more detail in the following chapters 3.1.2.1, 3.1.2.2, and 3.1.2.3.

#### 3.1.2.1 CAZyme fractions of *Rheinheimera* sp. GKU

Secreted, periplasmic and intracellular protein fractions were collected from cultures cultivated for 48 h in media containing either glucose or YCW as a carbon source. The scheme (Fig. 3.4) represents the samples collected for proteomic analysis. The samples were compared in pairs with respective protein fractions (secreted, periplasmic, or intracellular) obtained under different carbon sources – glucose or YCW.

A total of 362 proteins were identified across all tested samples. When total identified protein lists were summarised, the dbCAN3 tool (Zheng et al., 2023) was used to extract CAZymes from a general list of identified proteins. A total of 15 CAZymes (Appendix 5) were identified. Among 15 identified CAZymes, ten were GHs, two PLs, two AAs, and one CBM. The candidate

GHs for characterisation in this study were selected following analysis of proteomics results for carbon-source-dependent CAZymes (Appendix 5).

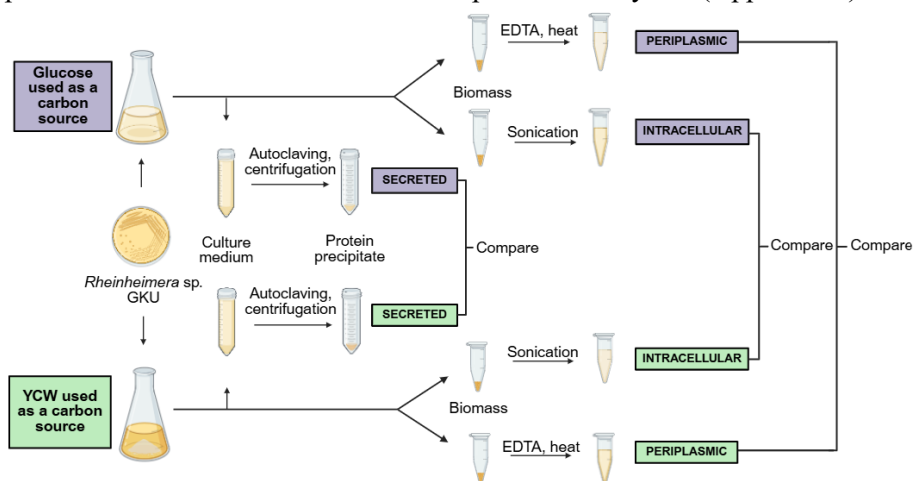


**Figure 3.4.** Overview of sample collection procedures for bottom-up proteomics analysis of carbon-source-dependent protein presence. *Rheinheimera* sp. GKU was cultivated in a mineral medium containing glucose or YCW as a carbon source. Culture medium was collected to prepare secreted protein samples. Biomass was resuspended, and cells were lysed using sonication or treated with heat and EDTA to collect intracellular and periplasmic protein fractions, respectively. All fractions were subjected to FASP digestion, and the resulting peptides were analysed using LC-MS/MS.

Four GHs with putative activity towards  $\beta$ -1,3/1,6-glucans were induced only when YCW was used as a carbon source instead of glucose. However, only three recombinant GHs showed hydrolytic activity in cell-free extracts and were further purified and characterised: GH3\_3456, GH16\_306, and GH16\_3455 (Fig. 3.5). Other CAZymes were not selected, due to the absence of correlation between protein abundance and the carbon source used, or having putative functions other than  $\beta$ -1,3/1,6-glycosidases. These CAZymes were not further explored, as they were beyond the scope of this study.

Analysis of the selected GHs sequences using SMART (Letunic et al., 2021) and SignalP-5.0 (Almagro Armenteros et al., 2019) web tools showed that all proteins of interest had a secretory signal peptide. Additionally, the modular architecture of selected GHs was predicted using the SMART tool (Fig. 3.5). Based on identified catalytic domains, selected GHs belong to the GH3 and GH16 families. In addition to the GH16 catalytic domain, the GH16\_306 enzyme had two non-catalytic domains annotated as internal repeats. According to the SMART tool, these domains have no defined function within the confidence threshold. An annotation as a CBM60 module was below the confidence threshold; the InterPro tool, however, annotated these domains as galactose-binding domain-like. These results suggest that

these internal repeats may play a role in binding polysaccharide substrates in the GH16\_306 enzyme. The truncated versions GH16\_306\_CBM\_CBM, GH16\_306\_CBM\_1, and GH16\_CBM\_2 (Fig. 3.5) of a full-length GH16\_306 enzyme were also synthesised and investigated for CBM function. The putative CBM function was evaluated by analysing the 3D structure model of GH16\_306 and conducting functional pull-down assays for truncated variants of the GH16\_306 protein. Amino acid sequences of all six recombinant proteins depicted in Figure 3.5 can be found in Appendix 10.

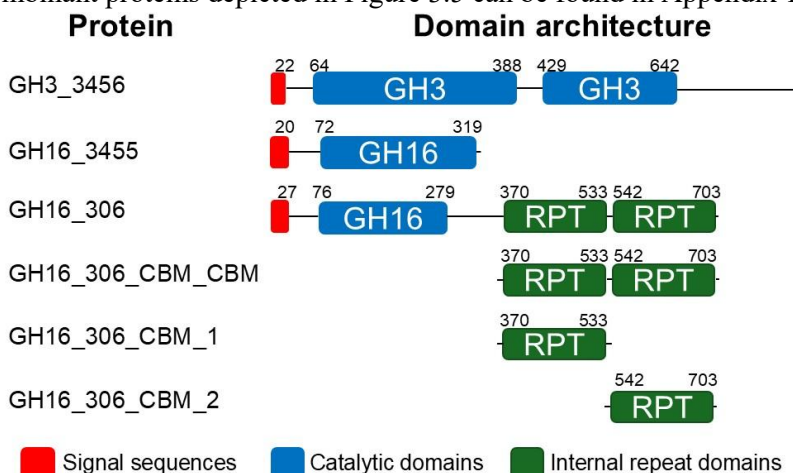


**Figure 3.5.** *Rheinheimera* sp. GKU GHs selected as targets for characterisation in this study. A schematic representation of the domain architecture of the investigated recombinant proteins is presented.

Based on modular structure analysis, all three GHs were expected to hydrolyse  $\beta$ -1,3/1,6-glucans and  $\beta$ -1,3/1,6-oligosaccharides. Therefore, various substrates and YCW were used as substrates to characterise the activities of these enzymes (Chapter 3.2.1).

### 3.1.2.2 Secretome of *Paenibacillus* sp. GKG

Proteomic analysis of the secreted protein fraction of *Paenibacillus* sp. GKG resulted in the identification of 710 proteins. Many of these proteins were homologous. Clustering these proteins at 90% identity resulted in the identification of 163 proteins in the cell-free culture medium of *Paenibacillus* sp. GKG. Most of the identified proteins were not related to carbohydrate degradation. A manually curated list of 24 CAZyme proteins (Appendix 3) revealed the presence of several GHs in the secretome of *Paenibacillus* sp. GKG. This selection was made based on available assigned EC numbers and protein descriptions.

According to the published data, the GH30 and GH81 families include a small number of characterised bacterial enzymes. Therefore, a putative GHs

belonging to family GH81, further referred to as PsLam81A (UniProt accession: A0A5Q2NJK9) and GH30, further referred to as PsGly30A (UniProt accession: A0A2N5N256) were selected for further analysis, to broaden knowledge about these groups of enzymes (Chapter 3.2.2) and to evaluate the possibility of applying these enzymes for the bioconversion of YCW to laminaribiose and gentiobiose, respectively (Chapter 3.3).

### 3.1.2.3 Secretomes of *Cellulosimicrobium* sp. GKH

Since the 16S rRNA gene sequence analysis showed that *Cellulosimicrobium* sp. GKH isolate had 100% identity with *Cellulosimicrobium cellulans* strain MC29 (accession no. ON629814.1), the proteomic analysis of the secreted protein fraction of *Cellulosimicrobium* sp. GKH was performed using the *Cellulosimicrobium cellulans* UniProt reference proteome (UniProt ID: UP000319068). Two secreted protein fractions were analysed: secreted proteins present in hydrolysis zones of agar plates and secreted proteins in liquid culture medium (Fig. 3.6). The dbCAN3 server (Zheng et al., 2023) was used to screen the lists of identified proteins for potential CAZymes. This tool identified 33 CAZymes in the secretome of the solid medium and 37 CAZymes in the secretome of the liquid medium (data not shown). Both secretome fractions contained a single representative of yeast-lytic endo-1,3- $\beta$ -glucanases belonging to the GH16 and GH64 families (Appendix 6). Sequence coverage (%) and identification confidence score (-10lgP) indicated that the sample collected from the liquid medium produced higher-quality results.

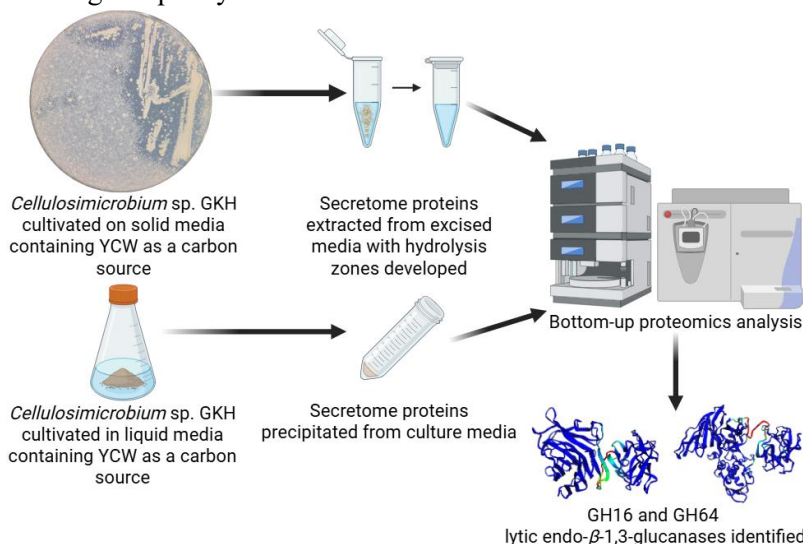


**Figure 3.6.** Principal scheme of the experimental design for the analysis of secreted proteins of *Cellulosimicrobium* sp. GKH. Both samples analysed contained GH16 and GH64 enzymes, which are yeast lytic endo- $\beta$ -1,3-glucanases.

In addition to the catalytic GH16 and GH64 modules, these enzymes both contained CBM13 non-catalytic modules, which have been reported to be crucial for yeast lytic efficiency. Shen and colleagues have demonstrated that a truncated enzyme variant lacking the CBM module lost the ability to lyse living yeast cells, but retained the ability to hydrolyse  $\beta$ -glucan polysaccharides (Shen et al., 1991).

A previous review of the variety and properties of *Cellulosimicrobium* yeast-lytic endo-1,3- $\beta$ -glucanases (Ferrer, 2006) was used as a reference when selecting *Cellulosimicrobium* enzymes for lysing living yeast cells. Two enzymes with high lytic activity, GH16\_LLG109 (Salazar et al., 2006) and GH64\_21606 (Shen et al., 1991), were selected for comparison with the lytic enzymes GH16\_CELCE and GH64\_CELCE, which were experimentally identified at the proteome level in this study. This comparison revealed two pairs, one representing the GH16 family and one representing the GH64 family of lytic endo-1,3- $\beta$ -glucanases (Fig. 3.7). The GH16 enzyme pair showed 97.97% amino acid sequence identity, while the GH64 enzyme pair showed 100% amino acid sequence identity and the same domain structure. Due to their high degree of identity and the availability of data from previous investigations, two sequences were selected from the previously reported data to synthesise the gene sequences encoding the GH16\_LLG109 (Salazar et al., 2006) and GH64\_21606 (Shen et al., 1991) enzymes. These enzymes are referred to as GH16 and GH64 in the following text, respectively. The characterisation data of GH16 and GH64 enzymes are presented further in Chapter 3.2.3.

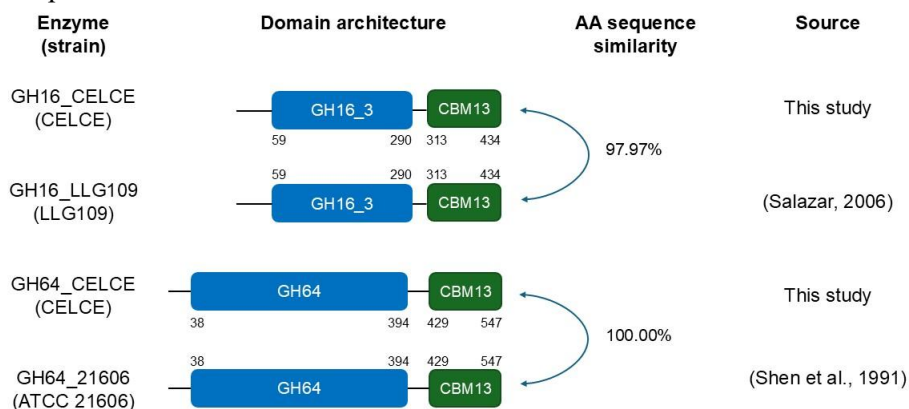


**Figure 3.7.** Comparison of the sequence identity of the four lytic endo-1,3- $\beta$ -glucanases investigated in this study. The GH16\_CELCE and GH64\_CELCE enzyme sequences were obtained from the UniProt reference proteome of *Cellulosimicrobium cellulans* (UniProt ID: UP000319068), while the GH16\_LLG109 and GH64\_21606 enzyme sequences were obtained from gene sequences published in previous studies.

Overall, seven proteins were identified as potential YCW-degrading enzymes and were subjected to recombinant synthesis in *E. coli* and characterisation. PsLam81A and PsGly30A enzymes were derived from *Paenibacillus* sp. GKG; GH3\_3456, GH16\_306, and GH16\_3455 were derived from *Rheinheimera* sp. GKU; GH16 and GH64 from *Cellulosimicrobium* bacterium. The characterisation data is presented in the following Chapter 3.2.

## 3.2 Characterisation of YCW-targeting recombinant GHs

### 3.2.1 YCW utilisation-ensuring GHs from *Rheinheimera*

The experimental design of proteomic analysis (Chapter 3.1.1.1) allowed the identification of YCW-induced and YCW-degrading CAZymes in *Rheinheimera* sp. GKU intracellular, periplasmic, and secreted protein fractions. The following Chapters 3.2.1.1 and 3.2.1.2 investigate the properties of the identified YCW-dependent GHs.

#### 3.2.1.1 Characterisation of GHs

After proteomic analysis, YCW-induced and putative YCW-degrading GHs (GH3\_3456, GH16\_306, GH16\_3455) were selected for further characterisation. Genes lacking signal peptide nucleotide sequences were amplified and cloned into expression vectors. Proteins were synthesised in *E. coli* cells and extracted using sonication. After purification using Ni-NTA chromatography, GHs were analysed using SDS-PAGE (Fig. 3.8).

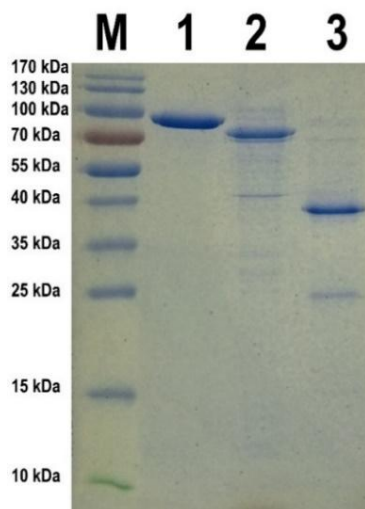


**Figure 3.8.** SDS-PAGE analysis of recombinant GHs from *Rheinheimera* sp. GKU, produced in *E. coli* after Ni-NTA purification. Lane M – molecular mass standard, lane 1 – GH3\_3456, lane 2 – GH16\_306, lane 3 – GH16\_3455.

Protein bands corresponded to the theoretical molecular weights, deduced from the amino acid sequence: GH3\_3456  $M_w = 89$  kDa, GH16\_306  $M_w = 75$  kDa, GH16\_3455  $M_w = 36$  kDa. The enzymatic activities of recombinant GHs were evaluated using various polysaccharides and oligosaccharides. TLC analysis was used to evaluate hydrolysis products (Appendix 7). The polysaccharides selected for testing as substrates were chosen based on the results of proteomic analysis (Chapter 3.1.1.2), which indicated a potential  $\beta$ -1,3-glycosidase activity for all three GHs tested. All three tested enzymes hydrolysed laminarin and yeast  $\beta$ -glucan. For other substrates, the specificity and efficiency patterns were different (Table 3.1, Appendix 7).

GH3\_3456 was the universal  $\beta$ -glucosidase, capable of disaccharide, laminaribiose and gentiobiose, hydrolysis. However, cellobiose hydrolysis was incomplete. Another  $\beta$ -glucosidase-specific trait was only trace activity on long linear polysaccharides (curdlan, pachyman, pustulan and barley  $\beta$ -glucan). In the case of branched polysaccharides (laminarin, yeast  $\beta$ -glucan, and YCW), hydrolysis was more efficient. All substrates were hydrolysed down to glucose as the main hydrolysis product. This activity pattern is similar to other GH3 family members. GH3A, GH3B, GH3C, GH3D enzymes from *Cellvibrio japonicus* were capable of laminaribiose, gentiobiose and cellobiose hydrolysis (Nelson et al., 2017).

**Table 3.1.** Substrate specificity of recombinant GHs from *Rheinheimera* sp. GKU.

| Substrates<br>(glycosidic bonds)                     | Glycosidases |          |           |
|------------------------------------------------------|--------------|----------|-----------|
|                                                      | GH3_3456     | GH16_306 | GH16_3455 |
| Laminarin ( $\beta$ -1,3/ $\beta$ -1,6)              | Yes          | Yes      | Yes       |
| Yeast $\beta$ -glucan ( $\beta$ -1,3/ $\beta$ -1,6)  | Yes          | Yes      | Yes       |
| YCW ( $\beta$ -1,3/ $\beta$ -1,6)                    | Yes          | tr       | Yes       |
| Curdlan ( $\beta$ -1,3)                              | tr           | Yes      | Yes       |
| Pachyman ( $\beta$ -1,3)                             | tr           | Yes      | Yes       |
| Pustulan ( $\beta$ -1,6)                             | tr           | No       | No        |
| Barley $\beta$ -glucan ( $\beta$ -1,3/ $\beta$ -1,4) | tr           | Yes      | Yes       |
| Laminaribiose ( $\beta$ -1,3)                        | Yes          | No       | pr        |
| Laminaritetraose ( $\beta$ -1,3)                     | Yes          | Yes      | Yes       |
| Gentiobiose ( $\beta$ -1,6)                          | Yes          | No       | No        |
| Cellobiose ( $\beta$ -1,4)                           | pr           | No       | No        |
| Carboxymethyl cellulose ( $\beta$ -1,4)              | No           | No       | No        |
| Microcrystalline cellulose ( $\beta$ -1,4)           | No           | No       | No        |
| $\beta$ -1,2-glucan ( $\beta$ -1,2)                  | No           | No       | No        |

Yes – hydrolysis products detected; No – hydrolysis products not detected; tr – trace amount of hydrolysis products detected; pr – partial hydrolysis.

GH16\_306 and GH16\_3455 enzymes acted as endo-1,3- $\beta$ -glucanases, as they were capable of efficient hydrolysis of linear (curdlan, pachyman, barley  $\beta$ -glucan) and branched polysaccharides (laminarin, yeast  $\beta$ -glucan, YCW). As expected, the hydrolysis of disaccharides was absent, or efficiency was lower than that exhibited by GH3\_3456 glucosidase. Overall, GH16\_306 and GH16\_3455 GHs showed similar hydrolysis patterns. The key difference was that GH16\_3455 partially hydrolysed laminaribiose, and YCW hydrolysis was more efficient. Compared with other GH16 members, previously reported activity patterns align with the results of this study. PglA from *Paenibacillus* sp. S09 hydrolysed laminarin, pachyman, zymosan (yeast  $\beta$ -glucan), and barley  $\beta$ -glucan (Cheng et al., 2013).

Overall, the substrate specificity patterns showed that GH3\_3456 has  $\beta$ -glucosidase activity, while GH16\_306 and GH16\_3455 have endo-1,3- $\beta$ -glucanase and endo-1,3/4- $\beta$ -glucanase activity.

#### 3.2.1.2 YCW conversion to glucose

Further, the ability of *Rheinheimera* sp. GKU's utilisation of YCW as a carbon source was proven. This was demonstrated by showing that three GHs can produce glucose from YCW, either individually or in combination. Experiments with pure substrates, such as laminarin and yeast  $\beta$ -glucan, showed that GH3\_3456, GH16\_306, and GH16\_3455 could hydrolyse  $\beta$ -1,3/1,6-glucans (Table 3.1, Appendix 7). The efficiency of laminarin and pure yeast  $\beta$ -glucan hydrolysis was higher than for YCW. The most likely reason is the reduced accessibility of  $\beta$ -glucan in YCW due to crosslinking with other polymers, such as mannoproteins and chitin. Additionally, the purity of  $\beta$ -1,3/1,6-glucan in YCW substrate is lower, and water solubility is worse than in laminarin and yeast  $\beta$ -glucan.

These differences are demonstrated by comparing the hydrolysis products of laminarin (Fig. 3.9a) and YCW (Fig. 3.9b). The differences in hydrolysis product patterns show that all GHs produced a higher amount of oligosaccharides from laminarin than YCW. Nevertheless, the combination of all three GHs improved YCW hydrolysis, while on laminarin hydrolysis this effect was not observed. This data is in agreement with proteomic analysis results (Fig. 3.9a), as they showed that GH3\_3456, GH16\_306 and GH16\_3455 are induced by YCW. The set of these three enzymes ensures the conversion of YCW to a common carbon source – glucose.

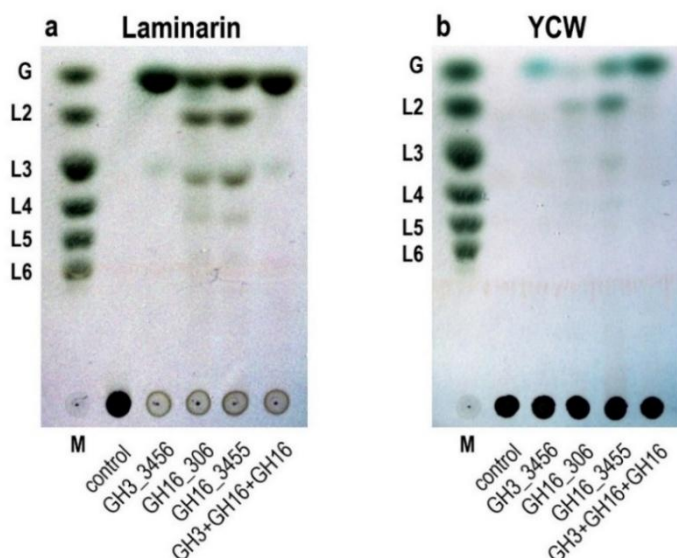


**Figure 3.9.** Ability of glycosidases from *Rheinheimera* sp. GKU to produce glucose from  $\beta$ -1,3/1,6 glucans. a – laminarin; b – YCW. M – standard mixture of G – glucose, L2 – laminaribiose, L3 – laminaritriose, L4 – laminaritetraose, L5 – laminaripentaose, L6 – laminarihexaose. Control - substrate with no enzymes. GH3\_3456, GH16\_306, GH16\_3455 – represent hydrolysis products of separate GHs. GH3+GH16+GH16 – represents synergistic action of GH3\_3456, GH16\_306, and GH16\_3455.

Moreover, amino acid sequence analysis showed that among the three investigated GHs, GH16\_306 has two additional non-catalytic domains (Fig. 3.5). SMART server results showed that these non-catalytic domains are annotated as internal repeat domains.

### 3.2.1.3 Ability to bind $\beta$ -1,3-glucans

In order to investigate whether non-catalytic domains of GH16\_306 protein, annotated as “Internal repeat domains”, have CBM properties experimentally, truncated versions GH16\_306\_CBM\_CBM, GH16\_306\_CBM\_1, and GH16\_306\_CBM\_2 of a full-length GH16\_306 enzyme were synthesised (Fig. 3.5). The hypothesis was confirmed experimentally by performing pull-down (for insoluble carbohydrates) and pull-down inhibition (for soluble carbohydrates) assays, which are used to investigate protein-carbohydrate interactions. Truncated protein GH16\_306\_CBM\_CBM containing both internal repeats was able to bind  $\beta$ -1,3-glucan polysaccharides (yeast  $\beta$ -glucan and curdlan), but not  $\beta$ -1,6 polysaccharide pustulan (Fig. 3.10a). Surprisingly,  $\beta$ -1,3-glucan polysaccharide pachyman did not bind to GH16\_306\_CBM\_CBM protein. Overall, this result indicated that these putative CBMs bind  $\beta$ -1,3-glucans rather than  $\beta$ -1,6-glucans.

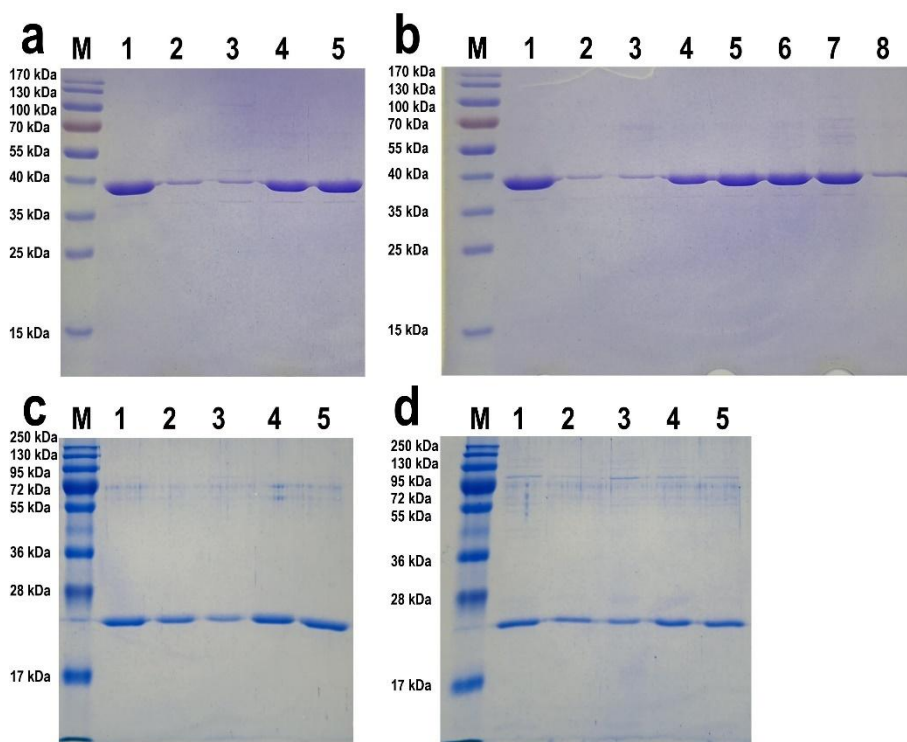


**Figure 3.10.** Pull-down assay to determine protein-carbohydrate interactions between putative CBM domains of GH16\_306 protein and various  $\beta$ -glucans. a – pull-down assay of GH16\_306\_CBM\_CBM protein, b – pull-down inhibition assay of GH16\_306\_CBM\_CBM protein, c – pull-down assay of GH16\_306\_CBM\_1 protein, d – pull-down assay of GH16\_306\_CBM\_2 protein. Samples for gels 3.10a, 3.10c, 3.10d: 1 –  $\beta$ -glucan absent (control), 2 – yeast  $\beta$ -glucan, 3 – curdlan, 4 – pachyman, 5 – pustulan, M – protein molecular weight standard. Samples for gel 3.10b: 1 – no carbohydrate (control), 2 – yeast  $\beta$ -glucan (control), 3 – yeast  $\beta$ -glucan and glucose, 4 – yeast  $\beta$ -glucan and laminaribiose, 5 – yeast  $\beta$ -glucan and laminaritetraose, 6 – yeast  $\beta$ -glucan and laminarihexaose, 7 – yeast  $\beta$ -glucan and laminarin, 8 – yeast  $\beta$ -glucan and gentiobiose, M – protein molecular weight standard.

Pull-down inhibition assay confirmed this pattern, as soluble  $\beta$ -1,3-carbohydrates (Fig. 3.10b, lanes 4–7) were able to inhibit protein-carbohydrate interactions between GH16\_306\_CBM\_CBM and yeast  $\beta$ -glucan, while  $\beta$ -1,6-disaccharide gentiobiose did not (Fig. 3.10b, lane 8). In addition, in the presence of a glucose pull-down inhibition did not occur as well (Fig. 3.10b, lane 3). When two putative CBM domains were synthesised as separate proteins: GH16\_306\_CBM\_1 and GH16\_306\_CBM\_2, the ability to bind  $\beta$ -1,3-glucans decreased. GH16\_306\_CBM\_1 protein bound to curdlan the best (Fig. 3.10c, lane 3), and the ability to bind yeast  $\beta$ -glucan was retained as well (Fig. 3.10c, lane 2). GH16\_306\_CBM\_2 protein retained the ability to bind yeast  $\beta$ -glucan and curdlan at similar efficiencies (Fig. 3.10d,

lanes 2, 3), but less efficiently than GH16\_306\_CBM\_CBM protein (Fig. 3.10a, lanes 2, 3).

One possible reason the pull-down assay did not show binding to linear  $\beta$ -1,3-glucan pachyman (Fig. 3a, 3b, 3c, lane 4) as efficiently as to linear  $\beta$ -1,3-glucan curdlan (Fig. 3a, 3b, 3c, lane 3) could be structural differences among polysaccharides, leading to differences in physical properties and accessibility to binding sites. Hettle and colleagues have investigated CBM6 and CBM56 modules' abilities to bind  $\beta$ -1,3-glucans, e.g. laminarin, laminari-oligosaccharides, curdlan, and pachyman (Hettle et al., 2017). Similarly to this study, Hettle and colleagues observed different binding properties among  $\beta$ -1,3-glucans. For example, the CBM6 module was able to bind pachyman, but not curdlan. The other difference was observed for the CBM56 domain – it could bind  $\beta$ -1,3-glucans with DPs higher than 25, but not  $\beta$ -1,3-oligosaccharides with DPs lower than 7. These differences were explained by the different number of binding sites on each carbohydrate and their ability to form different 3D structures.

In the experimental part, physical differences between substrates were also observed. Both yeast  $\beta$ -glucan and curdlan hydrate well and form uniform suspensions with sedimented particles, whereas pachyman hydrates poorly, and the particles float on the surface of the buffer and stick to the walls of the centrifugal tube. In addition, GH16\_306 enzyme showed lower hydrolysis efficiency on pachyman (Appendix 7e) in comparison to GH16\_3455 enzyme. Whereas the hydrolysis patterns on yeast  $\beta$ -glucan (Appendix 7b) and curdlan (Appendix 7d) were similar for both enzymes.

In addition to the experimental pull-down assays, the interactions between the GH16\_306 protein and linear  $\beta$ -1,3-glucan oligosaccharide laminarihexaose were simulated using 3D structure models (Fig. 3.11). The structural modelling of the multidomain GH16\_306 protein using Chai-1 predicted a modular architecture comprising a GH domain and two putative CBMs connected by linker regions with a pTM score of 0.6582 (Figure 3.11a). The docking of three laminarihexaose molecules has shown potential carbohydrate-binding sites within the GH domain and both putative CBMs. This suggests that multiple regions of the protein, the active site of GH and carbohydrate-binding sites of putative CBMs, may participate in substrate recognition. The predicted CBM binding sites are lined by aromatic and polar amino acid residues (Figure 3.11b), consistent with carbohydrate-binding interfaces in which aromatic stacking and hydrogen-bonding interactions contribute to ligand recognition.

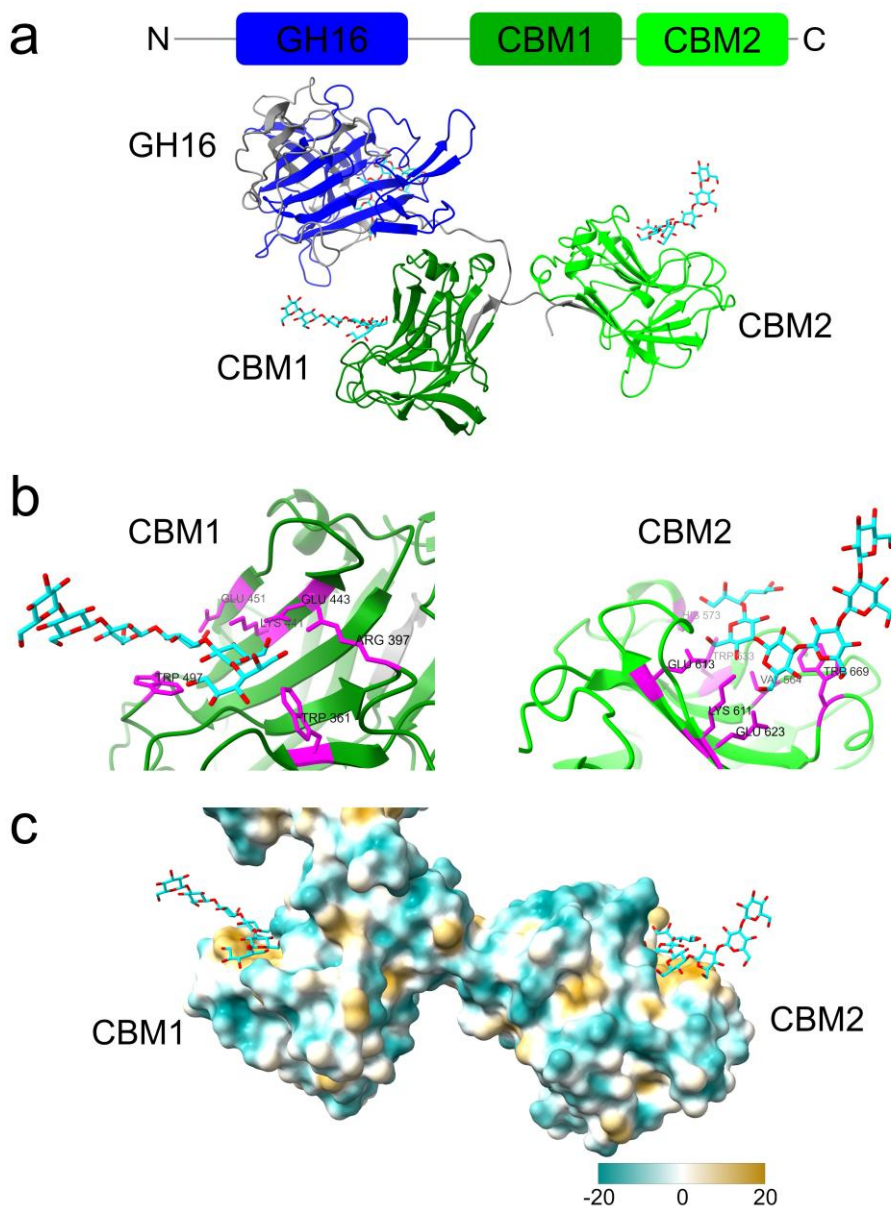


**Figure 3.11.** The 3D structural model of the multidomain GH16\_306 protein and predicted carbohydrate-binding sites were generated using Chai-1 <https://www.chaidiscovery.com/>. (a) A ribbon plot showing the overall domain architecture of GH16\_306, with the GH domain coloured blue and putative CBMs coloured green and lime. Regions not assigned to GH or CBM domains are shown in grey. Predicted laminarihexaose ligands are shown in cyan and red. (b) A closer view of predicted ligand binding sites within two putative CBMs, highlighting hydrophobic and polar predicted binding residues (magenta) proposed to mediate ligand recognition through stacking and hydrogen-bonding interactions. (c) Surface representation of two putative CBMs coloured according to hydrophobicity, illustrating the location of docked Laminarihexaose molecules within surface pockets enriched in hydrophobic regions (tan).

The importance of aromatic and polar residues for CBMs of families 4, 15, 17, 28 was shown in previous studies (Kognole and Payne, 2018; Pell et al., 2003). Within predicted binding sites of putative CBMs of GH16\_306 protein several aromatic and polar residues were found (Trp361, Arg397, Lys441, Glu443, Glu451, and Trp497 for CBM1; Trp533, His573, Lys611, Glu613, Glu623, and Trp669 for CBM2). Further analysis of hydrophobicity surface representations indicated that the docked ligands occupy shallow pockets that are enriched in hydrophobic residues (Figure 3.11c). This finding provides further support for the proposed carbohydrate-binding function of these domains. Collectively, these structural predictions and previously presented experimental results (Fig. 3.10) indicate that putative CBM modules of the GH16\_306 protein encompass carbohydrate-binding regions that may enhance  $\beta$ -glucan recognition and processing. Considering practical applications of these properties, CBMs with appropriate selectivities can be used as ligands in affinity chromatography, e.g. to separate xylo-oligosaccharides from cello-oligosaccharides (Johansson et al., 2006). In the context of this study, such properties could be adapted to separate  $\beta$ -1,3-glucan oligosaccharides from  $\beta$ -1,6-glucan oligosaccharides. Further investigation of CBM function should focus on experimentally characterising the carbohydrate-binding sites of each putative CBM critical for carbohydrate binding, and on testing a wider range of glucans. However, this is beyond the scope of this study.

Overall, the analysis of *Rheinheimera* sp. GKU YCW-induced enzymes demonstrated the bacteria's capacity to degrade and utilise YCW substrate. Furthermore, the GH16 enzyme was discovered to be a multi-domain GH enzyme. To date, its internal repeat domains have not been assigned a CBM function, but they may represent novel CBM domains. Regardless, the properties of *Rheinheimera* sp. GKU GHs did not align with the main objective of this study – to convert YCW into a functional, bioactive oligosaccharides. GH3\_3456 hydrolysed YCW to glucose as a sole hydrolysis product, while GH16\_306 and GH16\_3455 hydrolysed YCW to laminaribiose and glucose, but with very low efficiency. Therefore, further characterisation of these enzymes was not carried out.

### 3.2.2 Disaccharides-producing GHs from *Paenibacillus*

Compared with GHs from *Rheinheimera* sp. GKU, endo-1,3- $\beta$ -glucanase PsLam81A from *Paenibacillus* sp. GKG showed superior efficiency in YCW hydrolysis. Therefore, two enzymes, endo-1,3- $\beta$ -glucanase PsLam81A and endo-1,6- $\beta$ -glucanase PsGly30A, from *Paenibacillus* sp. GKG were

investigated in more detail with the focus of determining optimum hydrolysis reaction conditions and industrially relevant property – thermal stability.

### 3.2.2.1 PsLam81A is an endo-1,3- $\beta$ -glucanase

Up to date, three bacterial endo-1,3- $\beta$ -glucanases from the GH81 family have been characterised: TfLam81A from *Thermobifida fusca* (McGrath and Wilson, 2006), BH026 from *Bacillus halodurans* (Pluvinae et al., 2017), and CtLam81A from *Clostridium thermocellum* (Kumar et al., 2018). The latter two have their crystal structures determined as well. Several bacterial endo-1,3- $\beta$ -glucanases have different additional domains, but none have the same domain organisation as PsLam81A, which is reported in this study (Fig. 3.12). According to the bioinformatic analysis, PsLam81A consists of two domains: a catalytic GH81 domain and a CBM56 module. BH026 has a three-domain organisation: catalytic GH81, CBM6 and CBM56 modules, CtLam81A has a two-domain organisation, while TfLam81A has only a catalytic domain.

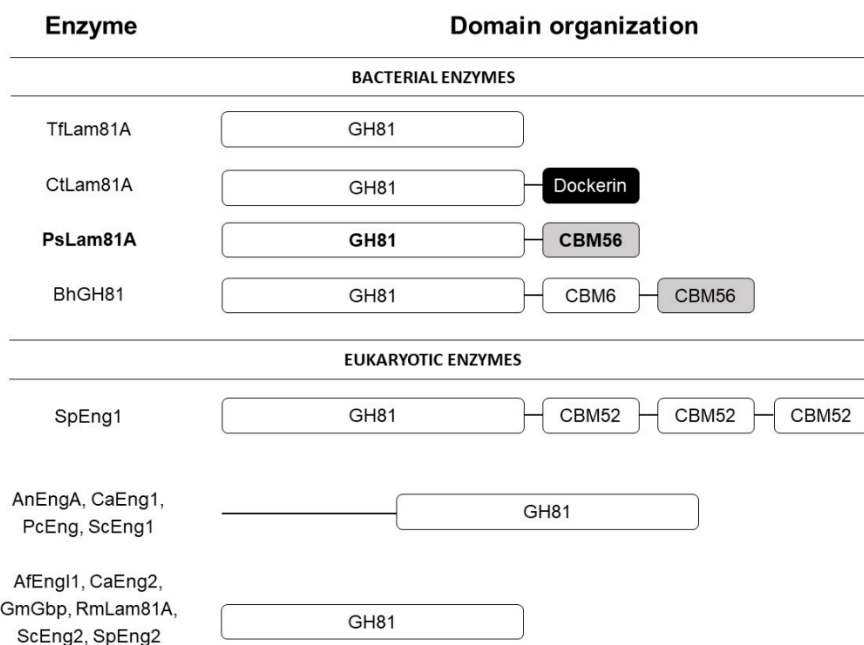


**Figure 3.12.** Modular schematics of arrangement of domains in characterised enzymes from GH81 family according to CAZy and InterPro databases. PsLam81A enzyme reported in this study is marked in bold. Domains with a white background are referenced in the CAZy database and are found using a Pfam search. Domains with a grey background are referenced in the CAZy database, but are not found using a Pfam search. Domain with a black background is found using a Pfam search, but is not referenced in the CAZy database. Modular boundaries in the picture are unified for illustration purposes and do not represent the exact sizes of domains.

So far, there are 11 characterised members of the GH81 family of microorganisms of eukaryotic origin. Only one GH from

*Schizosaccharomyces pombe* (SpEng1) has three CBM52 domains besides a catalytic domain (Martín-Cuadrado et al., 2008). AnEngA from *Aspergillus nidulans* (Szilágyi et al., 2010), CaEng1 from *Candida albicans* (Esteban et al., 2005), PcEng from *Pneumocystis carinii* (Kutty et al., 2015), and ScEng1 from *Saccharomyces cerevisiae* (Baladrón et al., 2002) has a catalytic domain and a hypothetical non-cytoplasmic domain. AfEng11 from *Aspergillus fumigatus* (Fontaine et al., 1997), CaEng2 from *Candida albicans* (Esteban et al., 2005), GmGbp from *Glycine max* (Fliegmann et al., 2005), RmLam81A from *Rhizomucor miehei* (Zhou et al., 2013), ScEng2 from *Saccharomyces cerevisiae* (Martín-Cuadrado et al., 2008), and SpEng2 from *Schizosaccharomyces pombe* (Martín-Cuadrado et al., 2008) have only catalytic domains.

One task was to determine whether the CBM56 module enhances the enzymatic activity of PsLam81A. The two gene variants of the selected glycoside hydrolase from family 81 (coding for the full-length PsLam81A and the PsLam81A $\Delta$ CBM56, which lack a carbohydrate-binding module, proteins) were cloned into a pLATE31 plasmid and expressed in *E. coli* strain BL21(DE3) cells. Both enzymes were soluble independently of induction at either 20 °C or 30 °C temperatures (Appendix 9). Small amounts of target proteins were detected in the culture medium, suggesting a low-efficiency secretion. The presence of an N-terminal signal peptide sequence (1–23 aa) in the PsLam81A protein was predicted using the SignalP 6.0 server (Teufel et al., 2022). Both enzymes had C-terminal 6  $\times$  His tags and were purified successfully using immobilised metal ion affinity chromatography, then desalted by size-exclusion chromatography. The obtained recombinant enzymes were analysed by SDS-PAGE (Fig. 3.13). The molecular weights of both enzymes PsLam81A and PsLam81A $\Delta$ CBM56 were comparable to the ones deduced from theoretical amino acid sequences (Appendix 10). However, the resolution was too low to determine the presence or absence of the signal peptides.

For this reason, the purified recombinant PsLam81A enzyme was analysed using LC-MS/MS. The PsLam81A protein was separately digested with two proteases: trypsin and chymotrypsin. The sequence coverage obtained after tryptic and chymotryptic digestion was 86% and 78%, respectively (Appendix 8). Overlaying the sequences of both digestions provided 96% coverage of the recombinant protein sequence. One region of the sequence (1–27 amino acids (aa)) remained uncovered. This region included a predicted signal peptide sequence (1–23 aa), as determined using the SignalP 6.0 server (Teufel et al., 2022). The absence of peptides from this region in both digests indicated that the signal peptide was cleaved off in the *E. coli* cells.

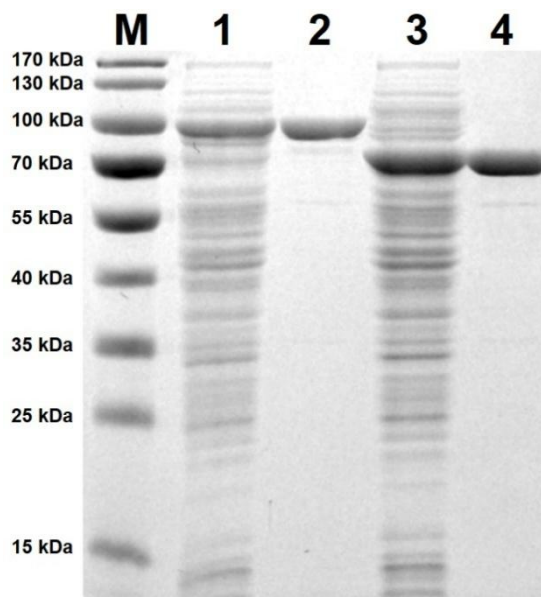


**Figure 3.13.** SDS-PAGE analysis of induced and purified recombinant PsLam81A and PsLam81A $\Delta$ CBM56 proteins. Lane M – Molecular mass, lane 1 – soluble protein fraction of induced *E. coli* strain BL21 (DE3) cell-free extracts carrying pLATE31-PsLam81A plasmid, lane 2 – purified PsLam81A protein, lane 3 – soluble protein fraction of induced *E. coli* strain BL21 (DE3) cell-free extracts carrying pLATE31-PsLam81A $\Delta$ CBM56 plasmid, lane 4 – purified PsLam81A $\Delta$ CBM56 protein. Soluble protein fractions were obtained by centrifugation of cell-free extracts at 16,000 $\times$  g for 20 min at 4 °C.

Only a small amount of the recombinant enzymes was detected in the cell-free culture medium (Appendix 9). As the majority of the induced proteins remained in the cell with their signal sequences removed, it is probable that they remained in the periplasmic space of *E. coli* cells. Previous findings support the prediction that the presence of the native *Paenibacillus* sp. GKG signal peptide results in the periplasmic secretion of PsLam81A and PsLam81A $\Delta$ CBM56 in *E. coli* BL21(DE3) cells. For example, the endoglucanase EG5B (a full-length enzyme with a native signal peptide) from *Paenibacillus* sp. IHB B 3084 was transported to the periplasm of *E. coli* BL21(DE3) cells (Dhar et al., 2015). Moreover, the xylanase XynA from *Paenibacillus* sp. DG-22 heterologously produced in *E. coli* M15 (pREP4) cells was also located in the periplasm when the host cells were carrying the plasmid pXA8 with a gene encoding a full-length protein including a native signal peptide, or in the cytoplasm when the host cells were carrying the plasmid pQE60-XynA with a truncated gene (the sequence encoding a native signal peptide was removed) (Lee et al., 2007).

### 3.2.2.2 Optimal reaction conditions

The purified recombinant protein PsLam81A was active towards YCW (Fig. 3.26a). Therefore, the effects of pH and temperature on the enzyme's activity and stability were tested using laminarin as a standard substrate. The enzyme showed a broad pH optimum, with 97% of the maximum activity within 6.5–8.0 and 80% within 6.0–9.0 (Fig. 3.14a). Similar optimum pH ranges of 80% of the maximum activity have been reported for two previously characterised bacterial GH81 hydrolases: a pH range from 5.5 to 8.0 for CtLam81A from *Clostridium thermocellum* (*Acetivibrio thermocellus*) (Kumar et al., 2018) and a pH range from 5.5 to 10.0 for TtLam81A from *Thermobifida fusca* (McGrath and Wilson, 2006).

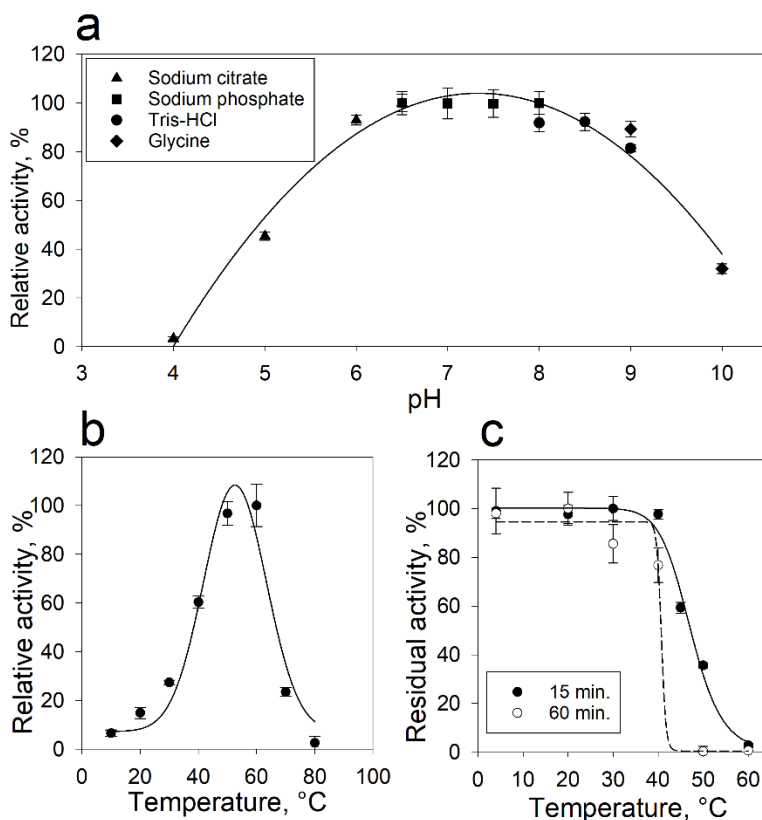


**Figure 3.14.** Effect of pH and temperature on the activity of the PsLam81A enzyme. (a) Effect of pH on enzyme activity. (b) Effect of temperature on enzyme activity. (c) Effect of temperature on enzyme stability after incubation at the indicated temperatures for 15 and 60 minutes. Results are presented as average values of at least three measurements. The bars represent SD.

The highest activity of the PsLam81A enzyme was recorded at 60 °C (Fig. 3.14b). In comparison, the optimal temperatures were 75 °C for CtLam81A

(Kumar et al., 2018) and 50 °C for TfLam81A (McGrath and Wilson, 2006). However, PsLam81A showed low-temperature stability (Fig. 3.14c). After 15 min of incubation at temperatures below 40 °C, the enzyme retained 97% of its maximum activity, whereas at 45 °C, only 59% was retained. Moreover, after 60 min of incubation, the enzyme retained 77% of its maximum activity at 40 °C and showed no activity at temperatures above 50 °C. Previously characterised members of the GH81 family are generally more thermostable. In comparison, CtLam81A retained 93% of its maximum activity after incubation for 60 min at 75 °C (Kumar et al., 2018), while TfLam81A retained approximately 90% of its maximum activity after incubation for 18 h at 50 °C (McGrath and Wilson, 2006).

Literature research revealed that the removal of CBM modules can result in either improved (Cheng et al., 2019; Wang et al., 2009) or decreased (Couturier et al., 2011; Winterhalter et al., 1995) thermal stability of an enzyme. Moreover, both increased and decreased thermal stability can be observed across different truncation variants of the same enzyme. For example, the truncated endo-1,4- $\beta$ -xylanase rXTMA $\Delta$ NC, which lacks all four CBM modules ( $2 \times$  CBM22,  $2 \times$  CBM9), shows lower thermal stability. In contrast, the truncated rXTMA $\Delta$ C, with only two out of four CBM modules ( $2 \times$  CBM22) removed, shows higher thermal stability compared to the native enzyme rXTMA, where all four CBM modules are present (Verjans et al., 2010).

In this study, we observed a slight improvement in the thermal stability of the PsLam81A enzyme upon removal of the CBM56 module (PsLam81A $\Delta$ CBM56 enzyme). After 60 min of incubation at 45 °C, PsLam81A retained  $24.0 \pm 2.7\%$  of maximum activity, while PsLam81A $\Delta$ CBM56 retained  $35.6 \pm 1.0\%$  of maximum activity. After 60 min incubation at 50 °C, PsLam81A retained no detectable activity, while PsLam81A $\Delta$ CBM56 showed a trace of activity. The fusion of the homologous CBM56 module with chitosanases improved the thermal stability of the fusion enzymes compared to the native enzymes (Lin et al., 2019). However, the thermal stability-enhancing effect differed between the two chitosanases: CBM56-GsCsn46A showed improved thermal stability only at 35 °C, whereas CBM56-BaCsn46A was more stable across the 40–55 °C temperature range. These observations support the idea that the effect of the CBM56 module on temperature stability is case-dependent, and that there is no general rule for predicting whether the presence of a CBM module will improve or decrease the thermal stability of a truncated or fused enzyme.

The temperature of 40 °C and pH 7.0 were selected for the substrate specificity reactions (Section 3.2.2.3). This is a compromise between an increasing reaction rate (Fig. 3.14b) and the decreasing thermal stability of the enzyme (Fig. 3.14c) as the reaction temperature increases.

### 3.2.2.3 CBM56 improves the efficiency of hydrolysis

The substrate specificity of the recombinant PsLam81A and PsLam81AΔCBM56 enzymes was determined by testing various polysaccharides (Appendix 2). The specific activity of each enzyme for the polysaccharide substrates was calculated by quantifying the amount of reducing sugars released during the hydrolysis reaction. Both enzyme variants hydrolysed laminarin, yeast  $\beta$ -glucan, curdlan and pachyman (Table 3.2).

**Table 3.2.** Substrate specificity of PsLam81A and PsLam81AΔCBM56 enzymes.

| Substrate             | $U \times 10^9/mol$ |                |
|-----------------------|---------------------|----------------|
|                       | PsLam81A            | PsLam81AΔCBM56 |
| Laminarin             | $10.1 \pm 0.2$      | $10.1 \pm 0.2$ |
| Yeast $\beta$ -glucan | $4.4 \pm 0.1$       | $5.1 \pm 0.2$  |
| Curdlan               | $4.5 \pm 0.4$       | $1.9 \pm 0.1$  |
| Pachyman              | $2.5 \pm 0.2$       | $0.7 \pm 0.1$  |
| Other*                | ND                  | ND             |

\*barley  $\beta$ -glucan,  $\beta$ -1,2-glucan, pustulan, carboxymethyl cellulose, microcrystalline cellulose. ND—not detected.

The full-length enzyme variant (PsLam81A) showed the highest activity towards laminarin, followed by yeast  $\beta$ -glucan and curdlan with very similar specific activities and was the least active on pachyman. This data is in agreement with previously published data on the specificity of the GH81 hydrolases (Baladrón et al., 2002; Fontaine et al., 1997; Kumar et al., 2018; Ma et al., 2022; Martín-Cuadrado et al., 2008; McGrath and Wilson, 2006; Pluvinaige et al., 2017). PsLam81A exhibited lower specific activity towards pachyman compared to laminarin (~4.1-fold lower activity) and curdlan (~1.8-fold lower activity). These ratios differ from previously reported data. An ~8.5-fold lower specific activity of TfLam81A towards pachyman compared to laminarin (McGrath and Wilson, 2006), and a > 15-fold lower activity of BH026 on pachyman relative to laminarin, as well as a > 10-fold lower activity on pachyman versus curdlan (Hettle et al., 2017) have been reported previously.

The truncated variant PsLam81AΔCBM56 hydrolysed the same substrates as the full-length enzyme PsLam81A. The specific activity of PsLam81AΔCBM56 towards the water-soluble  $\beta$ -1,6 branches containing

polysaccharides, such as laminarin and yeast  $\beta$ -glucan, was similar or higher in comparison to a full-length counterpart. However, PsLam81A $\Delta$ CBM56 had lower specific activity in the presence of water-insoluble linear  $\beta$ -1,3-glucans such as curdlan and pachyman. The importance of CBM modules on curdlan hydrolysis by the GH81 family enzyme was also demonstrated in a previous study (Hettle et al., 2017). Moreover, the removal of a CBM1 module from the GH45 hydrolase affected its activity towards polysaccharides (Couturier et al., 2011). The current understanding of such activity enhancement profile by CBM on insoluble substrates, but not on soluble substrates, is observed because of the increased local enzyme concentration on the surface of the polysaccharide particles via CBM binding (Hettle et al., 2017). However, another study suggests that CBM removal can simultaneously decrease enzymatic activity on both soluble and insoluble substrates. The activity of endo-1,3- $\beta$ -glucanase TnLam16A, which contains two CBM4 modules, decreased by approximately 70% on laminarin and pachyman, and 60% on curdlan, after removal of both CBM modules. Moreover, the enzyme with one N-terminal CBM4 module removed only showed a higher activity loss towards laminarin than curdlan and pachyman (Zverlov et al., 2001).

Deleting the C-terminal CBM56 module from the BH026 enzyme resulted in  $\sim$ 3-fold lower activity, and the subsequent removal of the second module CBM6 resulted in  $\sim$ 6-fold lower activity on curdlan relative to a native BH026 enzyme (Hettle et al., 2017). In this study, deleting the CBM56 module from PsLam81A resulted in a  $\sim$ 2.4-fold lower activity towards curdlan. These results show that the CBM56 module is functional in the full-length PsLam81A enzyme and affects its specific activity when insoluble polysaccharides are used as substrates.

Barley  $\beta$ -glucan,  $\beta$ -1,2-glucan, pustulan, carboxymethyl cellulose and microcrystalline cellulose were not hydrolysed by any of the tested enzyme variants (Table 3.2). The majority of these substrates were reported to be non-hydrolysable by GH81 family enzymes, and only a minor hydrolytic activity towards barley  $\beta$ -glucan (Fontaine et al., 1997; McGrath and Wilson, 2006), pustulan (Baladrón et al., 2002; McGrath and Wilson, 2006) and carboxymethyl cellulose (Baladrón et al., 2002; Fontaine et al., 1997; Kumar et al., 2018; McGrath and Wilson, 2006) was detected.

#### 3.2.2.4 Laminaribiose is produced from $\beta$ -glucans

Analysis of the hydrolysis products of laminarin, yeast  $\beta$ -glucan, curdlan and pachyman by both PsLam81A and PsLam81A $\Delta$ CBM56 enzymes showed similar profiles (Fig. 3.15a, 3.15b), with laminaribiose being the main product. In the presence of linear  $\beta$ -1,3-glucans (curdlan, pachyman), the enzymes

yielded laminaribiose as the main product, followed by glucose as the second most abundant product.

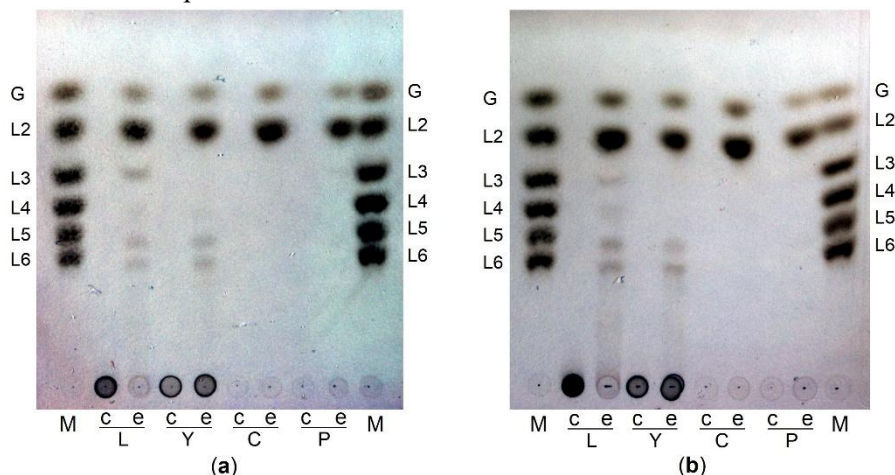


**Figure 3.15.** TLC analysis of hydrolysis products. Hydrolysis of laminarin (L), yeast  $\beta$ -glucan (Y), curdlan (C), and pachyman (P) using PsLam81A (a) and PsLam81A $\Delta$ CBM56 (b). Carbohydrate standards (M): glucose (G), laminaribiose (L2), laminaritriose (L3), laminaritetraose (L4), laminaripentaose (L5) and laminarihexaose (L6). Reactions were carried out in the presence (e) and absence (c) of the enzyme.

The same pattern was observed in the case of  $\beta$ -1,6-branches containing polysaccharides (laminarin and yeast  $\beta$ -glucan). However, some other hydrolysis products corresponding to compounds with a higher degree of DPs were also detected. Two spots near the L6 standard were detected for both branched polysaccharides. Similar differences between laminarin and curdlan hydrolysis products after a prolonged hydrolysis reaction were reported earlier (Pluvinage et al., 2017). These additional spots are most likely linear  $\beta$ -1,3 glucan oligosaccharides with  $\beta$ -1,6 branches. They appear as a result of incomplete hydrolysis, which is limited by  $\beta$ -1,6 bonds. Another difference between the hydrolysis products of these two groups of polysaccharides is possibly visible due to their different terminal monosaccharide compositions. The hydrolysis products corresponding to the L3 and L4 standards are present in laminarin, but not in yeast  $\beta$ -glucan. Yeast  $\beta$ -glucan contains glucose in the  $\beta$ -1,3-glucan backbone, whereas the terminal sugar of laminarin can be either mannitol or glucose (Read et al., 1996). Therefore, laminarin may contain longer terminal oligosaccharides that are non-hydrolysable due to the presence of mannitol compared to oligosaccharides with the same DP containing only glucose.

Analysis of the hydrolysis products of laminari-oligosaccharides produced by PsLam81A and PsLam81A $\Delta$ CBM56 showed that both enzymes were able to degrade laminarihexaose (L6), laminaripentaose (L5), laminaritetraose

(L4), laminaritriose (L3) to laminaribiose (L2) and glucose (Fig. 3.16a, 3.16b). Laminaritetraose, laminaripentaose and laminarihexaose were completely hydrolysed after two-hour incubation, while laminaritriose was degraded not completely.

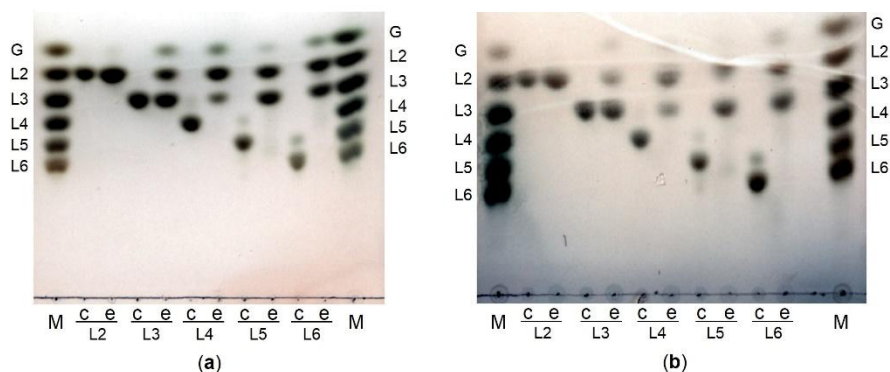


**Figure 3.16.** TLC analysis of hydrolysis products. The hydrolysis of laminari-oligosaccharides (laminaribiose (L2), laminaritriose (L3), laminaritetraose (L4), laminaripentaose (L5), laminarihexaose (L6) was carried out using PsLam81A (a) and PsLam81AΔCBM56 (b). Carbohydrate standards (M): glucose (G), laminaribiose (L2), laminaritriose (L3), laminaritetraose (L4), laminaripentaose (L5) laminarihexaose (L6). Reactions were carried out in the presence (e) and absence (c) of the enzyme.

However, the prolonged incubation of 18 h of PsLam81A and PsLam81AΔCBM56 with curdlan and pachyman resulted in the production of glucose and laminaribiose without the accumulation of laminaritriose (Fig. 3.15a, 3.15b). These observations indicate that the tested enzymes have lower activity towards shorter oligosaccharides than towards longer ones. This is consistent with previously reported data on the endo-1,3- $\beta$ -hydrolytic activity of the GH81 hydrolases, where a decrease in activity correlated with a decreased oligosaccharide length (Fliegmann et al., 2005; Fontaine et al., 1997; Ma et al., 2022; Martín-Cuadrado et al., 2008).

Neither PsLam81A nor PsLam81AΔCBM56 degraded laminaribiose, indicating that laminaritriose was the minimal substrate for both enzymes. Another endo-1,3- $\beta$ -glucanase belonging to the GH81 family has been reported to use laminaritriose as a minimal substrate (Pluvinage et al., 2017). Moreover, the disappearance of laminaritriose during the hydrolysis of laminarihexaose suggests that laminaritriose can be used as a substrate by endo-1,3- $\beta$ -glucanase Lam81A from *Thermobifida fusca* (McGrath and Wilson, 2006). Also, the decreasing proportion of laminaritriose with increasing curdlan hydrolysis time indicates that laminaritriose is a substrate of the enzyme from *Clostridium thermocellum* (Kumar et al., 2018). However, the ability to hydrolyse laminaritriose cannot be used as the only criterion,

since low activity on laminaritriose does not necessarily indicate that it is a substrate for GH81 family enzymes (Fliegmann et al., 2005; Martín-Cuadrado et al., 2008).

All this data showed that, like several other hydrolases from the GH81 family, PsLam81A can act on laminaritriose as a minimal substrate. In addition, the identical results on hydrolysable polysaccharides and oligosaccharides for both enzymes PsLam81A and PsLam81A $\Delta$ CBM56 indicated that the CBM56 module does not affect the substrate specificity for oligosaccharides. However, despite having no influence on substrate specificity, the CBM56 module improves the hydrolysis efficiency of water-insoluble  $\beta$ -glucans and slightly alters the thermostability of the PsLam81A enzyme.

### 3.2.2.5 PsGly30A acts as endo-1,6- $\beta$ -glucanase

Based on the LC-MS/MS proteomics data (Appendix 3) “glycosyl hydrolase, family 30” from *Paenibacillus pasadenensis* (UniProt ID: A0A2N5N256) appeared as the nearest homolog, which was used to design the primers “Fw PsGly30A” and “Rv PsGly30A” (Appendix 1) and to clone the enzyme PsGly30A encoding gene from *Paenibacillus* sp. GKG.

Phylogenetic analysis of the amino acid sequences of the catalytic domains with endo- $\beta$ -1,6-glucanase (EC 3.2.1.75) and  $\beta$ -1,6-glucosidase (EC 3.2.1.-) activities belonging to the GH5 and GH30 families revealed that PsGly30A belonged to the GH30 family (Appendix 11). To date, several GHs that act on  $\beta$ -1,6-glucans and belong to GH30\_1 and GH30\_3 subfamilies have been characterised. GH30\_1 subfamily contains only one bacterial representative, and the GH30\_3 subfamily contains ten such GHs, of which four are of bacterial origin.

Gly30B from *Saccharophagus degradans* was the first bacterial  $\beta$ -1,6-glycosidase discovered in the GH30\_3 subfamily (Wang et al., 2018). Like most other GH30 family enzymes, PsGly30A has no auxiliary domains. As shown in Appendix 12, the GH30 family contains a diverse group of enzymes based on amino acid sequence identity, with identity percentages ranging from 26% to 98%. According to the CAZymes classification (Drula et al., 2022), the closest homologues of PsGly30A belong to the GH30\_3 subfamily. The closest homologue of PsGly30A for which a crystal structure (PDB ID: 5NGK) has been determined is endo-1,6- $\beta$ -glucanase BT3312 from *Bacteroides thetaiotaomicron* (Temple et al., 2017), with a sequence identity of 32%, and the closest characterised member of the GH30 family is CpGlu30A from *Chitinophaga pinensis* (Lu et al., 2023), which belongs to the GH30\_3 subfamily, with 40% sequence identity.

The recombinant PsGly30A protein containing the 6×His tag was produced heterologously and successfully purified using the pLATE31 vector and either the *Escherichia coli* BL21 (DE3) or LOBSTR strains. The expression conditions were selected based on the ability of the tested fractions to yield the highest concentration of reducing sugars during hydrolysis of pustulan (Fig. 3.17b) and to produce hydrolysis zones on solid medium plates containing YCW as the substrate (Fig. 3.17a).

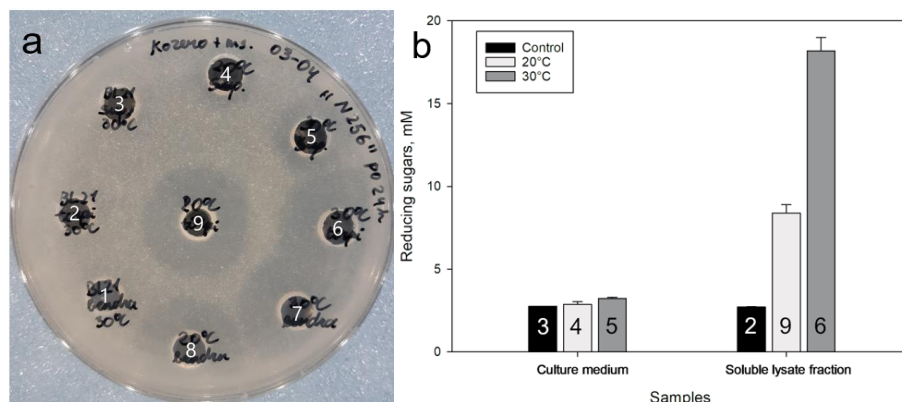


**Figure 3.17.** (a) Hydrolysis zones were produced on an agar plate with the YCW substrate. The *E. coli* BL21(DE3) strain was used. 1 – control sample total protein fraction of cell-free extract; 2 – control sample soluble protein fraction of cell-free extract; 3 – control sample culture medium fraction; 4 – culture medium fraction, PsGly30A expression induced at 20 °C; 5 – culture medium fraction, PsGly30A expression induced at 30 °C; 6 – cell-free extract soluble protein fraction, PsGly30A expression induced at 30 °C; 7 – cell-free extract total protein fraction, PsGly30A expression induced at 30 °C; 8 – cell-free extract total protein fraction, PsGly30A expression induced at 20 °C; 9 – cell-free extract soluble protein fraction, PsGly30A expression induced at 20 °C. The cell-free extract total protein fraction was obtained after ultrasonic cell disruption. A soluble protein fraction was obtained from the ultrasonically disrupted cell-free extract by centrifugation at 30,000× g for 30 min at 4 °C. Evaluation of the hydrolytic activity of the PsGly30A enzyme towards YCW. (b) Amount of reducing sugars produced after 75 min of 10 mg/mL pustulan (50 µL) hydrolysis using culture medium and soluble cell-free extract (lysate) samples (50 µL) collected after PsGly30A expression induction at 20 °C and 30 °C temperatures. The numbers in the graph columns represent the fraction of the numbers tested on the agar plate.

The homogeneity of the heterologously produced and purified PsGly30A enzyme was assessed using SDS-PAGE (Fig. 3.18). A band corresponding to the molecular weight (55 kDa) of PsGly30A was excised from an acrylamide gel and digested with trypsin according to the in-gel protein digestion protocol. LC-MS/MS analysis of the tryptic digest revealed 92% sequence coverage (Appendix 13) of the PsGly30A sequence deduced from the nucleotide sequence.

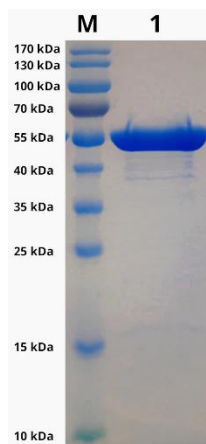


**Figure 3.18.** SDS-PAGE analysis of PsGly30A protein produced in *E. coli*. Lane M – molecular mass standard, lane 1 – the purified PsGly30A protein.

The signal peptide sequence predicted by SignalP v. 6.0 (1–31 aa) accounted for the majority of the non-covered region (1–30 aa), in which no peptides were experimentally identified. The low level of enzymatic activity in cell-free culture medium, indicating limited secretion efficiency (Fig. 3.17b), and the LC-MS/MS results showing the cleavage of signal peptide (Appendix 13) in the *E. coli* cells suggest that the PsGly30A is primarily located in the periplasm of *E. coli*.

### 3.2.2.6 Substrate specificity and optimal conditions

The specific activity of PsGly30A towards a wide range of  $\beta$ -glucans is shown in Table 3.3. Since the solubility of  $\beta$ -1,6-glucans has been reported to be affected by the temperature (e.g., pustulan is insoluble in cold water, but it is soluble in hot water) (Isbell, 1973), two sets of hydrothermally treated and hydrothermally untreated substrates were used to evaluate the activity of Ps30GlyA. Pustulan is insoluble in water, but becomes soluble after undergoing hydrothermal treatment (Chapter 2.2.8.2). Instead of the hydrothermal treatment applied to all substrates, the YCW were autoclaved to inactivate endogenous yeast enzymes.

Based on existing data, the GH30 family  $\beta$ -1,6-glycosidases can have endo- $\beta$ -1,6-glucanase (EC 3.2.1.75) and  $\beta$ -1,6-glucosidase (EC 3.2.1.-) activities (Drula et al., 2022). To determine the regioselectivity and mode of action of PsGly30A, a variety of  $\beta$ -glucans (Table 3.3) was tested. The DNS-based reducing sugar assay (Table 3.3) showed that PsGly30A was only active on polysaccharides with consecutive  $\beta$ -1,6-glycosidic bonds present – pustulan, yeast  $\beta$ -glucan and YCW.

**Table 3.3.** Substrate specificity of PsGly30A.

| Substrate                                                                                                                                                                      | Specific activity<br>(U/mg) | Relative activity<br>(%) |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------|--------------------------|
| Pustulan <sup>[a]</sup>                                                                                                                                                        | 1262±82                     | 100                      |
| Pustulan <sup>[b]</sup>                                                                                                                                                        | 322±22                      | 26                       |
| Yeast $\beta$ -glucan <sup>[a]</sup>                                                                                                                                           | 241±12                      | 19                       |
| Yeast $\beta$ -glucan <sup>[b]</sup>                                                                                                                                           | 214±7                       | 17                       |
| YCW <sup>[c]</sup>                                                                                                                                                             | 45±6                        | 4                        |
| YCW <sup>[b]</sup>                                                                                                                                                             | 28±1                        | 2                        |
| <i>p</i> NPG2 <sup>[b]</sup>                                                                                                                                                   | 365±19                      | 29                       |
| Laminarin, barley $\beta$ -glucan, carboxymethyl cellulose, $\beta$ -1,2-glucan, microcrystalline cellulose, <i>p</i> NP- $\alpha$ -glucoside, <i>p</i> NP- $\beta$ -glucoside | not detected                | -                        |

<sup>[a]</sup> Hydrothermally pre-treated and <sup>[b]</sup> untreated. <sup>[c]</sup> Autoclaved at 1 atm, 121 °C for 30 minutes. Measurements were performed in quadruplicate and expressed as (mean±SD).

The PsGly30A enzyme showed a 3.9-fold higher specific activity towards hydrothermally treated pustulan than towards untreated pustulan (Table 3.3). This difference is most likely due to a decrease in pustulan crystallinity in the hydrothermally treated water-soluble form. Therefore, water-soluble pustulan is more easily accessible to the active site of the PsGly30A. Polysaccharides tend to form crystalline regions due to hydrogen bonding between adjacent chains, and crystallinity is more prevalent in linear polysaccharides, with cellulose being the most common example (Yazdi et al., 2021). Crystalline regions form when the interactions between chains become stronger than those between the chains and water molecules. Crystallisation of polysaccharides can be controlled by the temperature treatment (Seidi et al., 2022). Similarly, the presence of CBMs appended to GH modules is thought to increase enzyme-substrate proximity and reduce the crystallinity of insoluble substrates. There are multiple cases in which CBMs improve catalytic efficiency on insoluble substrates more significantly, but have little to no effect on the hydrolysis of soluble substrates (Boraston et al., 2004), indicating that soluble substrates are more accessible and do not require the additional assistance of CBMs.

The time course of the pustulan hydrolysis was also examined (Fig. 3.19a). TLC analysis of pustulan hydrolysis showed the formation of a series of oligosaccharides of high DPs at initial stages of reaction, and gentiobiose and glucose were identified as ultimate products after a prolonged incubation (Fig. 3.19a). These results indicated a random cleavage of  $\beta$ -1,6-bonds along the

pustulan chain – a feature typical of the mode of action of endo-1,6- $\beta$ -glucanases. Furthermore, PsGly30A was not active against laminarin, as indicated by reducing-sugar analysis (Table 3.3) and TLC (Fig. 3.20a). In addition, PsGly30A did not hydrolyse gentiobiose efficiently (Fig. 3.20b). These results demonstrated that PsGly30A is an endo-1,6- $\beta$ -glucanase (EC 3.2.1.75) but not a laminarin-debranching  $\beta$ -1,6-glucosidase (EC 3.2.1.-).

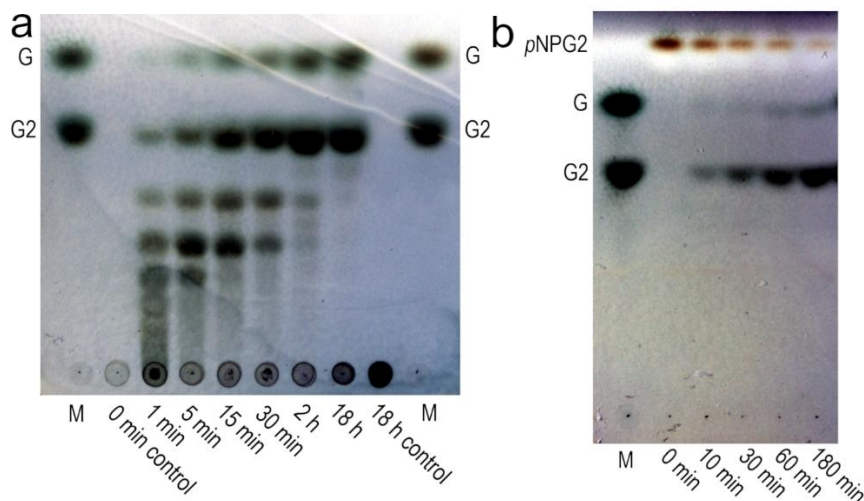


**Figure 3.19.** TLC analysis of hydrolysis products of hydrothermally treated pustulan, pNPG2, laminarin and gentiobiose after incubation with PsGly30A. (a) Pustulan hydrolysis. Reactions were carried out in the presence (1 min – 18 h) and absence (0 min control and 18 h control) of the PsGly30A. (b) pNPG2 hydrolysis. Reactions were carried out in the presence (10–180 min) and absence (0 min) of the PsGly30A enzyme. Carbohydrate standards (M): glucose (G), gentiobiose (G2).

Similarly, other recently reported endo-1,6- $\beta$ -glucanases, e.g., CpGlu30A from *Chitinophaga pinensis* (Lu et al., 2023) and GH30A from *Coprinosia cinerea* (Liu et al., 2020), showed activity against pustulan but not against laminarin from *Laminaria digitata*. However, Sde\_2994 from *Saccharophagus degradans* (Wang et al., 2017), FbGH30 from *Formosa bacterium* (Becker et al., 2017) and GH30A from *C. cinerea* (Liu et al., 2020) hydrolysed laminarin from *Eisenia bicyclis*.

Most likely, these differences are observed due to structural differences between laminarin from *Laminaria digitata* with  $\beta$ -1,3- bonds as a backbone and  $\beta$ -1,6 bonds as side branches (Read et al., 1996) and *Eisenia bicyclis* with  $\beta$ -1,3 and  $\beta$ -1,6 bonds as a backbone in a ratio of 3:2 (Nobe et al., 2003). An example confirming this difference was presented in a study published by Liu and colleagues, where endo-1,6- $\beta$ -glucanase GH30A from *C. cinerea* did not hydrolyse laminarin from *Laminaria digitata*, but hydrolysed one from *Eisenia bicyclis* (Liu et al., 2020).

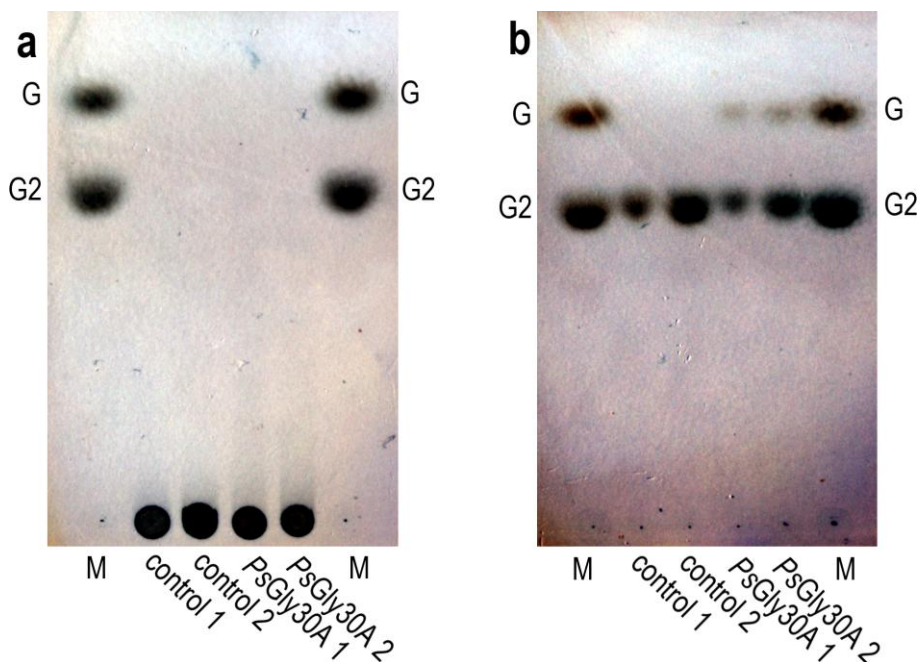


**Figure 3.20.** TLC analysis of hydrolysis products after incubation of (a) laminarin and (b) gentiobiose with PsGly30A at 37 °C for 18 hours. Carbohydrate standards (M): glucose (G), gentiobiose (G2). Reactions were carried out in the presence (PsGly30A 1, PsGly30A 2) and absence (control 1, control 2) of the PsGly30A enzyme. M lanes contain 5 µg each of loaded carbohydrate standards, control 1 and PsGly30A 1 lanes contain 9 µg of laminarin or 2.25 µg of gentiobiose, control 2 and PsGly30A 2 lanes contain 18 µg of laminarin or 4.5 µg of gentiobiose.

Among all tested substrates, pustulan was the most efficiently hydrolysed substrate. This observation was consistent with the previous reports on GH30  $\beta$ -1,6-glycosidases (Boisramé and Gaillardin, 2009; De La Cruz et al., 1995; Konno and Sakamoto, 2011; Liu et al., 2020; Lu et al., 2023; Wang et al., 2017). In addition, the specific activity ( $1262 \pm 82$  U/mg) of PsGly30A towards pustulan was the highest one comparing to previously characterised GH30 family  $\beta$ -1,6-glycosidases:  $776.50 \pm 1.02$  U/mg for GH30A from *C. cinerea* (Liu et al., 2020), 86 U/mg for  $\beta$ -1,6-glucanase from *Trichoderma harzianum* (De La Cruz et al., 1995); 83 U/mg for AfNeg1 from *Aspergillus fumigatus* (Boisramé and Gaillardin, 2009); and 14.8 U/mg for LePus30A from *Lentinula edodes* (Konno and Sakamoto, 2011). The majority of studies did not specify whether hydrothermally treated or untreated pustulan was used; also, the structure of pustulan can vary greatly between the same and different source microorganism species and storage conditions (Narui et al., 1999). Nevertheless, even when the PsGly30A enzyme was used to hydrolyse an untreated pustulan, the specific activity ( $322 \pm 22$  U/mg) exceeded the majority of previously reported values for the GH30 family (Boisramé and Gaillardin,

2009; De La Cruz et al., 1995; Konno and Sakamoto, 2011). The lower activity of PsGly30A towards yeast  $\beta$ -glucan (Table 3.3) as well as towards a crude preparation of YCW compared to pustulan corresponded to previously published data on GH30 family enzymes (Liu et al., 2020; Lu et al., 2023). Overall, the high specific activity of PsGly30A compared to that of previously characterised GH30 family  $\beta$ -1,6-glycosidases indicates its potential for application in converting  $\beta$ -1,6-glucans to gentiobiose.

To evaluate the dependence of PsGly30A activity on pH and temperature, pustulan was chosen as the substrate. This polysaccharide is a linear  $\beta$ -1,6-glucan extracted from *Umbilicariaceae* fungi (Zeković et al., 2005). Pustulan from *Lasallia pustulata* has an average molecular mass of 20 kDa and is the most commonly used substrate for the characterisation of  $\beta$ -1,6-glycosidases. The highest activity of PsGly30A towards this substrate was observed at pH 5.5 in a 50 mM MES buffer (Fig. 3.21a). In addition, a small deviation in activity was observed at the same pH when buffers of different chemical composition were used.

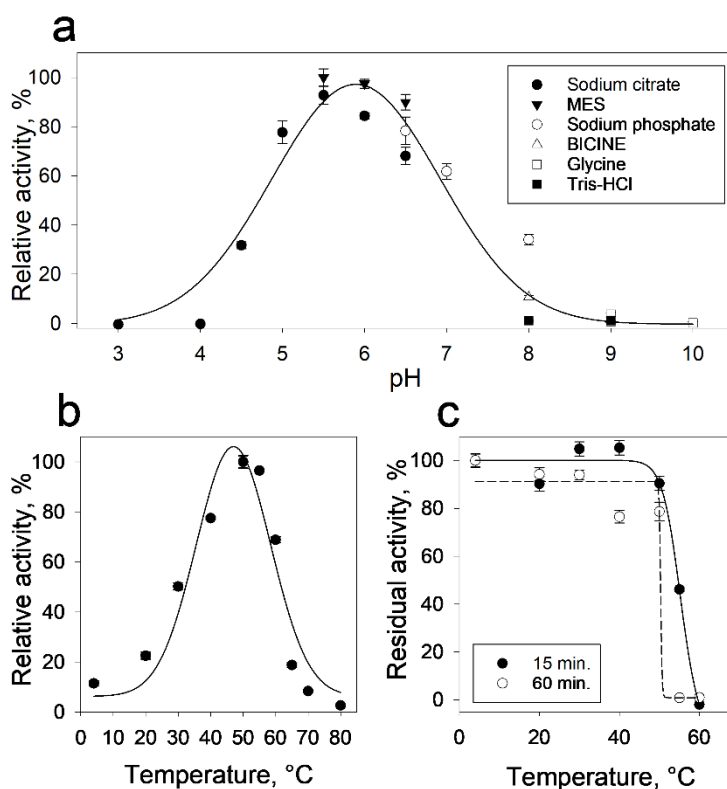


**Figure 3.21.** Effect of (a) pH and (b) temperature on PsGly30A activity. (c) Effect of temperature on PsGly30A stability after pre-incubation at indicated temperatures. Measurements were performed in quadruplicate. Error bars represent SD.

One of the main disadvantages of buffers such as citrate, phosphate, Tris, and Bicine is considered to be the formation of complexes with polyvalent metal cations, making them unavailable to the enzymes, while the MES buffer does not form such complexes (Ferreira et al., 2015). However, it appears that the complex formation may not be the main reason in this case, since the activity of PsGly30A is among the highest in citrate and phosphate buffers and among the lowest in Tris. Other GHs from the GH30\_3 subfamily show either increased activity, e.g. GH30A from *C. cinerea* (Liu et al., 2020), or decreased activity, e.g. Gly30B from *Saccharophagus degradans* (Wang et al., 2018), when reactions were supplemented with different metal cations.

In comparison, the optimum pH of the previously characterised GH30  $\beta$ -1,6-glycosidases ranges from pH 4.5 for AfNeg1 from *Aspergillus fumigatus* (Boisramé and Gaillardin, 2009) to pH 7.0 for Gly30B from *Saccharophagus degradans* (Wang et al., 2017) and FbGH30 from *Formosa* sp. Hell\_33\_131 (Becker et al., 2017).

Regarding optimal temperature, the highest PsGly30A activity was observed at 50 °C (Fig. 3.21b). In comparison, up-to-date characterised GH30 family  $\beta$ -1,6-glycosidases showed the highest temperature activity between 40 °C for Gly30B from *Saccharophagus degradans* (Wang et al., 2017) and 60 °C for GH30A from *C. cinerea* (Liu et al., 2020).

PsGly30A retained more than 75% of its initial activity after 15 min and 60 min pre-incubation at temperatures below 50 °C, but lost activity after pre-incubation at 60 °C (Fig. 3.21c). In comparison, GH30A from *C. cinerea* (Liu et al., 2020) showed the highest thermal stability, retaining 88% of residual activity after 1 h of preincubation at 60 °C. In contrast, Gly30B from *Saccharophagus degradans* (Wang et al., 2017) showed the lowest thermal stability, retaining more than 90% of activity after 1 h of pre-incubation at temperatures below 40 °C and retaining approximately 20% of activity after 1 h of pre-incubation at temperatures between 50 °C and 70 °C. In conclusion, PsGly30A exhibits a thermostability characteristic of mesophilic enzymes, which is moderate compared with other GH30 family  $\beta$ -1,6-glycosidases.

#### 3.2.2.7 Gentiobiose is produced from $\beta$ -glucans

Some enzymes in the GH30 family, such as FbGH30 from the *Formosa* bacterium (Becker et al., 2017) and Gly30B from *S. degradans* (Wang et al., 2017) can efficiently hydrolyse gentiobiose. In addition, the commercial product “Lysing enzymes from *T. harzianum*” completely hydrolysed gentiobiose to glucose when  $\beta$ -1,3/1,6-glucan from *A. pullulans* was used as a substrate (Hirabayashi et al., 2016). Such enzymes are suitable for hydrolysing polysaccharides to glucose or for the determination of the composition of

polysaccharides containing mixed-linkage  $\beta$ -glucans (Becker et al., 2017). However, such GHs are not the most effective tools for producing gentiobiose, which is only present at the initial stage of hydrolysis and is efficiently hydrolysed down to glucose during prolonged incubation (Hirabayashi et al., 2016; Wang et al., 2017). PsGly30A produced only trace amounts of glucose after 18 h of incubation with gentiobiose (Fig. 3.20b). Similar results have been reported for GH30A from *C. cinerea* – no evidence of gentiobiose hydrolysis was observed after 20 min of incubation (Liu et al., 2020) and for LePus30A from *L. edodes* showed no evidence of gentiobiose hydrolysis (Konno and Sakamoto, 2011).

The distinctive property of PsGly30A, which is its ability to produce gentiobiose at a more favourable gentiobiose-to-glucose ratio compared to other previously characterised GH30 family  $\beta$ -1,6-glycosidases, may be due to structural differences in the enzyme. Only one crystal structure of endo-1,6- $\beta$ -glucanase has been solved (5NGL, *B. thetaiotaomicron*) (Temple et al., 2017). The AlphaFold-predicted structure of PsGly30A can be superimposed on that of *B. thetaiotaomicron* with a root-mean-square deviation of 0.949 Å over 311 Ca carbon atoms (Fig. 3.22a). While there are high structural similarities in the active site environment, some structural differences can be observed in the predicted substrate binding region. Based on the 3D model and multiple sequence alignment (MSA) (Appendix 14), the catalytic residues can be predicted to be Glu240 (acid/base) and Glu340 (nucleophile) (Fig. 3.22b). The potential substrate-binding subsites –2 and –1 comprise the other highly conserved amino acids (Asp139, Trp189, Asn239, Tyr316, Trp346, and Trp378). It is difficult to predict the exact number of substrate-binding subsites from the structure alone. However, analysis of the products of pustulan hydrolysis by PsGly30A suggests that there are likely to be at least three subsites, since  $\beta$ -1,6-glucotriose is the smallest oligosaccharide towards which PsGly30A exhibits reasonable activity, and  $\beta$ -1,6-glucotetraose accumulates at the initial stages of hydrolysis.

Comparing the structure of 5NGL with the 3D model of PsGly30A reveals differences in the topology of the substrate-binding clefts, as well as in the electrostatic potential of these regions (Fig. 3.22c). We hypothesise that the profile of end products of pustulan hydrolysis is determined by the number of substrate-binding subsites of the enzyme and, consequently, its different affinity for and activity on oligosaccharides with different degrees of polymerisation.

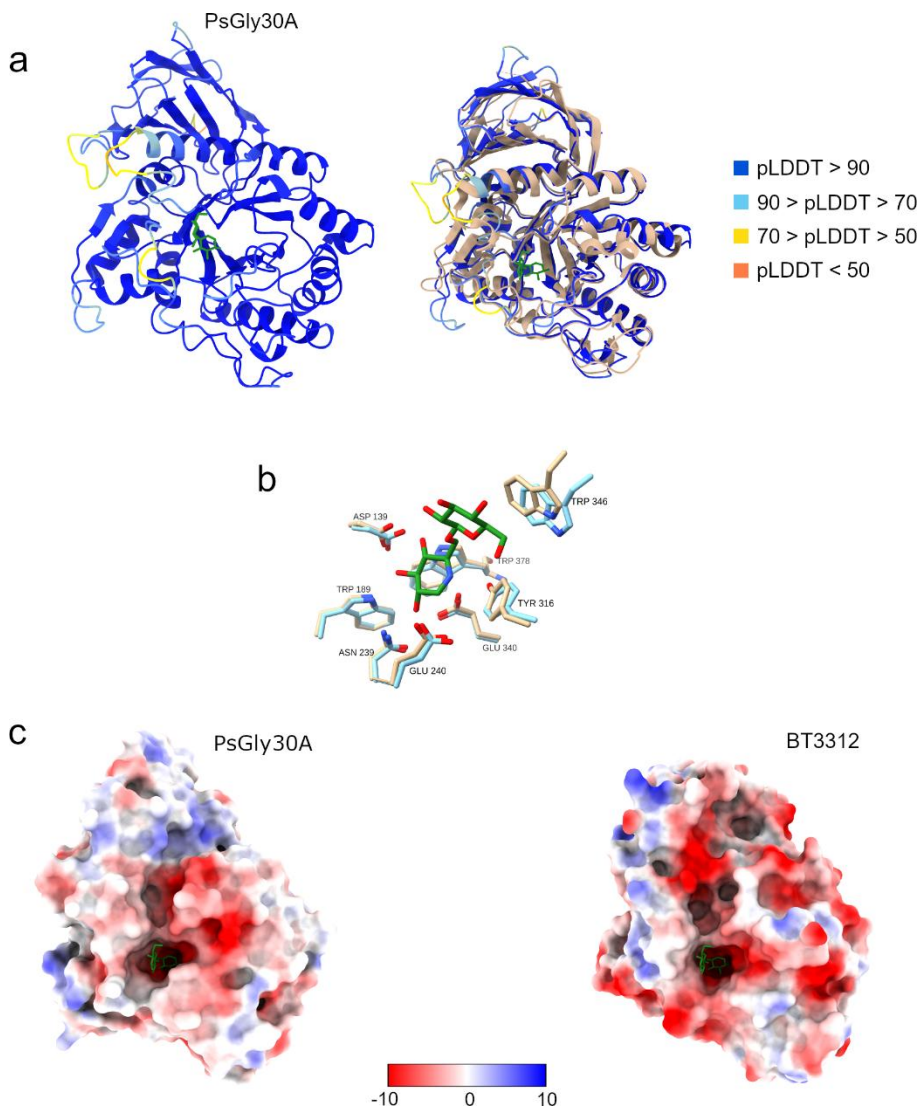


**Figure 3.22.** The 3D model structure of PsGly30A was generated using AlphaFold. (a) A ribbon plot showing the entire PsGly30A scaffold, with a colour gradient representing pLDDT values, and a superposition of PsGly30A and BT3312 (5NGL). (b) A closer view of the superposed residues in the active site environment, including the ligand  $\beta$ -glucosyl-1,6-deoxynojirimycin. (c) Surface models of PsGly30A and BT3312 showing the difference in electrostatic potential at the substrate-binding cleft. Dr. Justas Vaitekūnas carried out the bioinformatics analysis.

In terms of specific activity, pustulan is the optimal substrate for GH30 family endo-1,6- $\beta$ -glucanases. However, other substrates are more technologically relevant. Spent brewer's yeast, for example, is an industrial by-product of the brewing process, with an annual accumulation of 0.9 million tonnes (Avramia and Amariei, 2021). While the  $\beta$ -1,6-glucan content of YCW is lower than that of pustulan, there is potential to utilise yeast  $\beta$ -glucans to

produce gentiobiose (Rombouts and Phaff, 1976b). PsGly30A produced gentiobiose from three different substrates (Fig. 3.23a and 3.23b).

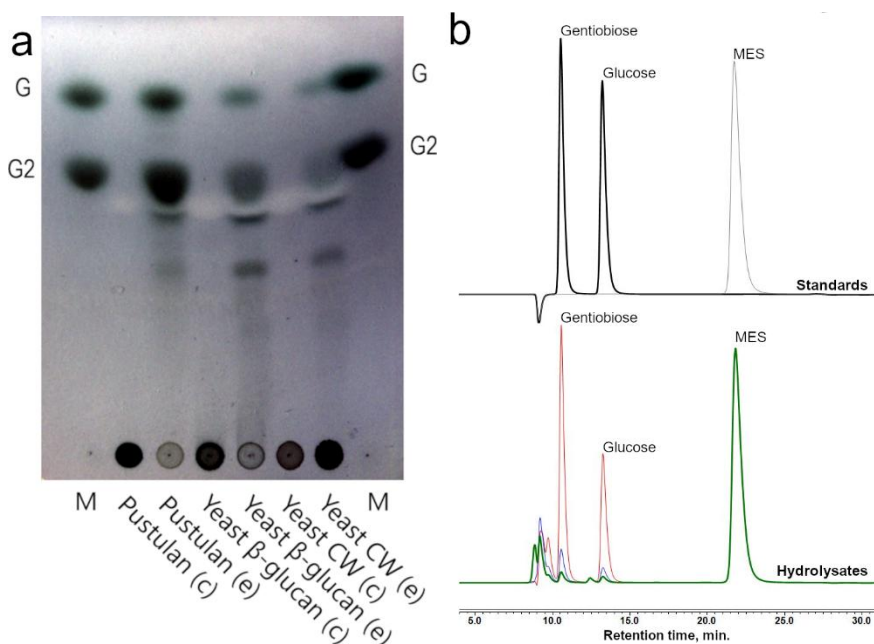


**Figure 3.23.** Production of gentiobiose from pustulan, yeast  $\beta$ -glucan and YCW after treatment with PsGly30A for 18 hours: (a) TLC analysis of hydrolysis products. Carbohydrate standards (M): glucose (G), gentiobiose (G2). Reactions were carried out in the presence (e) or absence (c) of PsGly30A. (b) HPAEC-RI analysis of the hydrolysis products. Standard chromatogram: 5 mg/mL glucose and gentiobiose standard mixture (1:1.01 glucose to gentiobiose area ratio), 50 mM MES buffer. The hydrolysate chromatogram shows pustulan (red), yeast  $\beta$ -glucan (blue) and YCW (green). The pustulan hydrolysis products resulted in a glucose:gentiobiose ratio of 1:1.68.

Analysis of the compounds obtained after the hydrolysis of hydrothermally treated pustulan using PsGly30A revealed that gentiobiose was the main product (Fig. 3.23b). By contrast, the major product obtained from pustulan after prolonged hydrolysis using BT3312 from *B. thetaiotaomicron* (Temple et al., 2017), CpGlu30A from *C. pinensis* (Lu et al., 2023), GH30A from *C. cinerea* (Liu et al., 2020), and Gly30B from *S. degradans* (Wang et al., 2017) was glucose.

As expected, the hydrolysis of yeast  $\beta$ -glucan and YCW using PsGly30A produced significantly lower concentrations of gentiobiose. Nevertheless, a similar pattern of additional products was observed compared to pustulan hydrolysis (Fig. 3.23a). While the removal of glucose is relatively straightforward using *Saccharomyces cerevisiae* yeast (Moreno et al., 2014), the separation of the remaining oligosaccharides may present a greater challenge and require additional treatment. Nevertheless, utilising yeast  $\beta$ -

glucans is a more promising prospect for the industrial production of gentiobiose than pustulan.

Overall, the PsGly30A enzyme shows a high specific activity on pustulan and a low hydrolytic activity towards gentiobiose, and these properties make PsGly30A a suitable candidate for the conversion of various  $\beta$ -1,6-glucans (e.g., pustulan, yeast  $\beta$ -glucan, YCW) to gentiobiose.

### 3.2.3 Yeast-lytic GHs from *Cellulosimicrobium*

The homogeneity of the heterologously produced and purified GH16 and GH64 enzymes was assessed using SDS-PAGE (Fig. 3.24). The bands corresponded to the theoretical molecular masses of the GH16 (42 kDa) and GH64 (56 kDa) enzymes, with their signal sequences removed – GH16 (1–52), GH64 (1–36 amino acids) according to the SignalP 6.0 server (Teufel et al., 2022).

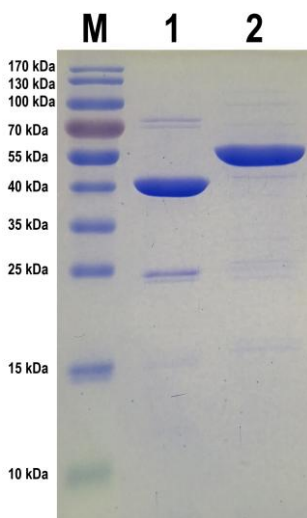


**Figure 3.24.** SDS-PAGE analysis of recombinant lytic endo-1,3- $\beta$ -glucanases produced in *E. coli*. Lane M – molecular mass standard, lane 1 – the GH16 protein after Ni-NTA purification, lane 2 – the GH64 protein after Ni-NTA purification.

The substrate specificity of the GH16 and GH64 enzymes was evaluated prior to testing their ability to lyse living yeast cells. The hydrolysis product patterns of the purified recombinant GH16 and GH64 enzymes, as well as the *Zymolyase* yeast lytic enzymatic preparation, were compared when used on laminarin (Fig. 3.25a) and yeast  $\beta$ -glucan (Fig. 3.25b).

The GH16 enzyme produced laminaribiose and glucose as the main hydrolysis products from laminarin and yeast  $\beta$ -glucan. Meanwhile, the GH64 enzyme produced laminaripentaose as the primary hydrolysis product, regardless of the substrate used. These patterns corresponded to those of GH16

family members that produce laminaribiose and GH64 family members that produce laminaripentaose (Drula et al., 2022). *Zymolyase* exhibited a hydrolysis product pattern similar to that of the GH16 enzyme. This pattern also matches *Zymolyase*'s enzymatic activities, as it exhibits both laminaribiose- and laminaripentaose-producing endo-1,3- $\beta$ -glucanase activities (Nacalai Tesque, Inc., 2025).

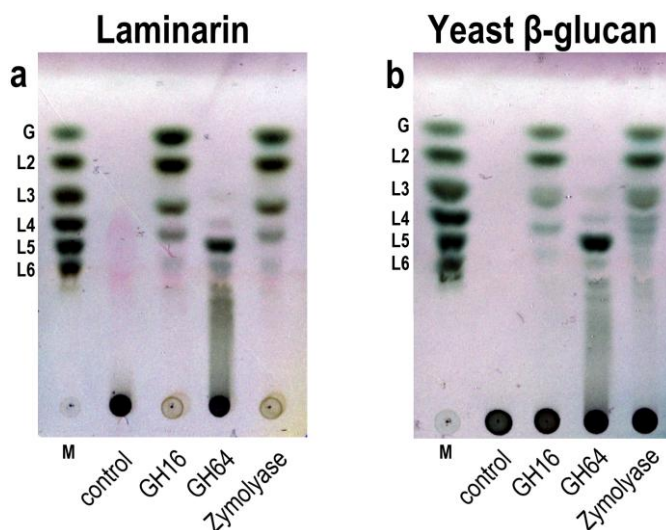


**Figure 3.25.** TLC analysis of the hydrolysis products of laminarin (a) and yeast  $\beta$ -glucan (b). Recombinant GH16 and GH64 glucanases as well as *Zymolyase*, were used to hydrolyse laminarin and yeast  $\beta$ -glucan. The carbohydrate standards (M): glucose (G), laminaribiose (L2), laminaritriose (L3), laminaritetraose (L4), laminaripentaose (L5) and laminarihexaose (L6). Control reactions were carried out in the absence of the enzyme.

Similar observations on the substrate specificity patterns of two different glucanases produced by *Arthrobacter* sp. D3 were reported in early studies (Doi and Doi, 1986). Glucanase I was found to rapidly solubilise YCW and produce laminaripentaose, whereas glucanase II only partially solubilised YCW and produced laminaribiose as the main hydrolysis product. However, these observations were based solely on YCW digestion and did not evaluate the ability to lyse living yeast cells.

Having confirmed that both the recombinant lytic endo-1,3- $\beta$ -glucanases and the *Zymolyase* exhibited the expected substrate specificity, the next step was to evaluate and compare their ability to lyse living yeast cells (Chapter 3.4).

### 3.3 Application of GHs to convert YCW to oligosaccharides

Investigation of secretome and YCW-induced proteins from *Rheinheimera* sp. GKU, *Paenibacillus* sp. GKG, and *Cellulosimicrobium* sp. GKH led to a list of seven GHs that produced four different sugars and oligosaccharides (laminaribiose, gentiobiose, laminaripentaose, and glucose) as the main hydrolysis products when used on yeast  $\beta$ -glucan and YCW. Out of seven GHs, three were selected for the production and quantitative evaluation of three functional and bioactive oligosaccharides (laminaribiose, gentiobiose, laminaripentaose) (Fig. 3.26a, c, d).

Four different enzymes, PsLam81A, GH16, GH16\_306, and GH16\_3455, produced laminaribiose, a disaccharide with prebiotic properties, among their major hydrolysis products. However, PsLam81A was selected as the most promising one (Fig. 3.15a). Based on initial qualitative assessment, PsLam81A enzyme solubilised the largest proportion of YCW (Fig. 3.26a). In contrast, the GH16 enzyme, which is also an endo-1,3- $\beta$ -glucanase, produced laminaribiose (Fig. 3.25b), but YCW solubilisation efficiency was lower (Fig. 3.26b) than that of PsLam81A (Fig. 3.26a). Similar YCW solubilisation efficiencies (data not shown) were obtained for other laminaribiose-producing (Fig. 3.9) GH16 family endo-1,3- $\beta$ -glucanases: GH16\_306 and GH16\_3455. In addition, PsLam81A enzyme produced a favourable high laminaribiose-to-glucose ratio (Fig 3.15). Other enzymes, GH16, GH16\_306, and GH16\_3455, also performed worse under these criteria (Fig. 3.9, 3.25b).

Endo-1,6- $\beta$ -glucanase PsGly30A was the only candidate that produced taste-enhancing disaccharide – gentiobiose (Fig. 3.23) and was selected for application trial. YCW solubilisation efficiency was also low (Fig. 3.26c). However, this can be explained by the low proportion of  $\beta$ -1,6-glucan in YCW.

Another candidate with sole laminaripentaose-producing endo-1,3- $\beta$ -glucanase activity was the GH64 enzyme. It produced a bioactive oligosaccharide laminaripentaose (Fig. 3.25) and was selected for the application trial. In addition, it solubilised YCW efficiently (Fig. 3.26d), in contrast to laminaribiose-producing GH16 (Fig. 3.26b). Similar YCW solubilisation difference was reported for laminaribiose- and laminaripentaose-producing endo-1,3- $\beta$ -glucanases from *Cellulosimicrobium* (also known as *Arthrobacter*) in a previous study (Doi and Doi, 1986).

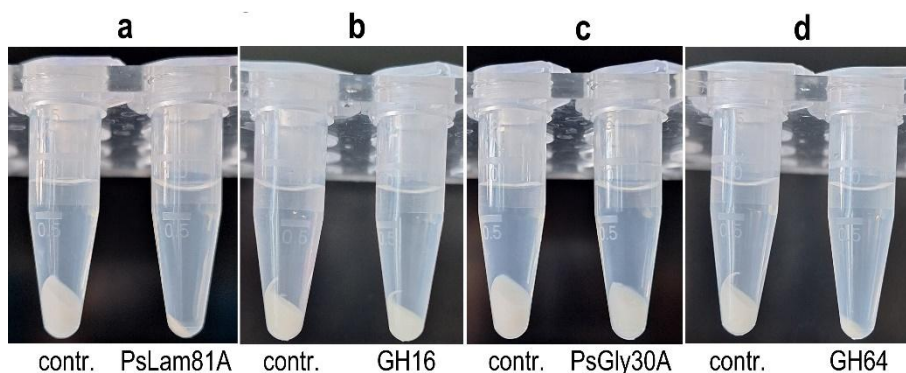


**Figure 3.26.** Qualitative evaluation of yeast CW hydrolysis efficiency. a – laminaribiose-producing endo-1,3- $\beta$ -glucanase PsLam81A from *Paenibacillus*, b – laminaribiose-producing endo-1,3- $\beta$ -glucanase GH16 from *Cellulosimicrobium*, c - gentiobiose-producing endo-1,6- $\beta$ -glucanase PsGly30A from *Paenibacillus*, d – laminaripentaose-producing endo-1,3- $\beta$ -glucanase GH64 from *Cellulosimicrobium*. Abbreviation “contr.” represents control reactions where YCW were incubated in the absence of enzymes.

$\beta$ -Glucosidase GH3\_3456 was not selected for application trials because it produced glucose rather than oligosaccharides (Fig. 3.9b). The next step was the quantitative determination of the concentrations of the target oligosaccharides and the calculation of the conversion yields of YCW  $\beta$ -glucans into laminaripentaose, laminaribiose, and gentiobiose.

### 3.3.1 Quantitative evaluation of oligosaccharide yield

The concentrations of laminaripentaose, laminaribiose, and gentiobiose in YCW hydrolysates were determined using an HPLC-RI method. Laminaripentaose was obtained after application of GH64, laminaribiose – after application of PsLam81A, and gentiobiose – after application of PsGly30A enzymes. The retention times ( $t_R$ ) of laminaripentaose ( $t_R=28.8$  min) and laminaribiose ( $t_R=41.0$  min) were different enough and peaks resolved well, whereas gentiobiose ( $t_R=39.9$  min) and laminaribiose ( $t_R=41.0$  min) were co-eluting (Fig. 3.27a). Two separate methods were applied: one for simultaneous quantification of laminaripentaose and laminaribiose and the other for quantification of gentiobiose. Both methods achieved good linearity ( $R^2>0.999$ ) (Fig. 3.27b).

The calculations were made using the previously reported (Klis et al., 2002) ratio of  $\beta$ -1,3-glucan (50–55%) to  $\beta$ -1,6-glucan (5–10%) in YCW, the experimentally determined  $\beta$ -glucan content ( $35.7\pm0.3\%$  w/w,  $n=2$ ), and dissolved solids content ( $91.2\pm0.1\%$ ,  $n=2$ ) in YCW determined in this study. The results are presented in Table 3.4.

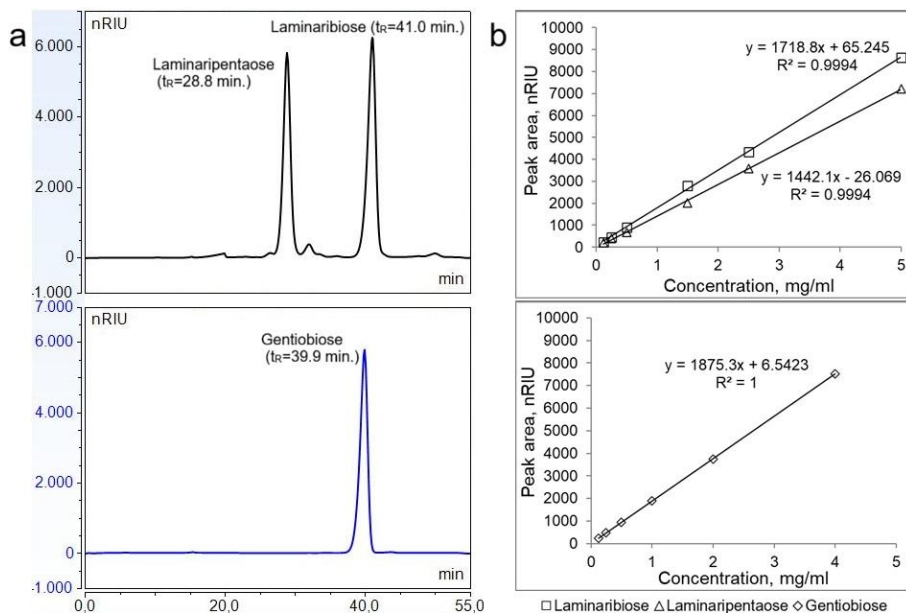


**Figure 3.27.** Quantitative HPLC-RI method for laminaribiose, laminaripentaose, and gentiobiose. a – chromatograms of standard compounds, b – calibration data.

A formula (1) was used to calculate the conversion yield for each of the oligosaccharides:

$$\text{Conversion yield (\%)} = \frac{OLIG}{YCW \times \frac{DS}{100} \times \frac{YBGC}{100} \times \frac{YBGP}{100}} \times 100\% \quad (1)$$

*OLIG* – concentration of oligosaccharide, determined by HPLC-RI (mg/mL);

*YCW* – concentration of YCW substrate (mg/mL);

*DS* – content of dissolved solids in YCW (%);

*YBGC* –  $\beta$ -glucan content in YCW (% w/w);

*YBGP* – theoretical proportion of target  $\beta$ -glucan in  $\beta$ -1,3/1,6-glucan polymer. 0.875 for laminaribiose and laminaripentaose ( $\beta$ -1,3-glucan), and 0.125 for gentiobiose ( $\beta$ -1,6-glucan).

The main factor limiting the conversion yield is the branched structure of yeast  $\beta$ -glucan and YCW. The  $\beta$ -1,3/1,6-branching degree of *Saccharomyces* yeast  $\beta$ -glucan is 7% (Aimanianda et al., 2009). In addition, cross-links between other YCW polymers are present as well (Fig. 1.3). These cross-links make yeast  $\beta$ -1,3/1,6-glucan inaccessible for hydrolysis with endo-1,3/1,6- $\beta$ -glucanases. Conversion yields could possibly be improved by using additional enzymes that debranch  $\beta$ -glucan or hydrolyse other YCW polymers. However, simultaneous conversion of  $\beta$ -1,3-glucans and  $\beta$ -1,6-glucans becomes unavailable, as one of the glucans must be hydrolysed to glucose. Another

possible measure would be chemical treatment of YCW to disrupt inter-polymer cross-links.

**Table 3.4.** Evaluation of  $\beta$ -glucan, present in the YCW, enzymatic conversion to oligosaccharides yield. Values presented as Average $\pm$ SD (n=2). Jurgita Kailiuvienė collected experimental data.

| Oligosaccharide  | Conversion yield, % |
|------------------|---------------------|
| Laminaribiose    | 78.1 $\pm$ 1.7      |
| Gentiobiose      | 72.5 $\pm$ 1.1      |
| Laminaripentaose | 51.4 $\pm$ 0.4      |

Generally, conversion yields look promising. From 51% to 78% of the  $\beta$ -1,3/1,6-glucan present in the low-value industrial-scale by-product YCW can be converted into bioactive and functional oligosaccharides using an environmentally friendly enzymatic hydrolysis method.

### 3.3.2 $p$ NP- $\beta$ -gentiobioside for residual activity measurement

From a practical standpoint, the enzyme must be used in a way that makes economic sense. The ability to trace residual activity after extended periods of hydrolysis allows for minimising the amount of enzyme used and considering enzyme reuse.

PsGly30A did not hydrolyse  $p$ NP- $\alpha$ -glucoside and  $p$ NP- $\beta$ -glucoside (Table 3.3), nor did GH30A from *C. cinerea* hydrolyse  $p$ NP- $\beta$ -glucoside (Liu et al., 2020). At the time of our investigation,  $p$ NP- $\beta$ -gentiobioside ( $p$ NPG2) was not commercially available.  $p$ NPG2 is typically synthesised using trans-glycosylation reactions (Arthornthurasuk et al., 2018; Hattori et al., 2013; Hrmova et al., 1998; Nakatani et al., 2014), but these methods produce various  $p$ NP-sugars and/or  $p$ NP-gentio-oligosaccharides (DP2–6) as by-products. Therefore,  $p$ NPG2 was synthesised chemically from gentiobiose using a novel approach, as described in our previous work (Plakys et al., 2024) and illustrated in Fig. 3.28.

PsGly30A released gentiobiose from  $p$ NPG2 (Fig. 3.19b). However, traces of the glucose appeared during the prolonged reaction, suggesting two possible side reactions. Firstly, the PsGly30A may hydrolyse the released gentiobiose to produce glucose. Secondly, the PsGly30A may hydrolyse  $p$ NPG2 to  $p$ NP- $\beta$ -glucoside and glucose rather than  $p$ NP and gentiobiose. Given the very low hydrolysis rate of gentiobiose (Fig. 3.20b), the second reaction is more likely. Nevertheless, this side reaction was considered negligible over the 30 min period that was used for further experiments with  $p$ NPG2 as a substrate. Compared to pustulan, the specific activity of

PsGly30A towards *p*NPG2 was 3.5-fold lower (Table 3.3). In contrast, a 430-fold difference between pustulan and *p*NPG2 was reported for endo-1,6- $\beta$ -glucanase from *Trichoderma harzianum* (De La Cruz et al., 1995).

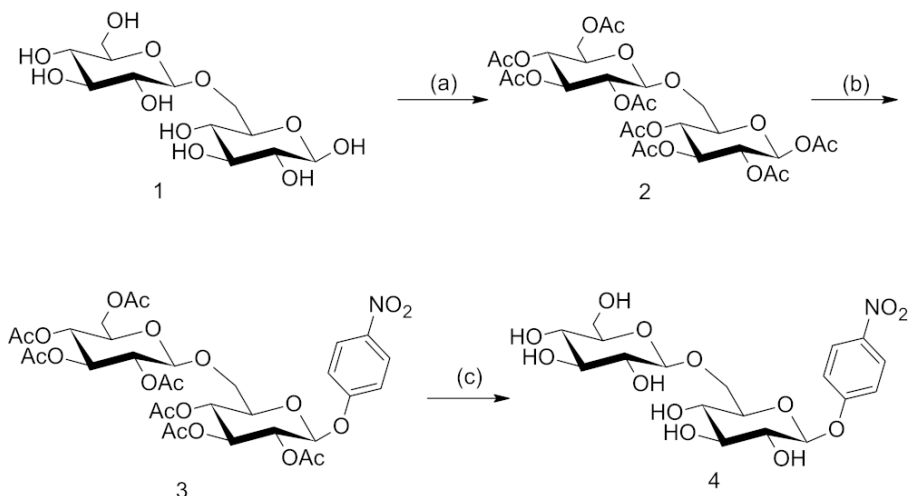


**Figure 3.28.** A principal scheme of synthesis of 4-nitrophenyl 6-O-( $\beta$ -D-glucopyranosyl)- $\beta$ -D-glucopyranoside (*p*NPG2) (compound 4). Gintaras Urbelis carried out the synthesis.

From a practical standpoint, it is necessary to monitor enzyme activity during the hydrolysis reaction to select the most efficient biocatalytic process. One of the most widely used methods for measuring GH enzyme activity is the DNS-based reducing sugar assay. However, this method is only applicable to a limited extent in biomass hydrolysates because the reducing sugars released from the biomass interfere with the measurement. As separating the enzyme from the mixture is challenging, methods that do not interfere with reducing sugars are preferable. Pro-chromogenic substrates containing sugars linked to 4-nitrophenol (*p*NP) are used to evaluate the substrate specificity and kinetic parameters of various GHs (Bastien et al., 2013).

Here, we used *p*NPG2 to measure the residual activity of PsGly30A in the YCW hydrolysate (Fig. 3.29). The data revealed that, following a 24-hour hydrolysis reaction, PsGly30A retained  $80.8 \pm 4.9\%$  of its initial activity. The data also showed that residual activity increased with prolonged hydrolysis duration. We hypothesise that this is due to competition between several substrates. Initially, the  $\beta$ -1,6-glucans present in the YCW hydrolysate compete with *p*NPG2. However, at later stages, the majority of the  $\beta$ -1,6-glucan has been hydrolysed to gentiobiose, thereby reducing competition with *p*NPG2.

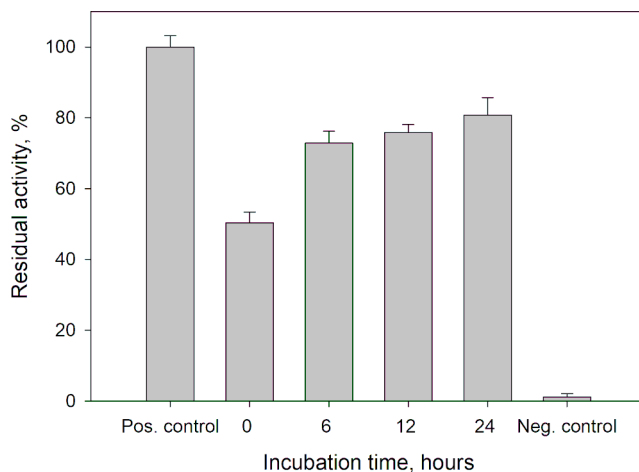


**Figure 3.29.** The residual activity of PsGly30A in the YCW hydrolysis reaction over a 24-hour period. Positive control: residual activity in the absence of the YCW substrate. The negative control is the residual activity in the hydrolysate after inactivation of PsGly30A at 80 °C for 10 minutes. Measurements were performed in triplicate. Error bars represent SD.

To sum up, the synthesised *p*NPG2 can be used to measure the residual activity of endo-1,6- $\beta$ -glucanases in various complex hydrolysates at later time points during hydrolysis.

### 3.4 Application of GHs to lyse living yeast cells

One of the objectives of this investigation was to identify conditions that would enable yeast cell lysis to be achieved by the action of endo-1,3- $\beta$ -glucanases without the use of proteases or the reducing agent  $\beta$ -ME. The main advantage of using purified endo-1,3- $\beta$ -glucanases is that they target  $\beta$ -1,3-glucans, disrupting YCW while leaving other cell wall and intracellular polymers intact. Intracellular proteins are particularly important, as they can undergo proteolysis if a lytic enzyme mixture contains proteases (e.g., *Zymolyase*). If the lysis mixture does not contain proteases, reducing agents are typically used instead. The main purpose of proteases and reducing agents is to disrupt the cell wall mannoprotein network. However, the non-specific action of these agents can alter the properties of intracellular proteins.

#### 3.4.1 Evaluation of yeast cell susceptibility to lysis

Interestingly, previous studies have only evaluated the yeast lytic activity of either GH16 (Saeki et al., 1994; Salazar et al., 2006) or GH64 (Shen et al., 1991) lytic glucanase per strain investigated. Some studies focused solely on the ability of YCW hydrolysis, and the lysis ability of living yeast cells was not observed (Doi et al., 1973; Doi and Doi, 1986; Fleet and Phaff, 1974;

Palumbo et al., 2003; Salazar et al., 2001). Additionally, the lysis of *Saccharomyces cerevisiae* living cells using lytic endo-1,3- $\beta$ -glucanase, without the addition of protease or  $\beta$ -ME, has been reported not to occur (Scott and Schekman, 1980). Based on this gap, we have decided to synthesise recombinant proteins for both GH16 and GH64 enzymes, compare their lytic activity, and evaluate their synergistic action.

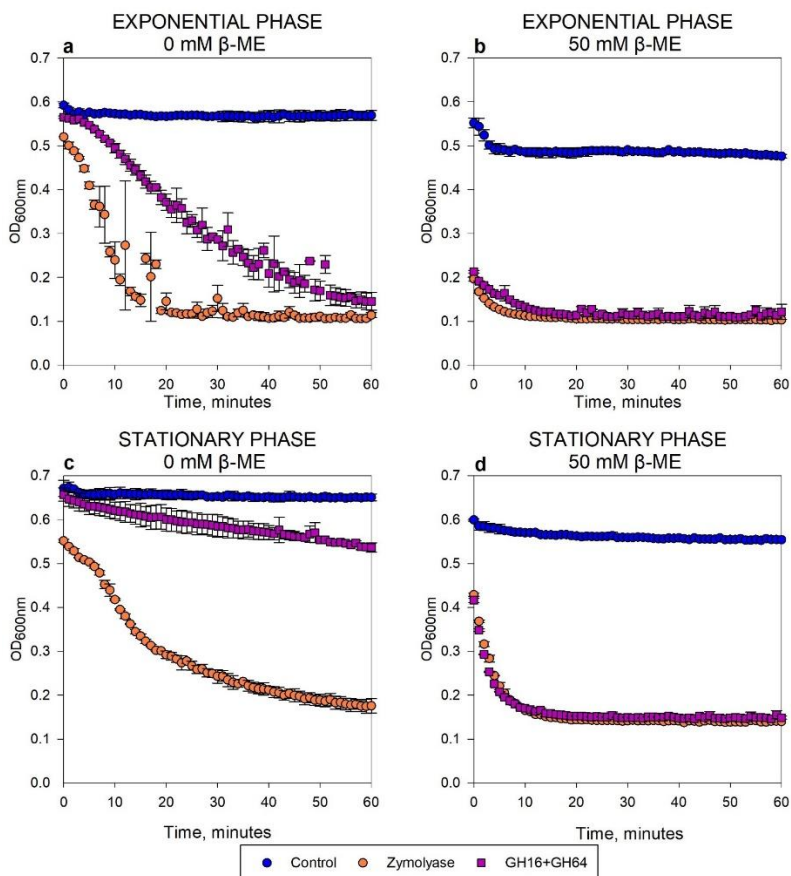


**Figure 3.30.** OD<sub>600nm</sub> dynamics during lysis of exponential phase and stationary phase *S. cerevisiae* BY4741 cells. a – cells collected during the exponential growth stage and lysed without  $\beta$ -ME additive, b – cells collected during the exponential growth stage and lysed with  $\beta$ -ME additive, c – cells collected during the stationary growth stage and lysed without  $\beta$ -ME additive, d – cells collected during the stationary growth stage and lysed with  $\beta$ -ME additive. Two enzymatic preparations were evaluated: *Zymolyase* and a combination of GH16 and GH64. Measurements were performed in duplicate and averaged. Error bars represent SD.

We initially selected the *Saccharomyces cerevisiae* BY4741 yeast strain. This strain was used to adjust the lysis conditions and the additive concentrations. First, we tested whether purified lytic GHs could lyse yeast cells at different growth stages. The results showed that, when a 50 mM  $\beta$ -ME

additive was used (Fig. 3.30b, 3.30d), the difference in lysis efficiency between cells collected at the exponential and stationary growth stages, which were lysed with *Zymolyase* vs a combination of GH16 and GH64, was less significant than when the  $\beta$ -ME additive was not used (Fig. 3.30a, 3.30c). Since purified lytic GHs lysed yeast cells efficiently only when collected at the exponential growth stage in the absence of the  $\beta$ -ME additive (Fig. 3.30a), subsequent experiments used yeast cells collected at that stage. However, it is important to note that when  $\beta$ -ME additive usage is acceptable, a combination of GH16 and GH64 can substitute for *Zymolyase*, even for the cells collected at the stationary growth stage.

### 3.4.2 Substitution of $\beta$ -ME with glycerol

As there was a significant difference in lysis efficiency between 0 mM and 50 mM  $\beta$ -ME, an osmotic shock treatment using glycerol was chosen as an alternative lysis-enhancing additive. Lysis reactions containing 2–50% glycerol concentrations were screened, and 5% glycerol usage was selected as the most suitable (Appendix 15).

Based on the detection of both lytic GHs, GH16 and GH64, in the secretome of *Cellulosimicrobium* sp. GKH (Fig. 3.6, Appendix 6), evaluation and comparison of the lytic activities of the sole enzymes and their synergistic action were made. To broaden the scope of study, the evaluation of a range of  $\beta$ -ME concentrations (0.5–50 mM) in combination with all available enzymatic preparations was carried out. The effect of different concentrations of  $\beta$ -ME and 5% glycerol on lytic efficiency was compared (Fig. 3.31). Four different enzymatic preparations were used: *Zymolyase*, GH16, GH64, and a combination of GH16 and GH64. The synergistic action of the GH16 and GH64 enzymes resulted in the most efficient lysis among the purified GH preparations. This resulted in complete yeast cell lysis, which was comparable in efficiency to *Zymolyase*. Most importantly, 5% glycerol ensured complete lysis (Fig. 3.31e) and was able to replace  $\beta$ -ME and proteases present in the preparation of *Zymolyase*. Additionally, complete yeast cell lysis could be achieved using 0.5 mM  $\beta$ -ME and a combination of GH16 and GH64 GHs. This demonstrates that high concentrations of  $\beta$ -ME (20–100 mM) (Hamouda et al., 2023; Karas et al., 2014; Kitamura and Yamamoto, 1981; Shen et al., 1991) are not always necessary when yeast is in the exponential growth stage, and a combination of two yeast lytic GHs is used as lytic enzymes.

Based on the previous study, the criteria for efficient yeast cell lysis were set as a 70% decrease in OD<sub>600nm</sub>. Efficient yeast cell lysis was indicated by a 70 % decrease in OD<sub>600nm</sub>, as no intact cells were observed under the

microscope (Kitamura and Yamamoto, 1981). In our study, a decrease in  $OD_{600nm}$  was used to evaluate lysis efficiency, and microscopy analysis was also used to confirm the efficient lysis of *S. cerevisiae* BY4741 strain cells when the  $OD_{600nm}$  decreased by more than 70% (Appendix 16).

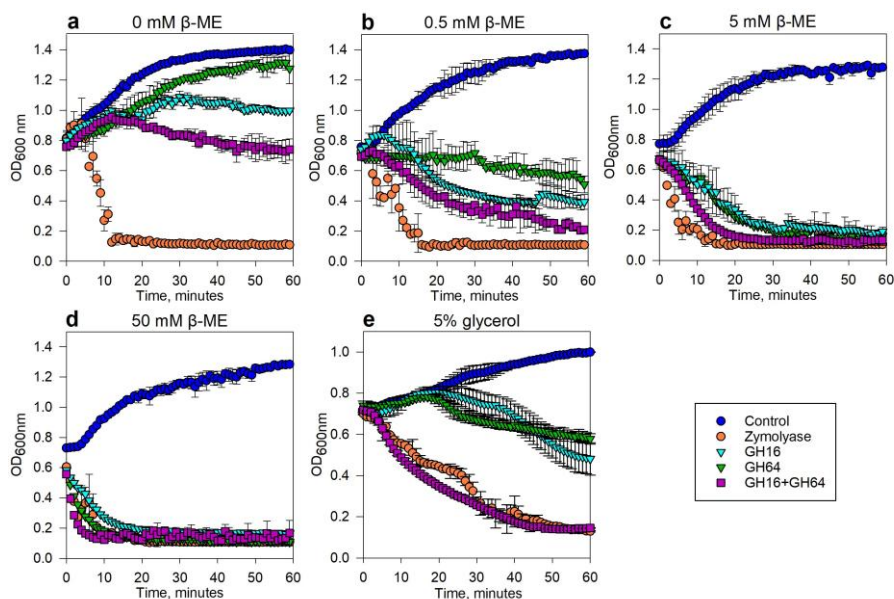


**Figure 3.31.**  $OD_{600nm}$  dynamics during lysis of *Saccharomyces cerevisiae* yeast. Different agents were used to enhance lysis efficiency: a – neither  $\beta$ -ME nor osmotic shock with 5% glycerol was used, b–d – 0.5–50 mM concentrations of  $\beta$ -ME were used, e – osmotic shock with 5% glycerol was used. Measurements were performed in duplicate (a–d) and triplicate (f), and the average values were plotted. Error bars represent SD. Dr. Ramunė Stanevičienė collected the experimental data.

Microscopy analysis showed that the combination of GH16 and GH64 enzymes could lyse yeast cells with similar efficiency in the presence of either 50 mM  $\beta$ -ME or 5% glycerol, which supports the dynamics of  $OD_{600nm}$  results.

### 3.4.3 Lysis of non-*Saccharomyces* yeast

The lysis conditions adjusted during testing on *Saccharomyces cerevisiae* yeast cells were also adapted for an additional 15 yeast strains. The following eight different yeast genera were selected for this experiment: *Aureobasidium*, *Candida*, *Hanseniaspora*, *Kazachstania*, *Lachancea*, *Metschnikowia*, *Pichia* and *Saccharomyces*. Each genus had two representative strains. In seven genera, the strains were from different species, while in *Aureobasidium*, the two strains were from the same species. Lysis experiments were performed at  $\beta$ -ME concentrations ranging from 0 mM to 50 mM. Treatment with 5%

glycerol was also used. Four enzymatic preparations were used: *Zymolyase*, GH16, GH64, and a combination of GH16 and GH64. The lysis results are summarised in Appendix 17.

In reactions containing *Zymolyase*, more than half of the tested strains were completely lysed without the use of  $\beta$ -ME. Both *Metschnikowia* strains were resistant to *Zymolyase* in the absence of  $\beta$ -ME. However, 5% glycerol can replace  $\beta$ -ME and still ensure complete lysis. A similar enhancement was observed in *Pichia anomala*. 5 mM  $\beta$ -ME was as effective as 50 mM  $\beta$ -ME, with only *Lachancea thermotolerans* maintaining resistance to complete lysis over a 60 min period. Based on the manufacturer's information for *Zymolyase-20T* used in this study, five genera (*Candida*, *Hanseniaspora*, *Metschnikowia*, *Pichia* and *Saccharomyces*) are susceptible to *Zymolyase*, while information for the other three genera (*Aureobasidium*, *Kazachstania*, and *Lachancea*) is unavailable (Nacalai Tesque, Inc., 2025).

None of the tested strains was completely lysed in reactions containing a combination of lytic GH16 and GH64 glucanases in the absence of  $\beta$ -ME. However, supplementation of reaction mixtures with 50 mM  $\beta$ -ME ensured the complete lysis of *Aureobasidium*, *Candida*, *Pichia* and *Saccharomyces* yeasts. Under the same conditions, the susceptibility of *Hanseniaspora*, *Kazachstania*, and *Lachancea* strains to complete lysis was strain-dependent, while both *Metschnikowia* strains were lysed with only average efficiency. The addition of 5% glycerol treatment improved the lysis of *Hanseniaspora osmophila*, *Kazachstania africana*, and *Saccharomyces cerevisiae* strains, increasing lysis efficiency from none to complete compared to lysis conditions without additives.

When comparing the lytic performance of individual GH16 and GH64 enzymes, we observe that the efficiency of the *Saccharomyces* yeast is remarkably similar. Lysis is not observed when 5% glycerol or  $\beta$ -ME are not used. Lysis is weak when 5% glycerol is used, while 5 mM of  $\beta$ -ME is sufficient for a complete lysis. When the full range of tested strains is compared, GH64 shows broader specificity than GH16, as it completely lysed more yeast strains. Nevertheless, the combination of both enzymes performed better than either enzyme alone. For reference, a commercial preparation containing GH64 yeast lytic glucanase, *Quantazyme*, was reported to exhibit different levels of efficiency with different yeast genera. *Saccharomyces* yeast was assigned as a genus with the highest susceptibility, while *Candida*, *Hanseniaspora* and *Metschnikowia* were less susceptible to *Quantazyme*, and *Pichia* showed strain-dependent susceptibility (Carvalho et al., 2021). For YCW hydrolysis, *Quantazyme* was supplemented with 60 mM of  $\beta$ -ME (Carvalho et al., 2021), whereas the recombinant GH64 from *Oerskovia*

*xanthineolytica* was originally evaluated for yeast lytic activity in the presence of 20 mM  $\beta$ -ME (Shen et al., 1991). The results of this study (Appendix 17) show that, when *Saccharomyces* yeast is collected at the exponential growth stage, lower concentrations of  $\beta$ -ME are sufficient.

The overall evaluation depends on which components of the lytic preparation are acceptable. Based on the data from this study, lysis efficiency is the highest when *Zymolyase* and  $\beta$ -ME are used together. If  $\beta$ -ME is removed from *Zymolyase*, or if additional enzymatic activities are removed by switching from *Zymolyase* to the GH16 and GH64 combination assisted with  $\beta$ -ME, the range of susceptible strains is similar, but not identical. The GH16 and GH64 combination, supplemented with 5% glycerol, can completely lyse *H. osmophila*, *K. africana*, and *S. cerevisiae*. However, complete lysis using this combination is not possible if neither the glycerol nor the  $\beta$ -ME additive is used. Finally, it is important to note that there are several intermediate stages between achieving high lysis efficiency and protecting yeast cell internal polymers. Therefore, the final decision on lysis conditions should depend on the tolerable level of lysis reaction specificity.

The suggested methodology provides a tool for disrupting yeast cells without altering the structure of intracellular proteins, since the use of additives such as proteases and reducing agents can be avoided. However, a fixation pre-treatment step is recommended for gene expression studies. This is because *Zymolyase*-induced enzymatic lysis of the cell wall activates cell wall integrity (CWI) and high osmolarity glycerol (HOG) pathways. These in turn, induce stress-responsive genes within minutes of cell wall digestion (Nadal-Ribelles et al., 2024). Purified lytic endo-1,3- $\beta$ -glucanases are also likely to induce similar responses in the CWI and HOG pathways. Based on a previous study investigating the relationship between individual *Zymolyase* enzymatic activities and the activation of CWI and HOG pathways, it has been shown that both endo-1,3- $\beta$ -glucanase and protease activities could be solely responsible for this activation (Rodríguez-Peña et al., 2013).

Given the aims of this study, *Zymolyase* side activities, particularly protease activity, were the least favourable option for the lytic enzyme preparation, as they encompass activities beyond YCW-targeted activities. The GH16 and GH64 combination with  $\beta$ -ME represented a compromise between a lytic reaction without side activities and a large scope with high lytic efficiency. The ultimate goal was to lyse yeast in the presence of either proteases or  $\beta$ -ME. Combining two lytic glucanases and using a 5% glycerol additive achieved this in *Hanseniaspora osmophila*, *Kazachstania africana*, and *Saccharomyces cerevisiae* yeast.

## FINAL REMARKS

An investigation reported in this study identified and characterised several YCW polysaccharide-degrading enzymes with endo-1,3- $\beta$ -glucanase, endo-1,6- $\beta$ -glucanase, and  $\beta$ -glucosidase activities. *Rheinheimera* sp. GKU enzymes GH3\_3456, GH16\_306, and GH16\_3455 were shown to be induced by YCW and to ensure YCW conversion to glucose. It was demonstrated that the internal repeat domains of the GH16\_306 enzyme act as CBM modules on  $\beta$ -1,3-glucans. *Paenibacillus* sp. GKG enzymes PsLam81A and PsGly30A hydrolysed YCW to laminaribiose and gentiobiose as main hydrolysis products, respectively. *Cellulosimicrobium* enzymes GH16 and GH64 hydrolysed YCW to either laminaribiose or laminaripentaose, respectively.

From an applicability standpoint, some of the identified enzymes are suitable for valorising the YCW, an industrial-scale by-product, or for lysing living yeast cells. PsLam81A enzyme can convert YCW to laminaribiose, PsGly30A to gentiobiose, while GH64 can be used to obtain laminaripentaose from YCW. All the listed oligosaccharides are reported to have functional or bioactive properties, according to previous studies. Laminaribiose has prebiotic activity, gentiobiose has taste-enhancing functionality, while laminaripentaose has prebiotic and anti-obesity therapeutic effects. Another area is the lysis of living yeast cells without adding proteases or reducing agents to the lysis reaction mixtures. The main benefit of the sole use of endo-1,3- $\beta$ -glucanases instead of mixtures of endo-1,3- $\beta$ -glucanases, proteases, and reducing agents is the ability to protect intracellular yeast proteins from degradation. The degradation is caused by externally added proteases or by externally added reducing agents that impair structure or activity. The conditions reported in this work allow for the lysis of yeast cells using purified endo-1,3- $\beta$ -glucanases, eliminating the need for proteases or reducing agents as additives. The usage of recombinantly produced and purified lytic endo-1,3- $\beta$ -glucanases instead of lytic enzyme mixtures obtained from secretomes offers additional benefit – the absence of nucleases. This trait becomes important when yeast cells are lysed to extract nucleic acids.

Further research in the YCW valorisation field should focus on confirming the bioactivity and functionality of laminaribiose, gentiobiose, and laminaripentaose. For bioactivity and functionality testing, such as prebiotic index determination, laminaripentaose can be used directly, whereas laminaribiose and gentiobiose hydrolysates should be further treated to remove glucose. Additionally, laminaribiose hydrolysates contain a small proportion of branched 3,6-linked oligosaccharides. These side products are particularly interesting due to their potential immunomodulatory activity.

Therefore, assessing the immunostimulatory properties of these branched side-products could lead to additional applications. The superior benefit would be expected if these branched side-products were equivalent or better in comparison to yeast  $\beta$ -glucans in immunostimulatory activity. Added that immunostimulatory activity would be consistent and predictable due to the simple structure of short, branched oligosaccharides, in contrast to  $\beta$ -glucans that need a particular 3D conformation to possess immunostimulatory activity.

For applications involving the lysis of viable yeast cells, further experiments should focus on demonstrating the absence of proteolytic activity in general and on specific proteins. The demonstration of a lack of nuclease activity would also be considerable. Up-to-date experiments showed efficient lysis only in exponential-stage cells when purified endo-1,3- $\beta$ -glucanases were combined with glycerol treatment. This may be the method's main limitation. However, if the use of a reducing agent is acceptable, lysis of stationary-phase cells is possible when purified endo-1,3- $\beta$ -glucanases are combined with a reducing agent. Therefore, further experiments could focus on adjusting the  $\beta$ -ME concentration required for lysis of stationary-phase cells.

## CONCLUSIONS

1. *Rheinheimera* sp. GKU, *Paenibacillus* sp. GKG, and *Cellulosmicrobium* sp. GKH bacteria degrade YCW into water-soluble compounds, which can be utilised as a carbon source.
2. *Rheinheimera* sp. GKU, *Paenibacillus* sp. GKG, and *Cellulosmicrobium* sp. GKH secrete CAZymes, mainly endo-1,3- $\beta$ -glucanases, sufficient for YCW conversion to DP2–DP5 oligosaccharides and glucose.
3. *Rheinheimera* sp. GKU secretes GHs that have distinctive roles in YCW degradation – GH16\_3455 and GH16\_306 release oligosaccharides, while GH3\_3456 hydrolyses oligosaccharides to glucose.
4. PsLam81A, PsGly30A and GH64 enzymes can convert from 51% to 78% of YCW  $\beta$ -glucans into bioactive and functional products – laminaribiose (78%), gentiobiose (72%) and laminaripentaose (51%), respectively.
5. Synergistic action of GH16 and GH64 endo-1,3- $\beta$ -glucanases and glycerol treatment allows protease and reducing agent-free lysis of living *Saccharomyces cerevisiae* BY4741 yeast cells.

## REFERENCES

1. Abi, A., Hartig, D., Vorländer, K., Wang, A., Scholl, S., Jördening, H., 2019. Continuous enzymatic production and adsorption of laminaribiose in packed bed reactors. *Engineering in Life Sciences* 19, 4–12. <https://doi.org/10.1002/elsc.201800110>
2. Aguilar-Uscanga, B., Francois, J.M., 2003. A study of the yeast cell wall composition and structure in response to growth conditions and mode of cultivation. *Lett Appl Microbiol* 37, 268–274. <https://doi.org/10.1046/j.1472-765X.2003.01394.x>
3. Aïmanianda, V., Clavaud, C., Simenel, C., Fontaine, T., Delepierre, M., Latgé, J.-P., 2009. Cell Wall  $\beta$ -(1,6)-Glucan of *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* 284, 13401–13412. <https://doi.org/10.1074/jbc.M807667200>
4. Aizawa, K., Ishikawa, T., Yamamoto, R., 2017. Gentiobiose-containing saccharide composition and taste improver. Japanese Patent JP2017184628A.
5. Almagro Armenteros, J.J., Tsirigos, K.D., Sønderby, C.K., Petersen, T.N., Winther, O., Brunak, S., Von Heijne, G., Nielsen, H., 2019. SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nat Biotechnol* 37, 420–423. <https://doi.org/10.1038/s41587-019-0036-z>
6. Ameri Shah Reza, M., Najafi, S., Kahfi, M., Safari, M., Mahjoor, M., 2025. Beta-glucans in oncology: revolutionizing treatment with immune power & tumor targeting. *Naunyn-Schmiedeberg's Arch Pharmacol*. <https://doi.org/10.1007/s00210-025-04519-8>
7. Arthornthurasuk, S., Jenkhetkan, W., Suwan, E., Chokchaichamnankit, D., Srisomsap, C., Wattana-Amorn, P., Svasti, J., Kongsaree, P.T., 2018. Molecular Characterization and Potential Synthetic Applications of GH1  $\beta$ -Glucosidase from Higher Termite *Microcerotermes annandalei*. *Appl Biochem Biotechnol* 186, 877–894. <https://doi.org/10.1007/s12010-018-2781-8>
8. Avramia, I., Amariei, S., 2021. Spent Brewer's Yeast as a Source of Insoluble  $\beta$ -Glucans. *IJMS* 22, 825. <https://doi.org/10.3390/ijms22020825>
9. Baek, K.-R., Rani Ramakrishnan, S., Kim, S.-J., Seo, S.-O., 2024. Yeast cell wall mannan structural features, biological activities, and production strategies. *Heliyon* 10, e27896. <https://doi.org/10.1016/j.heliyon.2024.e27896>
10. Bai, L., Kim, J., Son, K.-H., Shin, D.-H., Ku, B.-H., Kim, D.Y., Park, H.-Y., 2021. Novel Anti-Fungal d-Laminaripentaose-Releasing Endo- $\beta$ -1,3-glucanase with a RICIN-like Domain from *Cellulosimicrobium funkei* HY-13. *Biomolecules* 11, 1080. <https://doi.org/10.3390/biom11081080>

11. Baladrón, V., Ufano, S., Dueñas, E., Martín-Cuadrado, A.B., del Rey, F., Vázquez de Aldana, C.R., 2002. Eng1p, an Endo-1,3- $\beta$ -Glucanase Localized at the Daughter Side of the Septum, Is Involved in Cell Separation in *Saccharomyces cerevisiae*. *Eukaryot Cell* 1, 774–786. <https://doi.org/10.1128/EC.1.5.774-786.2002>
12. BarthHaas GmbH & Co. KG, 2025. BarthHaas Report 2024/25. BarthHaas GmbH & Co. KG, Nuremberg, Germany.
13. Bastien, G., Arnal, G., Bozonnet, S., Laguerre, S., Ferreira, F., Fauré, R., Henrissat, B., Lefèvre, F., Robe, P., Bouchez, O., Noirot, C., Dumon, C., O'Donohue, M., 2013. Mining for hemicellulases in the fungus-growing termite *Pseudacanthotermes militaris* using functional metagenomics. *Biotechnol Biofuels* 6, 78. <https://doi.org/10.1186/1754-6834-6-78>
14. Bastos, R., Oliveira, P.G., Gaspar, V.M., Mano, J.F., Coimbra, M.A., Coelho, E., 2022. Brewer's yeast polysaccharides — A review of their exquisite structural features and biomedical applications. *Carbohydrate Polymers* 277, 118826. <https://doi.org/10.1016/j.carbpol.2021.118826>
15. Becker, S., Scheffel, A., Polz, M.F., Hehemann, J.-H., 2017. Accurate Quantification of Laminarin in Marine Organic Matter with Enzymes from Marine Microbes. *Appl Environ Microbiol* 83, e03389-16. <https://doi.org/10.1128/AEM.03389-16>
16. Boisramé, A., Gaillardin, C., 2009. Heterologous expression and characterization of a  $\beta$ -1,6-glucanase from *Aspergillus fumigatus*. *Appl Microbiol Biotechnol* 82, 663–669. <https://doi.org/10.1007/s00253-008-1780-z>
17. Boraston, A.B., Bolam, D.N., Gilbert, H.J., Davies, G.J., 2004. Carbohydrate-binding modules: fine-tuning polysaccharide recognition. *Biochemical Journal* 382, 769–781. <https://doi.org/10.1042/BJ20040892>
18. Borchani, C., Fonteyn, F., Jamin, G., Paquot, M., Blecker, C., Thonart, P., 2014. Enzymatic process for the fractionation of baker's yeast cell wall (*Saccharomyces cerevisiae*). *Food Chemistry* 163, 108–113. <https://doi.org/10.1016/j.foodchem.2014.04.086>
19. Boutros, J.A., Magee, A.S., Cox, D., 2022. Comparison of structural differences between yeast  $\beta$ -glucan sourced from different strains of *saccharomyces cerevisiae* and processed using proprietary manufacturing processes. *Food Chemistry* 367, 130708. <https://doi.org/10.1016/j.foodchem.2021.130708>
20. Brettrager, E.J., Frederick, A.J., Van Waardenburg, R.C.A.M., 2024. Zymolyase Treatment of *Saccharomyces cerevisiae* Affects Cellular Proteins and Degrades Tyrosyl-DNA Phosphodiesterase I. *DNA and Cell Biology* 43, 353–361. <https://doi.org/10.1089/dna.2024.0062>

21. Broadway, P., Carroll, J., Sanchez, N., 2015. Live Yeast and Yeast Cell Wall Supplements Enhance Immune Function and Performance in Food-Producing Livestock: A Review. *Microorganisms* 3, 417–427. <https://doi.org/10.3390/microorganisms3030417>
22. Carvalho, V.S.D., Gómez-Delgado, L., Curto, M.Á., Moreno, M.B., Pérez, P., Ribas, J.C., Cortés, J.C.G., 2021. Analysis and application of a suite of recombinant endo- $\beta$ (1,3)-d-glucanases for studying fungal cell walls. *Microb Cell Fact* 20, 126. <https://doi.org/10.1186/s12934-021-01616-0>
23. Caseiro, C., Dias, J.N.R., de Andrade Fontes, C.M.G., Bule, P., 2022. From Cancer Therapy to Winemaking: The Molecular Structure and Applications of  $\beta$ -Glucans and  $\beta$ -1, 3-Glucanases. *IJMS* 23, 3156. <https://doi.org/10.3390/ijms23063156>
24. Chen, F., Ye, J., Sista Kameshwar, A.K., Wu, X., Ren, J., Qin, W., Li, D.-W., 2020. A Novel Cold-Adaptive Endo-1,4- $\beta$ -Glucanase From *Burkholderia pyrrocinia* JK-SH007: Gene Expression and Characterization of the Enzyme and Mode of Action. *Front. Microbiol.* 10, 3137. <https://doi.org/10.3389/fmicb.2019.03137>
25. Cheng, K., Zhou, Y., Neelamegham, S., 2016. DrawGlycan-SNFG: a robust tool to render glycans and glycopeptides with fragmentation information. *Glycobiology* glycob;cwv115v2. <https://doi.org/10.1093/glycob/cwv115>
26. Cheng, R., Chen, J., Yu, X., Wang, Y., Wang, S., Zhang, J., 2013. Recombinant production and characterization of full-length and truncated  $\beta$ -1,3-glucanase PglA from *Paenibacillus* sp. S09. *BMC Biotechnol* 13, 105. <https://doi.org/10.1186/1472-6750-13-105>
27. Cheng, R., Cheng, L., Wang, L., Fu, R., Sun, X., Li, J., Wang, S., Zhang, J., 2019. Characterization of an alkali-stable xyloglucanase/mixed-linkage  $\beta$ -glucanase Pgl5A from *Paenibacillus* sp. S09. *International Journal of Biological Macromolecules* 140, 1158–1166. <https://doi.org/10.1016/j.ijbiomac.2019.08.210>
28. Ciobanu, L.T., Constantinescu-Aruxandei, D., Farcasanu, I.C., Oancea, F., 2024. Spent Brewer's Yeast Lysis Enables a Best Out of Waste Approach in the Beer Industry. *IJMS* 25, 12655. <https://doi.org/10.3390/ijms252312655>
29. Collart, M.A., Oliviero, S., 1993. Preparation of Yeast RNA. *CP Molecular Biology* 23. <https://doi.org/10.1002/0471142727.mb1312s23>
30. Constantin, O.E., Păcularu-Burada, B., Bahrim, G.-E., 2024. Yeast Strains from Sourdough as Potential Clean-Label Starters for Fermentation Processes, in: Ceresino, E.B., Juodeikiene, G., Miescher Schwenninger, S., Ferreira da Rocha, J.M. (Eds.), *Sourdough Microbiota and Starter Cultures for Industry*. Springer International Publishing, Cham, pp. 343–378. [https://doi.org/10.1007/978-3-031-48604-3\\_12](https://doi.org/10.1007/978-3-031-48604-3_12)

31. Contato, A.G., Borelli, T.C., Buckeridge, M.S., Rogers, J., Hartson, S., Prade, R.A., Polizeli, M.D.L.T.D.M., 2024. Secretome Analysis of *Thermothelomyces thermophilus* LMBC 162 Cultivated with *Tamarindus indica* Seeds Reveals CAZymes for Degradation of Lignocellulosic Biomass. *JoF* 10, 121. <https://doi.org/10.3390/jof10020121>
32. Couturier, M., Feliu, J., Haon, M., Navarro, D., Lesage-Meessen, L., Coutinho, P.M., Berrin, J.-G., 2011. A thermostable GH45 endoglucanase from yeast: impact of its atypical multimodularity on activity. *Microb Cell Fact* 10, 103. <https://doi.org/10.1186/1475-2859-10-103>
33. Da Silva, A.S., Molina, J.F., Teixeira, R.S.S., Valdivieso Gelves, L.G., Bon, E.P.S., Ferreira-Leitão, V.S., 2017. Synthesis of disaccharides using  $\beta$ -glucosidases from *Aspergillus niger*, *A. awamori* and *Prunus dulcis*. *Biotechnol Lett* 39, 1717–1723. <https://doi.org/10.1007/s10529-017-2409-z>
34. De La Cruz, J., Pintor-Toro, J.A., Benítez, T., Llobell, A., 1995. Purification and characterization of an endo-beta-1,6-glucanase from *Trichoderma harzianum* that is related to its mycoparasitism. *J Bacteriol* 177, 1864–1871. <https://doi.org/10.1128/jb.177.7.1864-1871.1995>
35. De Marco Castro, E., Calder, P.C., Roche, H.M., 2021.  $\beta$ -1,3/1,6-Glucans and Immunity: State of the Art and Future Directions. *Mol. Nutr. Food Res.* 65, 1901071. <https://doi.org/10.1002/mnfr.201901071>
36. Deng, J., Li, Z., Lv, X., Chen, J., Liu, L., 2026. Precision hydrolysis: tailored yeast processing enzymes for yeast-based products. *Appl Microbiol Biotechnol* 110, 5. <https://doi.org/10.1007/s00253-025-13669-x>
37. Dhar, H., Kasana, R.C., Gulati, A., 2015. Heterologous expression and characterization of detergent stable endoglucanase EG5B from *Paenibacillus* sp. IHB B 3084. *Journal of Molecular Catalysis B: Enzymatic* 120, 9–15. <https://doi.org/10.1016/j.molcatb.2015.06.009>
38. Doi, K., Doi, A., 1986. Cloning and expression in *Escherichia coli* of the gene for an *Arthrobacter* beta-(1-3)-glucanase. *J Bacteriol* 168, 1272–1276. <https://doi.org/10.1128/jb.168.3.1272-1276.1986>
39. Doi, K., Doi, A., Ozaki, T., Fukui, T., 1973. Heterogeneity of the Lytic Activity for Yeast Cell Wall Observed among the Components of an *Arthrobacter* Glucanase. *The Journal of Biochemistry* 73, 667–670. <https://doi.org/10.1093/oxfordjournals.jbchem.a130127>
40. Dou, H., Chang, Y., Zhang, L., 2019. *Coriolus versicolor* polysaccharopeptide as an immunotherapeutic in China, in: *Progress in Molecular Biology and Translational Science*. Elsevier, pp. 361–381. <https://doi.org/10.1016/bs.pmbts.2019.03.001>
41. Driscoll, M., Hansen, R., Ding, C., Cramer, D.E., Yan, J., 2009. Therapeutic potential of various  $\beta$ -glucan sources in conjunction with anti-tumor

- monoclonal antibody in cancer therapy. *Cancer Biology & Therapy* 8, 218–225. <https://doi.org/10.4161/cbt.8.3.7337>
42. Drula, E., Garron, M.-L., Dogan, S., Lombard, V., Henrissat, B., Terrapon, N., 2022. The carbohydrate-active enzyme database: functions and literature. *Nucleic Acids Research* 50, D571–D577. <https://doi.org/10.1093/nar/gkab1045>
  43. Dumville, J.C., Fry, S.C., 2003. Gentiobiose: a novel oligosaccharin in ripening tomato fruit. *Planta* 216, 484–495. <https://doi.org/10.1007/s00425-002-0869-3>
  44. Dunn, B., Wobbe, C.R., 1993. Preparation of Protein Extracts from Yeast. *CP Molecular Biology* 23. <https://doi.org/10.1002/0471142727.mb1313s23>
  45. Ecker, M., Deutzmann, R., Lehle, L., Mersa, V., Tanner, W., 2006. Pir Proteins of *Saccharomyces cerevisiae* Are Attached to  $\beta$ -1,3-Glucan by a New Protein-Carbohydrate Linkage. *Journal of Biological Chemistry* 281, 11523–11529. <https://doi.org/10.1074/jbc.M600314200>
  46. Elder, M.J., Webster, S.J., Chee, R., Williams, D.L., Hill Gaston, J.S., Goodall, J.C., 2017.  $\beta$ -Glucan Size Controls Dectin-1-Mediated Immune Responses in Human Dendritic Cells by Regulating IL-1 $\beta$  Production. *Front. Immunol.* 8, 791. <https://doi.org/10.3389/fimmu.2017.00791>
  47. El-Helow, E.R., Elbahloul, Y., El-Sharouny, E.E., Ali, S.R., Ali, A.A.-M., 2015. Economic production of baker's yeast using a new *Saccharomyces cerevisiae* isolate. *Biotechnology & Biotechnological Equipment* 29, 705–713. <https://doi.org/10.1080/13102818.2015.1038302>
  48. Erde, J., Loo, R.R.O., Loo, J.A., 2014. Enhanced FASP (eFASP) to Increase Proteome Coverage and Sample Recovery for Quantitative Proteomic Experiments. *J. Proteome Res.* 13, 1885–1895. <https://doi.org/10.1021/pr4010019>
  49. Esteban, P.F., Ríos, I., García, R., Dueñas, E., Plá, J., Sánchez, M., De Aldana, C.R.V., Del Rey, F., 2005. Characterization of the CaENG1 Gene Encoding an Endo-1,3- $\beta$ -Glucanase Involved in Cell Separation in *Candida albicans*. *Curr Microbiol* 51, 385–392. <https://doi.org/10.1007/s00284-005-0066-2>
  50. Falch, B.H., Espevik, T., Ryan, L., Stokke, B.T., 2000. The cytokine stimulating activity of (1 $\rightarrow$ 3)- $\beta$ -d-glucans is dependent on the triple helix conformation. *Carbohydrate Research* 329, 587–596. [https://doi.org/10.1016/S0008-6215\(00\)00222-6](https://doi.org/10.1016/S0008-6215(00)00222-6)
  51. Felsenstein, J., 1985. Confidence Limits on Phylogenies: An Approach Using the Bootstrap. *Evolution* 39, 783. <https://doi.org/10.2307/2408678>
  52. Fernández, F.J., Santos-López, J., Martínez-Barricarte, R., Querol-García, J., Martín-Merino, H., Navas-Yuste, S., Savko, M., Shepard, W.E., Rodríguez De Córdoba, S., Vega, M.C., 2022. The crystal structure of iC3b-CR3  $\alpha$ I

- reveals a modular recognition of the main opsonin iC3b by the CR3 integrin receptor. *Nat Commun* 13, 1955. <https://doi.org/10.1038/s41467-022-29580-2>
53. Ferreira, C.M.H., Pinto, I.S.S., Soares, E.V., Soares, H.M.V.M., 2015. (Un)suitability of the use of pH buffers in biological, biochemical and environmental studies and their interaction with metal ions – a review. *RSC Adv.* 5, 30989–31003. <https://doi.org/10.1039/C4RA15453C>
  54. Ferrer, P., 2006. Revisiting the *Cellulosimicrobium cellulans* yeast-lytic  $\beta$ -1,3-glucanases toolbox: A review. *Microb Cell Fact* 5, 10. <https://doi.org/10.1186/1475-2859-5-10>
  55. Fleet, G.H., Phaff, H.J., 1974. Lysis of Yeast Cell Walls: Glucanases from *Bacillus circulans* WL-12. *J Bacteriol* 119, 207–219. <https://doi.org/10.1128/jb.119.1.207-219.1974>
  56. Fliegmann, J., Montel, E., Djulić, A., Cottaz, S., Driguez, H., Ebel, J., 2005. Catalytic properties of the bifunctional soybean  $\beta$ -glucan-binding protein, a member of family 81 glycoside hydrolases. *FEBS Letters* 579, 6647–6652. <https://doi.org/10.1016/j.febslet.2005.10.060>
  57. Fontaine, T., Hartland, R.P., Beauvais, A., Diaquin, M., Latge, J., 1997. Purification and Characterization of an Endo-1,3- $\beta$ -Glucanase from *Aspergillus fumigatus*. *European Journal of Biochemistry* 243, 315–321. <https://doi.org/10.1111/j.1432-1033.1997.0315a.x>
  58. Free, S.J., 2013. Fungal Cell Wall Organization and Biosynthesis, in: *Advances in Genetics*. Elsevier, pp. 33–82. <https://doi.org/10.1016/B978-0-12-407677-8.00002-6>
  59. Freimund, S., Sauter, M., Käppeli, O., Dutler, H., 2003. A new non-degrading isolation process for 1,3- $\beta$ -d-glucan of high purity from baker's yeast *Saccharomyces cerevisiae*. *Carbohydrate Polymers* 54, 159–171. [https://doi.org/10.1016/S0144-8617\(03\)00162-0](https://doi.org/10.1016/S0144-8617(03)00162-0)
  60. Fujimoto, Y., Hattori, T., Uno, S., Murata, T., Usui, T., 2009. Enzymatic synthesis of gentiooligosaccharides by transglycosylation with  $\beta$ -glycosidases from *Penicillium multicolor*. *Carbohydrate Research* 344, 972–978. <https://doi.org/10.1016/j.carres.2009.03.006>
  61. Gao, F., Li, Q., Wei, W., Wang, Y., Song, W., Yang, X., Ji, H., Zhou, J., Xin, Y., Tan, Z., Pei, J., Shi, H., 2024. Preparation of Yeast Extract from Brewer's Yeast Waste and Its Potential Application as a Medium Constituent. *Appl Biochem Biotechnol* 196, 6608–6623. <https://doi.org/10.1007/s12010-024-04885-8>
  62. Gericke, M., Amaral, A.J.R., Budtova, T., De Wever, P., Groth, T., Heinze, T., Höfte, H., Huber, A., Ikkala, O., Kapuśniak, J., Kargl, R., Mano, J.F., Másson, M., Matricardi, P., Medronho, B., Norgren, M., Nypelö, T., Nyström, L., Roig, A., Sauer, M., Schols, H., Van Der Linden, J., Wrodnigg, T., Xu, C.,

- Yakubov, G., Kleinschek, K.S., Fardim, P., 2023. The European Polysaccharide Network of Excellence (EPNOE) research roadmap 2040: Advanced strategies for exploiting the vast potential of polysaccharides as renewable bioresources. *Carbohydrate Polymers* 121633. <https://doi.org/10.1016/j.carbpol.2023.121633>
63. Gonzalez, M., Lipke, P.N., Ovalle, R., 2009. Chapter 15 GPI Proteins in Biogenesis and Structure of Yeast Cell Walls, in: *The Enzymes*. Elsevier, pp. 321–356. [https://doi.org/10.1016/S1874-6047\(09\)26015-X](https://doi.org/10.1016/S1874-6047(09)26015-X)
64. Gow, N.A.R., Lenardon, M.D., 2023. Architecture of the dynamic fungal cell wall. *Nat Rev Microbiol* 21, 248–259. <https://doi.org/10.1038/s41579-022-00796-9>
65. Günal-Köroğlu, D., Karabulut, G., Mohammadian, F., Can Karaca, A., Capanoglu, E., Esatbeyoglu, T., 2025. Production of yeast cell wall polysaccharides- $\beta$ -glucan and chitin by using food waste substrates: Biosynthesis, production, extraction, and purification methods. *Comp Rev Food Sci Food Safe* 24, e70161. <https://doi.org/10.1111/1541-4337.70161>
66. Hamouda, H.I., Fan, Y.-X., Abdalla, M., Su, H., Lu, M., Li, F.-L., 2023. Distinct roles of carbohydrate-binding modules in multidomain  $\beta$ -1,3–1,4-glucanase on polysaccharide degradation. *Appl Microbiol Biotechnol* 107, 1751–1764. <https://doi.org/10.1007/s00253-023-12416-4>
67. Hao, M.-S., Mazurkewich, S., Li, H., Kvammen, A., Saha, S., Koskela, S., Inman, A.R., Nakajima, M., Tanaka, N., Nakai, H., Brändén, G., Bulone, V., Larsbrink, J., McKee, L.S., 2024. Structural and biochemical analysis of family 92 carbohydrate-binding modules uncovers multivalent binding to  $\beta$ -glucans. *Nat Commun* 15, 3429. <https://doi.org/10.1038/s41467-024-47584-y>
68. Hattori, T., Kato, Y., Uno, S., Usui, T., 2013. Mode of action of a  $\beta$ -(1→6)-glucanase from *Penicillium multicolor*. *Carbohydrate Research* 366, 6–16. <https://doi.org/10.1016/j.carres.2012.11.002>
69. He, H., Qin, Y., Chen, G., Li, N., Liang, Z., 2013. Two-Step Purification of a Novel  $\beta$ -Glucosidase with High Transglycosylation Activity and Another Hypothetical  $\beta$ -Glucosidase in *Aspergillus oryzae* HML366 and Enzymatic Characterization. *Appl Biochem Biotechnol* 169, 870–884. <https://doi.org/10.1007/s12010-012-9936-9>
70. Henrissat, B., 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* 280, 309–316. <https://doi.org/10.1042/bj2800309>
71. Hettle, A., Fillo, A., Abe, K., Massel, P., Pluvinae, B., Langelaan, D.N., Smith, S.P., Boraston, A.B., 2017. Properties of a family 56 carbohydrate-binding module and its role in the recognition and hydrolysis of  $\beta$ -1,3-glucan. *Journal of Biological Chemistry* 292, 16955–16968. <https://doi.org/10.1074/jbc.M117.806711>

72. Hirabayashi, K., Kondo, N., Hayashi, S., 2016. Characterization and enzymatic hydrolysis of hydrothermally treated  $\beta$ -1,3-1,6-glucan from *Aureobasidium pullulans*. *World J Microbiol Biotechnol* 32, 206. <https://doi.org/10.1007/s11274-016-2167-4>
73. Hirabayashi, K., Tashiro, Y., Kondo, N., Hayashi, S., 2017. Production of Gentiobiose from Hydrothermally Treated *Aureobasidium pullulans*  $\beta$ -1,3-1,6-Glucan: Production of Gentiobiose from  $\beta$ -1,3-1,6-Glucan. *Journal of Applied Glycoscience* 64, 33–37. [https://doi.org/10.5458/jag.jag.JAG-2017\\_002](https://doi.org/10.5458/jag.jag.JAG-2017_002)
74. Hodoniczky, J., Morris, C.A., Rae, A.L., 2012. Oral and intestinal digestion of oligosaccharides as potential sweeteners: A systematic evaluation. *Food Chemistry* 132, 1951–1958. <https://doi.org/10.1016/j.foodchem.2011.12.031>
75. Holan, Z., Beran, K., Miler, I., 1980. Preparation of zymosan from yeast cell walls. *Folia Microbiol* 25, 501–504. <https://doi.org/10.1007/BF02897218>
76. Hrmova, M., MacGregor, E.A., Biely, P., Stewart, R.J., Fincher, G.B., 1998. Substrate Binding and Catalytic Mechanism of a Barley  $\beta$ -d-Glucosidase/(1,4)- $\beta$ -d-Glucan Exohydrolase. *Journal of Biological Chemistry* 273, 11134–11143. <https://doi.org/10.1074/jbc.273.18.11134>
77. Hromádková, Z., Ebringerová, A., Sasinková, V., Šandula, J., Hříbalová, V., Omelková, J., 2003. Influence of the drying method on the physical properties and immunomodulatory activity of the particulate (1 $\rightarrow$ 3)- $\beta$ -d-glucan from *Saccharomyces cerevisiae*. *Carbohydrate Polymers* 51, 9–15. [https://doi.org/10.1016/S0144-8617\(02\)00110-8](https://doi.org/10.1016/S0144-8617(02)00110-8)
78. Isbell, H.S. (Ed.), 1973. *Carbohydrates in Solution*, *Advances in Chemistry*. American Chemical Society, Washington, D. C. <https://doi.org/10.1021/ba-1971-0117>
79. Jach, M.E., Serefko, A., Ziaja, M., Kieliszek, M., 2022. Yeast Protein as an Easily Accessible Food Source. *Metabolites* 12, 63. <https://doi.org/10.3390/metabo12010063>
80. Jacob, F.F., Striegel, L., Rychlik, M., Hutzler, M., Methner, F.-J., 2019. Spent Yeast from Brewing Processes: A Biodiverse Starting Material for Yeast Extract Production. *Fermentation* 5, 51. <https://doi.org/10.3390/fermentation5020051>
81. Jamois, F., Goffic, F.L., Yvin, J.C., Plusquellec, D., Ferrieres, V., 2008. How to Improve Chemical Synthesis of Laminaribiose on a Large Scale. *TOGLYJ* 1, 19–24. <https://doi.org/10.2174/1875398100801010019>
82. Jin, X., Wang, J.-K., Wang, Q., 2023. Microbial  $\beta$ -glucanases: production, properties, and engineering. *World Journal of Microbiology and Biotechnology* 39, 106. <https://doi.org/10.1007/s11274-023-03550-2>

83. Jofre, F.M., Queiroz, S.D.S., Sanchez, D.A., Arruda, P.V., Santos, J.C.D., Felipe, M.D.G.D.A., 2024. Biotechnological potential of yeast cell wall: An overview. *Biotechnology Progress* 40, e3491. <https://doi.org/10.1002/btpr.3491>
84. Johansson, R., Gunnarsson, L.C., Ohlin, M., Ohlson, S., 2006. Thermostable carbohydrate-binding modules in affinity chromatography. *J Mol Recognit* 19, 275–281. <https://doi.org/10.1002/jmr.794>
85. Jones, D.T., Taylor, W.R., Thornton, J.M., 1992. The rapid generation of mutation data matrices from protein sequences. *Bioinformatics* 8, 275–282. <https://doi.org/10.1093/bioinformatics/8.3.275>
86. Jones, E.W., 1984. The synthesis and function of proteases in *Saccharomyces*: genetic approaches. *Annu. Rev. Genet.* 18, 233–270. <https://doi.org/10.1146/annurev.ge.18.120184.001313>
87. Karas, B.J., Jablanovic, J., Irvine, E., Sun, L., Ma, L., Weyman, P.D., Gibson, D.G., Glass, J.I., Venter, J.C., Hutchison, C.A., Smith, H.O., Suzuki, Y., 2014. Transferring whole genomes from bacteria to yeast spheroplasts using entire bacterial cells to reduce DNA shearing. *Nat Protoc* 9, 743–750. <https://doi.org/10.1038/nprot.2014.045>
88. Kitamura, K., Kaneko, T., Yamamoto, Y., 1972. Lysis of viable yeast cells by enzymes of *Arthrobacter luteus*. *J. Gen. Appl. Microbiol.* 18, 57–71. <https://doi.org/10.2323/jgam.18.57>
89. Kitamura, K., Yamamoto, Y., 1981. Lysis of Yeast Cells Showing Low Susceptibility to Zymolyase. *Agricultural and Biological Chemistry* 45, 1761–1766. <https://doi.org/10.1080/00021369.1981.10864786>
90. Kitaoka, M., Sasaki, T., Taniguchi, H., 1993. Conversion of Sucrose into Laminaribiose Using Sucrose Phosphorylase, Xylose Isomerase and Laminaribiose Phosphorylase. *Journal of the Japanese Society of Starch Science* 40, 311–314. <https://doi.org/10.5458/jag1972.40.311>
91. Klis, F.M., Mol, P., Hellingwerf, K., Brul, S., 2002. Dynamics of cell wall structure in *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* 26, 239–256. <https://doi.org/10.1111/j.1574-6976.2002.tb00613.x>
92. Kognole, A.A., Payne, C.M., 2018. Cellulose-specific Type B carbohydrate binding modules: understanding oligomeric and non-crystalline substrate recognition mechanisms. *Biotechnology for Biofuels* 11, 319. <https://doi.org/10.1186/s13068-018-1321-7>
93. Kollár, R., Petráková, E., Ashwell, G., Robbins, P.W., Cabib, E., 1995. Architecture of the Yeast Cell Wall. *Journal of Biological Chemistry* 270, 1170–1178. <https://doi.org/10.1074/jbc.270.3.1170>
94. Kollár, R., Reinhold, B.B., Petráková, E., Yeh, H.J.C., Ashwell, G., Drgonová, J., Kapteyn, J.C., Klis, F.M., Cabib, E., 1997. Architecture of the

- Yeast Cell Wall. *Journal of Biological Chemistry* 272, 17762–17775. <https://doi.org/10.1074/jbc.272.28.17762>
95. Konno, N., Sakamoto, Y., 2011. An endo- $\beta$ -1,6-glucanase involved in *Lentinula edodes* fruiting body autolysis. *Appl Microbiol Biotechnol* 91, 1365–1373. <https://doi.org/10.1007/s00253-011-3295-2>
  96. Kulagina, N., Besseau, S., Godon, C., Goldman, G.H., Papon, N., Courdavault, V., 2021. Yeasts as Biopharmaceutical Production Platforms. *Front. Fungal Biol.* 2, 733492. <https://doi.org/10.3389/ffunb.2021.733492>
  97. Kumagai, Y., Ojima, T., 2010. Isolation and characterization of two types of  $\beta$ -1,3-glucanases from the common sea hare *Aplysia kurodai*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 155, 138–144. <https://doi.org/10.1016/j.cbpb.2009.10.013>
  98. Kumar, K., Correia, M.A.S., Pires, V.M.R., Dhillon, A., Sharma, K., Rajulapati, V., Fontes, C.M.G.A., Carvalho, A.L., Goyal, A., 2018. Novel insights into the degradation of  $\beta$ -1,3-glucans by the cellulosome of *Clostridium thermocellum* revealed by structure and function studies of a family 81 glycoside hydrolase. *International Journal of Biological Macromolecules* 117, 890–901. <https://doi.org/10.1016/j.ijbiomac.2018.06.003>
  99. Kurakake, M., Yamanouchi, Y., Kinohara, K., Moriyama, S., 2013. Enzymatic Properties of  $\beta$ -1,3-Glucanase from *Streptomyces* sp Mo. *Journal of Food Science* 78. <https://doi.org/10.1111/1750-3841.12076>
  100. Kutty, G., Davis, A.S., Ma, L., Taubenberger, J.K., Kovacs, J.A., 2015. *Pneumocystis* Encodes a Functional Endo- $\beta$ -1,3-glucanase That is Expressed Exclusively in Cysts. *The Journal of Infectious Diseases* 211, 719–728. <https://doi.org/10.1093/infdis/jiu517>
  101. Lamers, C., Plüss, C.J., Ricklin, D., 2021. The Promiscuous Profile of Complement Receptor 3 in Ligand Binding, Immune Modulation, and Pathophysiology. *Front. Immunol.* 12, 662164. <https://doi.org/10.3389/fimmu.2021.662164>
  102. Lee, C., Verma, R., Byun, S., Jeun, E.-J., Kim, G.-C., Lee, S., Kang, H.-J., Kim, C.J., Sharma, G., Lahiri, A., Paul, S., Kim, K.S., Hwang, D.S., Iwakura, Y., Speciale, I., Molinaro, A., De Castro, C., Rudra, D., Im, S.-H., 2021. Structural specificities of cell surface  $\beta$ -glucan polysaccharides determine commensal yeast mediated immuno-modulatory activities. *Nat Commun* 12, 3611. <https://doi.org/10.1038/s41467-021-23929-9>
  103. Lee, H.J., Park, B.-R., Chewaka, L.S., 2024. A Comparative Study of Composition and Soluble Polysaccharide Content between Brewer's Spent Yeast and Cultured Yeast Cells. *Foods* 13, 1567. <https://doi.org/10.3390/foods13101567>

104. Lee, H.J., Yong, H.I., Kim, M., Choi, Y.-S., Jo, C., 2020. Status of meat alternatives and their potential role in the future meat market — A review. *Asian-Australas J Anim Sci* 33, 1533–1543. <https://doi.org/10.5713/ajas.20.0419>
105. Lee, T.H., Lim, P.O., Lee, Y.-E., 2007. Cloning, characterization, and expression of xylanase A gene from *Paenibacillus* sp. DG-22 in *Escherichia coli*. *Journal of Microbiology and Biotechnology* 17.
106. Legentil, L., Paris, F., Ballet, C., Trouvelot, S., Daire, X., Vetvicka, V., Ferrières, V., 2015. Molecular Interactions of  $\beta$ -(1 $\rightarrow$ 3)-Glucans with Their Receptors. *Molecules* 20, 9745–9766. <https://doi.org/10.3390/molecules20069745>
107. Legras, J., Merdinoglu, D., Cornuet, J., Karst, F., 2007. Bread, beer and wine: *Saccharomyces cerevisiae* diversity reflects human history. *Molecular Ecology* 16, 2091–2102. <https://doi.org/10.1111/j.1365-294X.2007.03266.x>
108. Lesage, G., Bussey, H., 2006. Cell Wall Assembly in *Saccharomyces cerevisiae*. *Microbiology and Molecular Biology Reviews* 70, 317–343. <https://doi.org/10.1128/mmbr.00038-05>
109. Letunic, I., Khedkar, S., Bork, P., 2021. SMART: recent updates, new developments and status in 2020. *Nucleic Acids Research* 49, D458–D460. <https://doi.org/10.1093/nar/gkaa937>
110. Li, J., Karboune, S., 2019. Characterization of the composition and the techno-functional properties of mannoproteins from *Saccharomyces cerevisiae* yeast cell walls. *Food Chemistry* 297, 124867. <https://doi.org/10.1016/j.foodchem.2019.05.141>
111. Lin, S., Qin, Z., Chen, Q., Fan, L., Zhou, J., Zhao, L., 2019. Efficient Immobilization of Bacterial GH Family 46 Chitosanase by Carbohydrate-Binding Module Fusion for the Controllable Preparation of Chitoooligosaccharides. *J. Agric. Food Chem.* 67, 6847–6855. <https://doi.org/10.1021/acs.jafc.9b01608>
112. Lipke, P.N., Ovalle, R., 1998. Cell Wall Architecture in Yeast: New Structure and New Challenges. *J Bacteriol* 180, 3735–3740. <https://doi.org/10.1128/JB.180.15.3735-3740.1998>
113. Liu, X., Wang, R., Bi, J., Kang, L., Zhou, J., Duan, B., Liu, Z., Yuan, S., 2020. A novel endo- $\beta$ -1,6-glucanase from the mushroom *Coprinopsis cinerea* and its application in studying of cross-linking of  $\beta$ -1,6-glucan and the wall extensibility in stipe cell walls. *International Journal of Biological Macromolecules* 160, 612–622. <https://doi.org/10.1016/j.ijbiomac.2020.05.244>
114. Liu, Z., Tyo, K.E.J., Martínez, J.L., Petranovic, D., Nielsen, J., 2012. Different expression systems for production of recombinant proteins in *Saccharomyces*

- cerevisiae*. Biotech & Bioengineering 109, 1259–1268. <https://doi.org/10.1002/bit.24409>
115. Lodolo, E.J., Kock, J.L.F., Axcell, B.C., Brooks, M., 2008. The yeast *Saccharomyces cerevisiae* – the main character in beer brewing. FEMS Yeast Research 8, 1018–1036. <https://doi.org/10.1111/j.1567-1364.2008.00433.x>
  116. Lomolino, G., Vincenzi, S., Zannoni, S., Vegro, M., De Iseppi, A., 2024. White Sparkling Wine Proteins and Glycoproteins and Their Behavior in Foam Expansion and Stability. Beverages 10, 21. <https://doi.org/10.3390/beverages10010021>
  117. Lu, Z., Rämngård, C., Ergenlioğlu, İ., Sandin, L., Hammar, H., Andersson, H., King, K., Inman, A.R., Hao, M., Bulone, V., McKee, L.S., 2023. Multiple enzymatic approaches to hydrolysis of fungal  $\beta$ -glucans by the soil bacterium *Chitinophaga pinensis*. The FEBS Journal febs.16720. <https://doi.org/10.1111/febs.16720>
  118. Ma, C., Xia, S., Song, J., Hou, Y., Hao, T., Shen, S., Li, K., Xue, C., Jiang, X., 2024. Yeast protein as a novel protein source: Processing, functional properties, and potential applications in foods. Innovative Food Science & Emerging Technologies 93, 103606. <https://doi.org/10.1016/j.ifset.2024.103606>
  119. Ma, J., Jiang, Z., Yan, Q., Lv, A., Li, Y., Yang, S., 2024. Structural and functional analysis of SpGu64A: a novel glycoside hydrolase family 64 laminaripentaose-producing  $\beta$ -1,3-glucanase from *Streptomyces pratensis*. The FEBS Journal 291, 2009–2022. <https://doi.org/10.1111/febs.17094>
  120. Ma, J., Qin, Z., Zhou, P., Wang, R., Yan, Q., Jiang, Z., Yang, S., 2022. Structural insights into the substrate recognition and catalytic mechanism of a fungal glycoside hydrolase family 81  $\beta$ -1,3-glucanase. Enzyme and Microbial Technology 153, 109948. <https://doi.org/10.1016/j.enzmictec.2021.109948>
  121. Ma, L., Lu, Y., Yan, H., Wang, X., Yi, Y., Shan, Y., Liu, B., Zhou, Y., Lü, X., 2020. Screening of cellulolytic bacteria from rotten wood of Qinling (China) for biomass degradation and cloning of cellulases from *Bacillus methylotrophicus*. BMC Biotechnol 20, 2. <https://doi.org/10.1186/s12896-019-0593-8>
  122. Maehara, Y., Tsujitani, S., Saeki, H., Oki, E., Yoshinaga, K., Emi, Y., Morita, M., Kohnoe, S., Kakeji, Y., Yano, T., Baba, H., 2012. Biological mechanism and clinical effect of protein-bound polysaccharide K (Krestin®): review of development and future perspectives. Surg Today 42, 8–28. <https://doi.org/10.1007/s00595-011-0075-7>
  123. Manners, D.J., Masson, A.J., Patterson, J.C., Björndal, H., Lindberg, B., 1973. The structure of a  $\beta$ -(1→6)-D-glucan from yeast cell walls. Biochemical Journal 135, 31–36. <https://doi.org/10.1042/bj1350031>

124. Markovina, N., Banjari, I., Bucevic Popovic, V., Jelacic Kadic, A., Puljak, L., 2020. Efficacy and safety of oral and inhalation commercial beta-glucan products: Systematic review of randomized controlled trials. *Clinical Nutrition* 39, 40–48. <https://doi.org/10.1016/j.clnu.2019.01.003>
125. Marshall, J.J., Grand, R.J.A., 1975. Comparative studies on  $\beta$ -glucan hydrolases. *Archives of Biochemistry and Biophysics* 167, 165–175. [https://doi.org/10.1016/0003-9861\(75\)90453-1](https://doi.org/10.1016/0003-9861(75)90453-1)
126. Martín-Cuadrado, A.-B., Fontaine, T., Esteban, P.-F., Dedo, J.E.D., Medina-Redondo, M.D., Rey, F.D., Latgé, J.P., Aldana, C.R.V.D., 2008. Characterization of the endo- $\beta$ -1,3-glucanase activity of *S. cerevisiae* Eng2 and other members of the GH81 family. *Fungal Genetics and Biology* 45, 542–553. <https://doi.org/10.1016/j.fgb.2007.09.001>
127. Mata-Martínez, P., Bergón-Gutiérrez, M., Del Fresno, C., 2022. Dectin-1 Signaling Update: New Perspectives for Trained Immunity. *Front. Immunol.* 13, 812148. <https://doi.org/10.3389/fimmu.2022.812148>
128. McGrath, C.E., Wilson, D.B., 2006. Characterization of a *Thermobifida fusca*  $\beta$ -1,3-Glucanase (Lam81A) with a Potential Role in Plant Biomass Degradation. *Biochemistry* 45, 14094–14100. <https://doi.org/10.1021/bi061757r>
129. Miyajima, Y., Satoh, K., Umeda, Y., Makimura, K., 2009. Quantitation of Fungal DNA Contamination in Commercial Zymolyase and Lyticase Used in the Preparation of Fungi. *Nippon Ishinkin Gakkai Zasshi* 50, 259–262. <https://doi.org/10.3314/jjmm.50.259>
130. Moreno, F.J., Carrero-Carralero, C., Hernández-Hernández, O., Sanz, M. Luz, 2014. Fractionation of Food Bioactive Oligosaccharides, in: Moreno, F.J., Sanz, María Luz (Eds.), *Food Oligosaccharides*. Wiley, pp. 255–283. <https://doi.org/10.1002/9781118817360.ch15>
131. Mrsă, V., Seidl, T., Gentzsch, M., Tanner, W., 1997. Specific Labelling of Cell Wall Proteins by Biotinylation. Identification of Four Covalently Linked O-mannosylated Proteins of *Saccharomyces cerevisiae*. *Yeast* 13, 1145–1154. [https://doi.org/10.1002/\(SICI\)1097-0061\(19970930\)13:12%3C1145::AID-YEA163%3E3.0.CO;2-Y](https://doi.org/10.1002/(SICI)1097-0061(19970930)13:12%3C1145::AID-YEA163%3E3.0.CO;2-Y)
132. Müller, C., Ortmann, T., Abi, A., Hartig, D., Scholl, S., Jördening, H.-J., 2017. Immobilization and Characterization of *E. gracilis* Extract with Enriched Laminaribiose Phosphorylase Activity for Biezymatic Production of Laminaribiose. *Appl Biochem Biotechnol* 182, 197–215. <https://doi.org/10.1007/s12010-016-2320-4>
133. Mundada, P.A., Mahajan, H.S., 2025. Pharmaceutical application of beta-glucan: a comprehensive overview. *Futur J Pharm Sci* 11, 77. <https://doi.org/10.1186/s43094-025-00835-1>

134. Nacalai Tesque, Inc., 2025. Zymolyase<sup>TM</sup> (from *Arthrobacter luteus*) [WWW Document]. Nacalai Tescue, Inc. URL <https://www.nacalai.co.jp/> (accessed 10.21.25).
135. Nadal-Ribelles, M., Solé, C., De Nadal, E., Posas, F., 2024. The rise of single-cell transcriptomics in yeast. *Yeast* 41, 158–170. <https://doi.org/10.1002/yea.3934>
136. Nakatani, Y., Larsen, D.S., Cutfield, S.M., Cutfield, J.F., 2014. Major Change in Regiospecificity for the Exo-1,3- $\beta$ -glucanase from *Candida albicans* following Its Conversion to a Glycosynthase. *Biochemistry* 53, 3318–3326. <https://doi.org/10.1021/bi500239m>
137. Narui, T., Sawada, K., Culberson, C.F., Culberson, W.L., Shibata, S., 1999. Pustulan-Type Polysaccharides as a Constant Character of the Umbilicariaceae (Lichenized Ascomycotina). *Bryologist* 102, 80–85. <https://doi.org/https://doi.org/10.2307/3244464>
138. Nelson, C.E., Attia, M.A., Rogowski, A., Morland, C., Brumer, H., Gardner, J.G., 2017. Comprehensive functional characterization of the glycoside hydrolase family 3 enzymes from *Cellvibrio japonicus* reveals unique metabolic roles in biomass saccharification. *Environmental Microbiology* 19, 5025–5039. <https://doi.org/10.1111/1462-2920.13959>
139. Niu, K., Liu, Z., Feng, Y., Gao, T., Wang, Z., Zhang, P., Du, Z., Gao, D., Fang, X., 2020. A novel strategy for efficient disaccharides synthesis from glucose by  $\beta$ -glucosidase. *Bioresour. Bioprocess.* 7, 45. <https://doi.org/10.1186/s40643-020-00334-6>
140. Nobe, R., Sakakibara, Y., Fukuda, N., Yoshida, N., Ogawa, K., Suiko, M., 2003. Purification and Characterization of Laminaran Hydrolases from *Trichoderma viride*. *Bioscience, Biotechnology, and Biochemistry* 67, 1349–1357. <https://doi.org/10.1271/bbb.67.1349>
141. Noorbakhsh Varnosfaderani, S.M., Ebrahimzadeh, F., Akbari Oryani, M., Khalili, S., Almasi, F., Mosaddeghi Heris, R., Payandeh, Z., Li, C., Nabi Afjadi, M., Alagheband Bahrami, A., 2024. Potential promising anticancer applications of  $\beta$ -glucans: a review. *Bioscience Reports* 44, BSR20231686. <https://doi.org/10.1042/BSR20231686>
142. Novák, M., Synytsya, Andriy, Gedeon, O., Slepíčka, P., Procházka, V., Synytsya, Alla, Blahovec, J., Hejlová, A., Čopíková, J., 2012. Yeast  $\beta$ (1-3),(1-6)-d-glucan films: Preparation and characterization of some structural and physical properties. *Carbohydrate Polymers* 87, 2496–2504. <https://doi.org/10.1016/j.carbpol.2011.11.031>
143. Oliveira, A.S., Odila Pereira, J., Ferreira, C., Faustino, M., Durão, J., Pereira, A.M., Oliveira, C.M., Pintado, M.E., Carvalho, A.P., 2022. Spent Yeast Valorization for Food Applications: Effect of Different Extraction Methodologies. *Foods* 11, 4002. <https://doi.org/10.3390/foods11244002>

144. Orlean, P., 2012. Architecture and Biosynthesis of the *Saccharomyces cerevisiae* Cell Wall. *Genetics* 192, 775–818. <https://doi.org/10.1534/genetics.112.144485>
145. Paeschke, T.M., Aimutis, W.R. (Eds.), 2010. Nondigestible Carbohydrates and Digestive Health, 1st ed. Wiley. <https://doi.org/10.1002/9780470958186>
146. Palumbo, J.D., Sullivan, R.F., Kobayashi, D.Y., 2003. Molecular Characterization and Expression in *Escherichia coli* of Three  $\beta$ -1,3-Glucanase Genes from *Lysobacter enzymogenes* Strain N4-7. *J Bacteriol* 185, 4362–4370. <https://doi.org/10.1128/JB.185.15.4362-4370.2003>
147. Parapouli, M., Vasileiadis, A., Afendra, A.-S., Hatziloukas, E., 2020. *Saccharomyces cerevisiae* and its industrial applications. *AIMS Microbiol* 6, 1–31. <https://doi.org/10.3934/microbiol.2020001>
148. Park, Y.-J., Jeong, Y.-U., Kong, W.-S., 2018. Genome Sequencing and Carbohydrate-Active Enzyme (CAZyme) Repertoire of the White Rot Fungus *Flammulina elastica*. *IJMS* 19, 2379. <https://doi.org/10.3390/ijms19082379>
149. Pell, G., Williamson, M.P., Walters, C., Du, H., Gilbert, H.J., Bolam, D.N., 2003. Importance of Hydrophobic and Polar Residues in Ligand Binding in the Family 15 Carbohydrate-Binding Module from *Cellvibrio japonicus* Xyn10C. *Biochemistry* 42, 9316–9323. <https://doi.org/10.1021/bi0347510>
150. Perez-Riverol, Y., Bai, J., Bandla, C., García-Seisdedos, D., Hewapathirana, S., Kamatchinathan, S., Kundu, D.J., Prakash, A., Frericks-Zipper, A., Eisenacher, M., Walzer, M., Wang, S., Brazma, A., Vizcaíno, J.A., 2022. The PRIDE database resources in 2022: a hub for mass spectrometry-based proteomics evidences. *Nucleic Acids Research* 50, D543–D552. <https://doi.org/10.1093/nar/gkab1038>
151. Plakys, G., Gasparavičiūtė, R., Vaitekūnas, J., Rutkienė, R., Meškys, R., 2022. Characterization of *Paenibacillus* sp. GKG Endo- $\beta$ -1,3-Glucanase, a Member of Family 81 Glycoside Hydrolases. *Microorganisms* 10, 1930. <https://doi.org/10.3390/microorganisms10101930>
152. Plakys, G., Urbelienė, N., Urbelis, G., Vaitekūnas, J., Labanauskas, L., Mažonienė, E., Meškys, R., 2024. Conversion of  $\beta$ -1,6-Glucans to Gentiobiose using an endo- $\beta$ -1,6-Glucanase PsGly30A from *Paenibacillus* sp. GKG. *ChemBioChem* 25, e202400010. <https://doi.org/10.1002/cbic.202400010>
153. Pluvinaige, B., Fillo, A., Massel, P., Boraston, A.B., 2017. Structural Analysis of a Family 81 Glycoside Hydrolase Implicates Its Recognition of  $\beta$ -1,3-Glucan Quaternary Structure. *Structure* 25, 1348-1359.e3. <https://doi.org/10.1016/j.str.2017.06.019>
154. Pradeep, N.S., Edison, L.K. (Eds.), 2022. Microbial Beta Glucanases: Molecular Structure, Functions and Applications, Interdisciplinary

- Biotechnological Advances. Springer Nature Singapore, Singapore.  
<https://doi.org/10.1007/978-981-19-6466-4>
155. Prayogo, F.A., Budiharjo, A., Kusumaningrum, H.P., Wijanarka, W., Supriyadi, A., Nurhayati, N., 2020. Metagenomic applications in exploration and development of novel enzymes from nature: a review. *Journal of Genetic Engineering and Biotechnology* 18, 39. <https://doi.org/10.1186/s43141-020-00043-9>
  156. Qiao, Y., Xia, C., Liu, L., Tang, L., Wang, Jihong, Xu, C., Wang, Juying, Zhang, L., Ye, X., Huang, Y., Mao, D., Liang, Y., Zhoukun, L., Cui, Z., 2022. Structural characterization and emulsifier property of yeast mannoprotein enzymatically prepared with a  $\beta$ -1,6-glucanase. *LWT* 168, 113898. <https://doi.org/10.1016/j.lwt.2022.113898>
  157. Qin, Z., Yan, Q., Yang, S., Jiang, Z., 2016. Modulating the function of a  $\beta$ -1,3-glucanosyltransferase to that of an endo- $\beta$ -1,3-glucanase by structure-based protein engineering. *Appl Microbiol Biotechnol* 100, 1765–1776. <https://doi.org/10.1007/s00253-015-7057-4>
  158. Rakowska, R., Sadowska, A., Dybkowska, E., Świdorski, F., 2017. Spent yeast as natural source of functional food additives. *Rocz Panstw Zakl Hig* 68, 115–121.
  159. Rani, T.S., Madhuprakash, J., Podile, A.R., 2020. Chitinase-E from *Chitiniphilus shinanonensis* generates chitobiose from chitin flakes. *International Journal of Biological Macromolecules* 163, 1037–1043. <https://doi.org/10.1016/j.ijbiomac.2020.07.052>
  160. Read, S.M., Currie, G., Bacic, A., 1996. Analysis of the structural heterogeneity of laminarin by electrospray-ionisation-mass spectrometry. *Carbohydrate Research* 281, 187–201. [https://doi.org/10.1016/0008-6215\(95\)00350-9](https://doi.org/10.1016/0008-6215(95)00350-9)
  161. Rimek, D., Garg, A.P., Haas, W.H., Kappe, R., 1999. Identification of Contaminating Fungal DNA Sequences in Zymolyase. *J Clin Microbiol* 37, 830–831. <https://doi.org/10.1128/JCM.37.3.830-831.1999>
  162. Robert, X., Gouet, P., 2014. Deciphering key features in protein structures with the new ENDscript server. *Nucleic Acids Research* 42, W320–W324. <https://doi.org/10.1093/nar/gku316>
  163. Rodríguez-Peña, J.M., Díez-Muñiz, S., Bermejo, C., Nombela, C., Arroyo, J., 2013. Activation of the yeast cell wall integrity MAPK pathway by zymolyase depends on protease and glucanase activities and requires the mucin-like protein Hkr1 but not Msb2. *FEBS Letters* 587, 3675–3680. <https://doi.org/10.1016/j.febslet.2013.09.030>
  164. Rombouts, F.M., Phaff, H.J., 1976a. Lysis of Yeast Cell Walls: Lytic  $\beta$ -(1→3)-Glucanases from *Bacillus circulans* WL-12. *European Journal of*

- Biochemistry 63, 121–130. <https://doi.org/10.1111/j.1432-1033.1976.tb10214.x>
165. Rombouts, F.M., Phaff, H.J., 1976b. Lysis of Yeast Cell Walls Lytic  $\beta$ -(1 $\rightarrow$ 6)-Glucanase from *Bacillus circulans* WL-12: Lytic  $\beta$ -(1 $\rightarrow$ 6)-Glucanase from *Bacillus circulans* WL-12. European Journal of Biochemistry 63, 109–120. <https://doi.org/10.1111/j.1432-1033.1976.tb10213.x>
  166. Ruiz-Herrera, J., Victoria Elorza, M., Valentín, E., Sentandreu, R., 2006. Molecular organization of the cell wall of *Candida albicans* and its relation to pathogenicity. FEMS Yeast Research 6, 14–29. <https://doi.org/10.1111/j.1567-1364.2005.00017.x>
  167. Saeki, K., Iwata, J., Yamazaki, S., Watanabe, Y., Tamai, Y., 1994. Purification and characterization of a yeast lytic  $\beta$ -1,3-glucanase from *Oerskovia xanthineolytica* TK-1. Journal of Fermentation and Bioengineering 78, 407–412. [https://doi.org/10.1016/0922-338X\(94\)90038-8](https://doi.org/10.1016/0922-338X(94)90038-8)
  168. Salazar, O., Basso, C., Barba, P., Orellana, C., Asenjo, J.A., 2006. Improvement of the Lytic Properties of a  $\beta$ -1,3-Glucanase by Directed Evolution. MB 33, 211–220. <https://doi.org/10.1385/MB:33:3:211>
  169. Salazar, O., Molitor, J., Lienqueo, M.E., Asenjo, J.A., 2001. Overproduction, Purification, and Characterization of  $\beta$ -1,3-Glucanase Type II in *Escherichia coli*. Protein Expression and Purification 23, 219–225. <https://doi.org/10.1006/prep.2001.1497>
  170. Samuelsen, A.B.C., Schrezenmeir, J., Knutsen, S.H., 2014. Effects of orally administered yeast-derived beta-glucans: A review. Molecular Nutrition Food Res 58, 183–193. <https://doi.org/10.1002/mnfr.201300338>
  171. San Martín, D., Orive, M., Iñarra, B., Castelo, J., Estévez, A., Nazzaro, J., Iloro, I., Elortza, F., Zufía, J., 2020. Brewers' Spent Yeast and Grain Protein Hydrolysates as Second-Generation Feedstuff for Aquaculture Feed. Waste Biomass Valor 11, 5307–5320. <https://doi.org/10.1007/s12649-020-01145-8>
  172. Santovito, E., Greco, D., Logrieco, A.F., Avantaggiato, G., 2018. Eubiotics for Food Security at Farm Level: Yeast Cell Wall Products and Their Antimicrobial Potential Against Pathogenic Bacteria. Foodborne Pathogens and Disease 15, 531–537. <https://doi.org/10.1089/fpd.2018.2430>
  173. Sanz, M.L., Gibson, G.R., Rastall, R.A., 2005. Influence of Disaccharide Structure on Prebiotic Selectivity in Vitro. J. Agric. Food Chem. 53, 5192–5199. <https://doi.org/10.1021/jf050276w>
  174. Schiavone, M., François, J.M., Zerbib, D., Capp, J.-P., 2023. Emerging relevance of cell wall components from non-conventional yeasts as functional ingredients for the food and feed industry. Current Research in Food Science 7, 100603. <https://doi.org/10.1016/j.crfs.2023.100603>

175. Scott, J.H., Schekman, R., 1980. Lyticase: Endoglucanase and Protease Activities That Act Together in Yeast Cell Lysis. *J Bacteriol* 142, 414–423. <https://doi.org/10.1128/jb.142.2.414-423.1980>
176. Seidi, F., Yazdi, M.K., Jouyandeh, M., Habibzadeh, S., Munir, M.T., Vahabi, H., Bagheri, B., Rabiee, N., Zarrintaj, P., Saeb, M.R., 2022. Crystalline polysaccharides: A review. *Carbohydrate Polymers* 275, 118624. <https://doi.org/10.1016/j.carbpol.2021.118624>
177. Seidle, H.F., Huber, R.E., 2005. Transglucosidic reactions of the *Aspergillus niger* Family 3  $\beta$ -glucosidase: Qualitative and quantitative analyses and evidence that the transglucosidic rate is independent of pH. *Archives of Biochemistry and Biophysics* 436, 254–264. <https://doi.org/10.1016/j.abb.2005.02.017>
178. Shen, S.H., Chrétien, P., Bastien, L., Slilaty, S.N., 1991. Primary sequence of the glucanase gene from *Oerskovia xanthineolytica*. Expression and purification of the enzyme from *Escherichia coli*. *Journal of Biological Chemistry* 266, 1058–1063. [https://doi.org/10.1016/S0021-9258\(17\)35282-1](https://doi.org/10.1016/S0021-9258(17)35282-1)
179. Shinya, T., Ménard, R., Kozone, I., Matsuoka, H., Shibuya, N., Kauffmann, S., Matsuoka, K., Saito, M., 2006. Novel  $\beta$ -1,3-,1,6-oligoglucan elicitor from *Alternaria alternata* 102 for defense responses in tobacco. *The FEBS Journal* 273, 2421–2431. <https://doi.org/10.1111/j.1742-4658.2006.05249.x>
180. Steimbach, L., Borgmann, A.V., Gomar, G.G., Hoffmann, L.V., Rutckeviski, R., De Andrade, D.P., Smiderle, F.R., 2021. Fungal beta-glucans as adjuvants for treating cancer patients – A systematic review of clinical trials. *Clinical Nutrition* 40, 3104–3113. <https://doi.org/10.1016/j.clnu.2020.11.029>
181. Sun, S., Wei, X., You, C., 2019. The Construction of an In Vitro Synthetic Enzymatic Biosystem that Facilitates Laminaribiose Biosynthesis from Maltodextrin and Glucose. *Biotechnology Journal* 14, 1800493. <https://doi.org/10.1002/biot.201800493>
182. Szilágyi, M., Kwon, N.-J., Dorogi, C., Pócsi, I., Yu, J.-H., Emri, T., 2010. The extracellular  $\beta$ -1,3-endoglucanase EngA is involved in autolysis of *Aspergillus nidulans*: *Aspergillus nidulans*  $\beta$ -1,3-endoglucanase EngA. *Journal of Applied Microbiology* no-no. <https://doi.org/10.1111/j.1365-2672.2010.04782.x>
183. Takaloo, Z., Nikkhah, M., Nemati, R., Jalilian, N., Sajedi, R.H., 2020. Autolysis, plasmolysis and enzymatic hydrolysis of baker's yeast (*Saccharomyces cerevisiae*): a comparative study. *World Journal of Microbiology and Biotechnology* 36, 68. <https://doi.org/10.1007/s11274-020-02840-3>
184. Tamura, K., Stecher, G., Kumar, S., 2021. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution* 38, 3022–3027. <https://doi.org/10.1093/molbev/msab120>

185. Tao, Z., Yuan, H., Liu, M., Liu, Q., Zhang, S., Liu, H., Jiang, Y., Huang, D., Wang, T., 2023. Yeast Extract: Characteristics, Production, Applications and Future Perspectives. *J. Microbiol. Biotechnol.* 33, 151–166. <https://doi.org/10.4014/jmb.2207.07057>
186. Temple, M.J., Cuskin, F., Baslé, A., Hickey, N., Speciale, G., Williams, S.J., Gilbert, H.J., Lowe, E.C., 2017. A *Bacteroidetes* locus dedicated to fungal 1,6- $\beta$ -glucan degradation: Unique substrate conformation drives specificity of the key endo-1,6- $\beta$ -glucanase. *Journal of Biological Chemistry* 292, 10639–10650. <https://doi.org/10.1074/jbc.M117.787606>
187. Teufel, F., Almagro Armenteros, J.J., Johansen, A.R., Gíslason, M.H., Pihl, S.I., Tsirigos, K.D., Winther, O., Brunak, S., Von Heijne, G., Nielsen, H., 2022. SignalP 6.0 predicts all five types of signal peptides using protein language models. *Nat Biotechnol* 40, 1023–1025. <https://doi.org/10.1038/s41587-021-01156-3>
188. Thomas, S., Rezoagli, E., Abidin, I.Z., Major, I., Murray, P., Murphy, E.J., 2022.  $\beta$ -Glucans from Yeast—Immunomodulators from Novel Waste Resources. *Applied Sciences* 12, 5208. <https://doi.org/10.3390/app12105208>
189. Tomé, D., 2021. Yeast Extracts: Nutritional and Flavoring Food Ingredients. *ACS Food Sci. Technol.* 1, 487–494. <https://doi.org/10.1021/acsfoodscitech.0c00131>
190. van Zanten, G.C., Knudsen, A., Röytiö, H., Forssten, S., Lawther, M., Blennow, A., Lahtinen, S.J., Jakobsen, M., Svensson, B., Jespersen, L., 2012. The Effect of Selected Synbiotics on Microbial Composition and Short-Chain Fatty Acid Production in a Model System of the Human Colon. *PLoS ONE* 7, e47212. <https://doi.org/10.1371/journal.pone.0047212>
191. Varelas, V., Liouni, M., Calokerinos, A.C., Nerantzis, E.T., 2016. An evaluation study of different methods for the production of  $\beta$ -D-glucan from yeast biomass: An evaluation study of different methods for the production of  $\beta$ -D-glucan from yeast biomass. *Drug Test. Analysis* 8, 46–55. <https://doi.org/10.1002/dta.1833>
192. Verjans, P., Dornez, E., Segers, M., Van Campenhout, S., Bernaerts, K., Beliën, T., Delcour, J.A., Courtin, C.M., 2010. Truncated derivatives of a multidomain thermophilic glycosyl hydrolase family 10 xylanase from *Thermotoga maritima* reveal structure related activity profiles and substrate hydrolysis patterns. *Journal of Biotechnology* 145, 160–167. <https://doi.org/10.1016/j.jbiotec.2009.10.014>
193. Vetvicka, V., Vannucci, L., Sima, P., Richter, J., 2019. Beta Glucan: Supplement or Drug? From Laboratory to Clinical Trials. *Molecules* 24, 1251. <https://doi.org/10.3390/molecules24071251>
194. Wakarchuk, W.W., Brochu, D., Foote, S., Robotham, A., Saxena, H., Erak, T., Kelly, J., 2016. Proteomic Analysis of the Secretome of *Cellulomonas fimi*

- ATCC 484 and *Cellulomonas flavigena* ATCC 482. PLoS ONE 11, e0151186. <https://doi.org/10.1371/journal.pone.0151186>
195. Wang, D., Kim, D.H., Yun, E.J., Park, Y.-C., Seo, J.-H., Kim, K.H., 2017. The first bacterial  $\beta$ -1,6-endoglucanase from *Saccharophagus degradans* 2-40T for the hydrolysis of pustulan and laminarin. Appl Microbiol Biotechnol 101, 197–204. <https://doi.org/10.1007/s00253-016-7753-8>
  196. Wang, F., Wu, J., Chen, S., 2018. Preparation of gentiooligosaccharides using *Trichoderma viride*  $\beta$ -glucosidase. Food Chemistry 248, 340–345. <https://doi.org/10.1016/j.foodchem.2017.12.044>
  197. Wang, M., Pan, J., Xiang, W., You, Z., Zhang, Y., Wang, J., Zhang, A., 2024.  $\beta$ -glucan: a potent adjuvant in immunotherapy for digestive tract tumors. Front. Immunol. 15, 1424261. <https://doi.org/10.3389/fimmu.2024.1424261>
  198. Wang, Y., Yuan, H., Wang, J., Yu, Z., 2009. Truncation of the cellulose binding domain improved thermal stability of endo- $\beta$ -1,4-glucanase from *Bacillus subtilis* JA18. Bioresource Technology 100, 345–349. <https://doi.org/10.1016/j.biortech.2008.06.001>
  199. Wang, Y., Zhao, Y., Wang, X., Zhong, L., Fan, Q., Lan, Z., Ye, X., Huang, Y., Li, Z., Cui, Z., 2021. Functional Characterization of the Novel Laminaripentaose-Producing  $\beta$ -1,3-Glucanase *Mo* GluB and Its Biocontrol of *Magnaporthe oryzae*. J. Agric. Food Chem. 69, 9571–9584. <https://doi.org/10.1021/acs.jafc.1c03072>
  200. Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173, 697–703. <https://doi.org/10.1128/jb.173.2.697-703.1991>
  201. Winterhalter, C., Heinrich, P., Candussio, A., Wich, G., Liebl, W., 1995. Identification of a novel cellulose-binding domain the multidomain 120 kDa xylanase XynA of the hyperthermophilic bacterium *Thermotoga maritima*. Molecular Microbiology 15, 431–444. <https://doi.org/10.1111/j.1365-2958.1995.tb02257.x>
  202. Woo, T.H., Cheng, A.F., Ling, J.M., 1992. An application of a simple method for the preparation of bacterial DNA. Biotechniques 13, 696–698.
  203. Wood, I.P., Elliston, A., Ryden, P., Bancroft, I., Roberts, I.N., Waldron, K.W., 2012. Rapid quantification of reducing sugars in biomass hydrolysates: Improving the speed and precision of the dinitrosalicylic acid assay. Biomass and Bioenergy 44, 117–121. <https://doi.org/10.1016/j.biombioe.2012.05.003>
  204. Xia, W., Sheng, L., Mu, W., Shi, Y., Wu, J., 2022. Enzymatic Preparation of Gentiooligosaccharides by a Thermophilic and Thermostable  $\beta$ -Glucosidase at a High Substrate Concentration. Foods 11, 357. <https://doi.org/10.3390/foods11030357>

205. Xiao, R., Zeng, J., Bressler, E.M., Lu, W., Grinstaff, M.W., 2022. Synthesis of bioactive (1→6)- $\beta$ -glucose branched poly-amido-saccharides that stimulate and induce M1 polarization in macrophages. *Nat Commun* 13, 4661. <https://doi.org/10.1038/s41467-022-32346-5>
206. Xin, Y., Ji, H., Cho, E., Roh, K.-B., You, J., Park, D., Jung, E., 2022. Immune-enhancing effect of water-soluble beta-glucan derived from enzymatic hydrolysis of yeast glucan. *Biochemistry and Biophysics Reports* 30, 101256. <https://doi.org/10.1016/j.bbrep.2022.101256>
207. Xu, Y., Li, Y., Yan, Q., Mao, X., Yang, S., Jiang, Z., 2024. The Function and Mechanism of Laminaripentaose Prepared from Curdlan for the Amelioration of the Cognitive Dysfunctions in Obese Mice. *J. Agric. Food Chem.* 72, 19904–19919. <https://doi.org/10.1021/acs.jafc.4c05163>
208. Xu, Z., Wu, X.M., Luo, Y.B., Li, H., Zhou, Y.Q., Liu, Z.Q., Li, Z.Y., 2024. Exploring the therapeutic potential of yeast  $\beta$ -glucan: Prebiotic, anti-infective, and anticancer properties - A review. *International Journal of Biological Macromolecules* 283, 137436. <https://doi.org/10.1016/j.ijbiomac.2024.137436>
209. Yan, Q., Yang, H., Jiang, Z., Liu, E., Yang, S., 2018. A novel thermostable  $\beta$ -1,3-1,4-glucanase from *Thermoascus aurantiacus* and its application in oligosaccharide production from oat bran. *Carbohydrate Research* 469, 31–37. <https://doi.org/10.1016/j.carres.2018.08.017>
210. Yang, M., Chen, L., Wang, J., Msigwa, G., Osman, A.I., Fawzy, S., Rooney, D.W., Yap, P.-S., 2023. Circular economy strategies for combating climate change and other environmental issues. *Environ Chem Lett* 21, 55–80. <https://doi.org/10.1007/s10311-022-01499-6>
211. Yang, Q., Song, H., Su, G., Wang, X., Hu, H., Zhai, Z., Chen, M., Zhou, J., Yin, H., Gao, Y., Hu, D., 2025. Genomic and Transcriptomic Analysis to Explore the Biological Characteristics of *Cyclocybe chaxingu*. *Horticulturae* 11, 409. <https://doi.org/10.3390/horticulturae11040409>
212. Yazdi, M.K., Seidi, F., Jin, Y., Zarrintaj, P., Xiao, H., Esmaeili, A., Habibzadeh, S., Saeb, M.R., 2021. Crystallization of Polysaccharides, in: Inamuddin, Ahamed, M.I., Boddula, R., Altalhi, T. (Eds.), *Polysaccharides*. Wiley, pp. 283–300. <https://doi.org/10.1002/9781119711414.ch13>
213. Yin, Q.Y., De Groot, P.W.J., Dekker, H.L., De Jong, L., Klis, F.M., De Koster, C.G., 2005. Comprehensive Proteomic Analysis of *Saccharomyces cerevisiae* Cell Walls. *Journal of Biological Chemistry* 280, 20894–20901. <https://doi.org/10.1074/jbc.M500334200>
214. Zeković, D.B., Kwiatkowski, S., Vrvic, M.M., Jakovljević, D., Moran, C.A., 2005. Natural and Modified (1→3)- $\beta$ -D-Glucans in Health Promotion and Disease Alleviation. *Critical Reviews in Biotechnology* 25, 205–230. <https://doi.org/10.1080/07388550500376166>

215. Zhang, X., Duan, P., Shi, S., Sun, M., Liu, N., Cao, Z., Dong, J., 2025. Global characterization of GH3 family glycoside hydrolase genes in *Fusarium verticillioides* and functional analysis of FvGH3-6. *Front. Microbiol.* 16, 1543210. <https://doi.org/10.3389/fmicb.2025.1543210>
216. Zhang, Y., Li, S., Wang, X., Zhang, L., Cheung, P.C.K., 2011. Advances in lentinan: Isolation, structure, chain conformation and bioactivities. *Food Hydrocolloids* 25, 196–206. <https://doi.org/10.1016/j.foodhyd.2010.02.001>
217. Zheng, J., Ge, Q., Yan, Y., Zhang, X., Huang, L., Yin, Y., 2023. dbCAN3: automated carbohydrate-active enzyme and substrate annotation. *Nucleic Acids Research* 51, W115–W121. <https://doi.org/10.1093/nar/gkad328>
218. Zhou, P., Chen, Z., Yan, Q., Yang, S., Hilgenfeld, R., Jiang, Z., 2013. The structure of a glycoside hydrolase family 81 endo- $\beta$ -1,3-glucanase. *Acta Crystallogr D Biol Crystallogr* 69, 2027–2038. <https://doi.org/10.1107/S090744491301799X>
219. Zhou, T., Solis, N.V., Marshall, M., Yao, Q., Garleb, R., Yang, M., Pearlman, E., Filler, S.G., Liu, H., 2024. Hyphal Als proteins act as CR3 ligands to promote immune responses against *Candida albicans*. *Nat Commun* 15, 3926. <https://doi.org/10.1038/s41467-024-48093-8>
220. Zhu, Y., Kong, F., 2001. A facile and effective synthesis of  $\alpha$ -(1→6)-linked mannose di-, tri-, tetra-, hexa-, octa-, and dodecasaccharides, and  $\beta$ -(1→6)-linked glucose di-, tri-, tetra-, hexa-, and octasaccharides using sugar trichloroacetimidates as the donors and unprotected or partially protected glycosides as the acceptors. *Carbohydrate Research* 332, 1–21. [https://doi.org/10.1016/S0008-6215\(01\)00070-2](https://doi.org/10.1016/S0008-6215(01)00070-2)
221. Zlotnik, H., Fernandez, M.P., Bowers, B., Cabib, E., 1984. *Saccharomyces cerevisiae* mannoproteins form an external cell wall layer that determines wall porosity. *J Bacteriol* 159, 1018–1026. <https://doi.org/10.1128/jb.159.3.1018-1026.1984>
222. Zverlov, V.V., Volkov, I.Y., Velikodvorskaya, G.A., Schwarz, W.H., 2001. The binding pattern of two carbohydrate-binding modules of laminarinase Lam16A from *Thermotoga neapolitana*: differences in  $\beta$ -glucan binding within family CBM4. *Microbiology* 147, 621–629. <https://doi.org/10.1099/00221287-147-3-621>

## SANTRAUKA

### 1. SANTRUMPŲ SĄRAŠAS

$\beta$ -ME – beta-merkaptioetanolis

CAZymes – angliavandenius veikiantys fermentai (*angl.* carbohydrate-active enzymes)

CBM – angliavandenių surišimo modulis (*angl.* carbohydrate-binding module)

DP – polimerizacijos laipsnis (*angl.* degree of polymerisation)

FASP – filtruose atliekama hidrolizė (*angl.* filter-assisted sample preparation)

GH – glikozilhidrolazės (*angl.* glycosyl hydrolases)

HPLC-RI – efektyvioji skysčių chromatografija su lūžio rodiklio nustatymu (*angl.* high performance liquid chromatography-refractive index)

LC-MS/MS – skysčių chromatografija-tandeminė masių spektrometrija (*angl.* liquid chromatography-mass spectrometry/mass spectrometry)

NDS-PAAG – natrio dodecilsulfato poliakrilamidinio gelio elektroforezė

TLC – plonasluoksnė chromatografija (*angl.* thin-layer chromatography)

YCW – mielių sienelė (*angl.* yeast cell wall)

## 2. ĮVADAS

Jau nuo 6000–7000 metų pr. Kr. mielės *Saccharomyces cerevisiae* pradėtos taikyti fermentuotų maisto produktų ir gėrimų, pvz. duonos, alaus, vyno, gamybai. Mielių, kaip funkcionalių ląstelių, naudojimas fermentacijoje yra pagrindinė pritaikymo sritis. Pastaruoju metu pavieniai mielių ląstelės komponentai sulaukia vis daugiau dėmesio tobulėjant biotechnologijos metodams ir atsirandant naujiems rinkos poreikiams. Viduląsteliniai vandenyje tirpūs komponentai, vadinami mielių ekstraktu, pradėti taikyti kaip skonio stiprikliai ir aminorūgščių šaltinis. Tuo tarpu vandenyje netirpi mielių sienelių frakcija tapo  $\beta$ -gliukanų šaltiniu, kurie daugiausiai naudojami pašarams ir maisto papildams. Tiek mielių sienelės, tiek mielių  $\beta$ -gliukanai gali būti gaunami ir naudojami po ekstrakcijos iš kepimo ar panaudotų alaus mielių. Kita dažnai naudojama forma – kaip panaudotos alaus mielės. Tokiu atveju pritaikymas ekonomiškėsnis, nes nenaudojami ekstrakcijos ir gryninimo metodai.

Ir kepimo, ir panaudotos alaus mielės gali būti naudojamos kaip mielių sienelių arba mielių  $\beta$ -gliukanų šaltinis. Ar naudojamas produktas bus naudojamas kaip panaudotos alaus mielės, ar kaip mielių sienelės, ar kaip mielių  $\beta$ -gliukanai priklauso nuo pageidaujamo grynumo laipsnio ir išgavimui naudotų metodų. Mielių sienelės laikomos žemos vertės šalutiniu produktu, susidarančiu mielių ekstrakto gamybos metu. Tiek mielių sienelės, tiek panaudotos alaus mielės yra nebrangūs, netoksiški ir savo sudėtimi vertingi pramoninės skalės produktai. Remiantis Europos polisacharidų meistrystės tinklo (*angl.* European Polysaccharide Network of Excellence (EPNOE)) tyrimų gairėmis 2040, biomasėje esančių polisacharidų panaudojimas maiste ir mityboje turėtų būti vystomas siekiant kurti tvarią bioekonomiką. Taigi, mielių sienelių konvertavimas į maisto produktams ir mitybai tinkamus maisto priedus yra žingsnis link tvarumo principų įgyvendinimo.

Mielių  $\beta$ -gliukanų imunomoduliacinis poveikis tiriamas jau nuo 1980-ųjų metų, daug dėmesio skiriant vėžio terapijų pagerinimui. Nepaisant perspektyvių rezultatų gautų *in vitro* sąlygomis, ikiklinikinių ir klinikinių tyrimų rezultatai mažiau reikšmingi. Esminis proveržis iki šiol nebuvo pasiektas, kadangi beveik visi  $\beta$ -gliukanų produktai rinkoje egzistuoja kaip maisto papildai, o ne kaip vaistai. Vienintelė išimtis yra ne *Saccharomyces* mielių  $\beta$ -gliukanas, kurio klinikinis vartojimas patvirtintas Japonijoje. Egzistuoja dvi priežastys, ribojančios terapinį taikymą: nepastovi  $\beta$ -gliukanų struktūra juos išskiriant iš skirtingų šaltinių skirtinguose tyrimuose ir skirtumai tarp supaprastintų *in vitro* sistemų, gyvūnų modelių ir žmogaus

organizmo, kurie veda prie nepastovių klinikinių tyrimų, vertinančių terapinį poveikį, rezultatų.

Kaip alternatyva dabartiniam panaudojimui ir proveržio nesulaukiančiai vėžio terapijos kryptčiai, mielių sienelėje esantys  $\beta$ -gliukanai gali būti panaudojami kaip substratas konvertuojant juos į bioaktyvias ir maisto produktų funkcionalumą gerinančias molekules, pvz. oligosacharidus. Toks alternatyvus pritaikymas išspręstų nepastovų  $\beta$ -gliukanų biologinį aktyvumą, priklausantį nuo sudėtingos  $\beta$ -gliukanų polimero erdvinės struktūros. Ankstesnių tyrimų metu jau buvo parodyta, kad laminaripentozė, laminaribiozė ir gentiobiozė gali būti gaunamos iš mielių sienelių paveikus mielių sienelės hidrolizuojančiais fermentais. Šis pritaikymo būdas pasitarnauja ne tik kaip mielių sienelių valorizacija, bet ir suteikia ekonomišką pramoninės skalės žaliavą šių oligosacharidų gavimui. Gausiausias mielių sienelių polisacharidas –  $\beta$ -gliukanas, o bakterijos yra dažnai sutinkamas  $\beta$ -gliukanazių šaltinis.

Dar viena kai kurių mielių sienelės degraduojančių fermentų savybė – gebėjimas suardyti gyvas mielių ląsteles. Komerciškai prieinami įvairūs lizuojančių fermentų mišiniai, pvz. *Zymolyase*, *Lyticase* ir *Glusulase*. Plačiai naudojami *Zymolyase* ir *Lyticase* lizuojančių fermentų preparatai yra gaunami iš bakterijos *Cellulosimicrobium cellulans*. Svarbu, kad šie mišiniai sudėtyje turi proteazių, kurios gali hidrolizuoti ne tik mielių sienelių manoproteinus, bet ir viduląstelinius mielių baltymus, kuriuos norima išgauti išlaikiusius aktyvumą arba funkcionalumą. Mielės gali pasitarnauti kaip natyvių arba rekombinantinių baltymų šaltinis. Nespecifiniai mielės lizuojančių fermentų mišinių aktyvumai riboja tokių preparatų pritaikomumą, kai reikalingas išgaunamų baltymų struktūros išsaugojimas. Kaip alternatyva proteazėms, fermentiniuose lizavimo preparatuose gali būti naudojamas redukuojančio agento priedas, kuris gali suardyti mielių sienelių manoproteinų tinklą. Tuo pačiu, redukuojantis agentas taip pat paveiks ir viduląstelinius baltymus. Taigi, sąlygos, kurioms esant specifinės endo-1,3- $\beta$ -gliukanazės galėtų lizuoti gyvas mielių ląsteles be proteazių arba be redukuojančių agentų, būtų pageidaujamos, optimizuojant pramoninius procesus.

Kadangi pilnas fermentinis mielių sienelių suardymas reikalauja įvairių fermentinių aktyvumų, mielių sienelių mėginys pats yra puikus substratas siekiant atrinkti mikroorganizmus, kurie gebėtų sintetinti mielių sienelių polisacharidus veikiančius fermentus. Nauji mikroorganizmai, sintetinantys sienelės hidrolizuojančius fermentus, gali pasiūlyti sprendimus abiem problemoms: įrankius mielių lizei be proteazių ir redukuojančių agentų naudojimo ir mielių sienelių valorizacijos galimybes.

**Darbo tikslas** – identifikuoti ir pritaikyti naujus mielių sienelių polisacharidus skaidančius bakterinius fermentus aukštesnės pridėtinės vertės produktų gavimui.

Darbo tikslui pasiekti buvo iškelti šie uždaviniai:

1. Identifikuoti bakterijas, gebančias skaidyti mielių sienelių substratą.
2. Identifikuoti polisacharidus skaidančius fermentus, kurie atsakingi už mielių sienelių ardymą.
3. Nustatyti atrinktų fermentų rekombinantinių variantų substratinį specifiškumą ir hidrolizės produktus.
4. Įvertinti polisacharidus skaidančių fermentų tinkamumą mielių sienelių konvertavimui į funkcinius ir bioaktyvius oligosacharidus.
5. Identifikuoti glikozidazių kombinacijas ir mielių lizės sąlygas, kurios leistų pakeisti proteazes arba redukuojančius agentus.

### 3. MOKSLINIS NAUJUMAS IR PRAKTINĖ VERTĖ

Pirmiausia, šiame darbe pristatoma galimybė pramoninį šalutinį produktą, mielių sieneses, paversti funkciniais ir biologiškai aktyviais oligosacharidais pritaikant fermentinę hidrolizę. Darbe charakterizuoti mielių sieneses skaidantys fermentai iš *Paenibacillus* sp. GKG ir *Rheinheimera* sp. GKG bakterijų buvo pirmieji GH3, GH16, GH30 ir GH81 šeimų atstovai genties lygiu. Tarp charakterizuotų fermentų didžiausią mokslinę reikšmę dėl savo išskirtinės domenų struktūros turi GH16\_306 fermentas iš *Rheinheimera* sp. GKG bakterijos. Šis fermentas turi vieną katalizinį modulį, priskirtą GH16 šeimai, ir du nekatalizinius modulius, kurie priskiriami pasikartojančių motyvų turintiems domenams. Įdomu, kad erdvinės struktūros modelio analizė ir funkcionalumo eksperimentai parodė, kad šie nekataliziniai domenai pasižymi angliavandenių surišimo aktyvumu  $\beta$ -1,3-gliukanų substratams. Kadangi šie domenai nepanašūs į iki šiol aprašytus angliavandenių surišimo modulius (*angl.* carbohydrate binding modules (CBM)) šeimų atstovus, šie domenai yra potencialūs naujos CBM šeimos atstovai. Gauti rezultatai praplečia dabartines žinias apie CBM modulius ir jų specifiškumą įvairiems angliavandeniams. Tuo tarpu PsLam81A ir PsGly30A fermentai iš *Paenibacillus* sp. GKG bakterijos pasižymėjo išskirtine savybe iš mielių sienelių generuoti laminaribiozės ir gentiobiozės oligosacharidus susidarant nedideliu gliukozės kaip šalutinio produkto kiekiui. Be to, fermentas GH64 iš *Cellulosimicrobium* sp. GKH bakterijos generavo laminaripentozę kaip dominuojantį produktą.

Praktinio pritaikymo aspektu CBM moduliai gali būti pritaikomi afininėje chromatografijoje kaip ligandai siekiant išfrakcionuoti oligosacharidus, turinčius skirtingas savybes, pvz. celo-oligosacharidus ir ksilo-oligosacharidus. Šio darbo temos kontekste GH16\_306 baltymo CBM modulių savybės toliau gali būti nagrinėjamos norint sukurti sorbentus  $\beta$ -1,3-oligosacharidų atskyrimui nuo  $\beta$ -1,6-oligosacharidų. Tuo tarpu, PsLam81A, PsGly30A ir GH64 fermentai gali konvertuoti mielių sieneses į oligosacharidus su aukšta konversijos išeiga. Siekiamas šių oligosacharidų pritaikymas – įtraukimas į mitybą kaip bioaktyvių ar maisto funkcines savybes gerinančių priedų. Gentiobiozė pagerintų maisto produktų skonį, laminaribiozė veiktų kaip prebiotikas, o laminaripentozė veiktų kaip prebiotikas ir antsvorį ir jo pasekmes mažinantis priedas. Mielių ekstrakto produktai galėtų būti praturtinami funkciniais ir bioaktyviais oligosacharidais naudojant šiame darbe aprašytus fermentus mielių autolizės metu arba po jos, kuri taikoma mielių ekstrakto gamybos metu.

Visų antra, šiame darbe tiriamas endo-1,3- $\beta$ -gliukanazių gebėjimas sukelti gyvų mielių ląstelių lizę. Tam tikrų endo-1,3- $\beta$ -gliukanazių mieles lizuojantis aktyvumas buvo atrastas prieš kelis dešimtmečius. Deja, proteazės ir redukuojantys agentai reikalingi efektyviai lizei. Šiame darbe tiriamos sąlygos, kuriose *Saccharomyces cerevisiae* B4741 mielių lizė galėtų vykti nesant nei proteazių, nei redukuojančių agentų priedų – kas ankstesnių tyrimu metu nevyko.

Praktinio pritaikymo aspektu mielių lizė svarbi laboratorinėje ir komercinėje skalėje. Laboratorinės skalės taikymai apima mielių sienelių suardymą mielių sferoplastų gavimui ir viduląstelių baltymų ekstrakcijai. Nukleorūgščių padauginimui ir sekų nustatymo taikymams reikalingi DNR ir RNazių neturintys mielių lizei skirti preparatai. Komercinis taikymas apima rekombinantinių viduląstelių baltymų ekstrakciją, kurie naudojami kaip biofarmaciniai preparatai terapiniams tikslams. Dar viena besivystanti sritis – natyvių mielių baltymų ekstrakcija panaudojimui maisto pramonėje kaip aminorūgščių ar funkcinių maisto priedų šaltinio. Dar viena lizuojančių endo-1,3- $\beta$ -gliukanazių panaudojimo sritis, kuri nenagrinėjama šiame darbe, yra priešgrybiniai preparatai.

Apibendrinant, šiame darbe parodyta, kad baltymo GH16\_306 baltymo pasikartojančius motyvus turintys domenai yra tikėtini CBM domenai, kurie šiuo metu nėra priskiriami jokiai egzistuojančiai CBM šeimai. Be to, pademonstruota, kad PsLam81A, PsGly30A ir GH64 fermentai yra tinkami įrankiai mielių sienelių valorizacijai. Galiausiai, tyrime apibūdinamos *Saccharomyces cerevisiae* BY4741 mielių ląstelių lizės sąlygos, kai nenaudojami proteazių ir  $\beta$ -ME priedai. Šie rezultatai suteikia pagrindą tolesniems praktinio pritaikymo tyrimams.

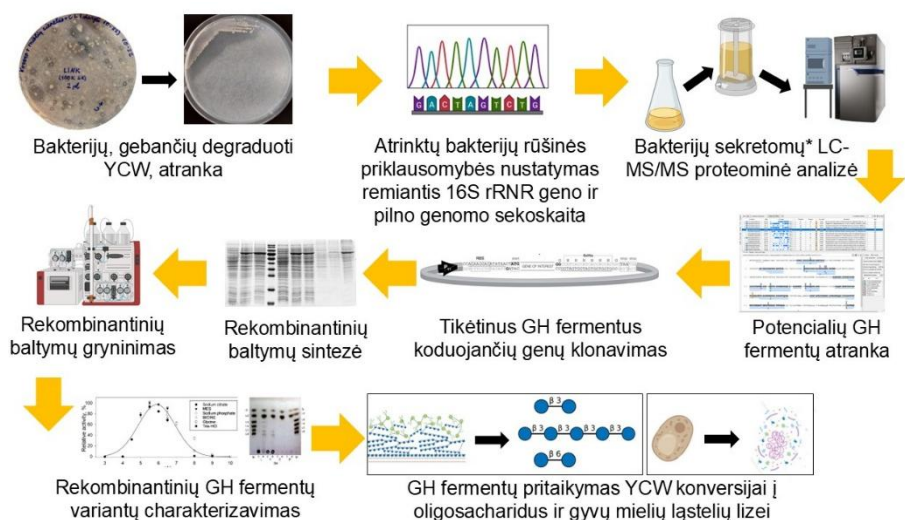
#### 4. GINAMIEJI TEIGINIAI

1. *Rheinheimera* sp. GKU, *Paenibacillus* sp. GKG ir *Cellulosmicrobium* sp. GKH bakterijos gali degraduoti mielių sienėles ir panaudoti jas kaip anglies šaltinį.
2. *Rheinheimera* sp. GKU, *Paenibacillus* sp. GKG ir *Cellulosmicrobium* sp. GKH sekretuoja polisacharidus skaidančius fermentus, kurie konvertuoja mielių sienėles į vandenyje tirpius oligosacharidus ir gliukozę.
3. Trys *Rheinheimera* sp. GKU GH fermentai, GH16\_3455, GH16\_306 ir GH3\_3456, yra atsakingi už mielių sienelių skaidymą iki gliukozės.
4. Trys charakterizuoti fermentai, PsLam81A, PsGly30A ir GH64, yra tinkami mielių sienelių  $\beta$ -gliukanų konversijai į bioaktyvius ir funkcionalius oligosacharidus – laminaribiozę, gentiobiozę ir laminaripentozę, atitinkamai.
5. Kombinuotas GH16 and GH64 fermentų veikimas leidžia lizuoti gyvas *Saccharomyces cerevisiae* BY4741 mielių ląsteles be proteazių ir redukuojančių priedų, kai vietoj jų naudojamas glicerolis.

## 5. METODAI

### 5.1. Mielių sieneles degraduojančių bakterijų ir fermentų identifikavimas

Darbe naudotos metodologijos principinė tyrimų schema pateikta 1 paveiksle. Mielių sieneles (YCW) degraduojančių bakterijų paieška ir atranka atlikta naudojantis standartiniais mikrobiologijos metodais. Bakterijų gebėjimas panaudoti YCW kaip anglies šaltinį ir formuoti hidrolizės zonas buvo panaudotas kaip atrankos kriterijus. Dirvožemio ir vandens telkinių mėginiai buvo kultivuojami ant standžios mitybinės terpės, papildytos vandenyje netirpiu YCW substratu. Bakterijos, gebėjusios aplink kolonijas suformuoti skaidrias hidrolizės zonas, buvo izoliuotos ir tiriamos toliau. Bakterijų rūšinė priklausomybė nustatyta atlikus genominės DNR išskyrimą bei 16S rRNR geno (*Paenibacillus* sp. GKG, *Cellulosimicrobium* sp. GKH) arba viso genomo sekoskaitą (*Rheinheimera* sp. GKU). „Sanger“ metodas naudotas 16S rRNR sekos nustatymui, o „Oxford Nanopore long-read sequencing“ metodas naudotas genomo sekos nustatymui.



**1 paveikslas.** Principinė darbe taikytos metodikos schema. \**Paenibacillus* sp. GKG ir *Cellulosimicrobium* sp. GKH bakterijų sekretomų frakcijos; *Rheinheimera* sp. GKU sekretomų, periplazminių ir viduląstelių baltymų frakcijos.

Fermentų, atsakingų už YCW degradaciją, identifikavimas atliktas pasitelkiant viduląstelių, periplazminių ir sekretuojamų baltymų frakcijų proteominę analizę. Baltymų frakcijos surinktos bakterijas kultivuojant skystoje mineralinėje terpėje, papildytoje gliukoze arba vandenyje netirpiu YCW anglies šaltiniu. Netirpios baltymų frakcijos iš kultūrinio skysčio surinktos temperatūrinės denatūracijos būdu. Baltymai renatūruoti

panaudojant 8 M koncentracijos karbamidą. Renatūruoti baltymai hidrolizuoti tripsinu filtruose atliekamos hidrolizės (*angl.* filter-assisted sample preparation (FASP)) metodu. Proteominė analizė atlikta duomenis užrašius skysčių chromatografijos-tandeninės masių spektrometrijos metodu (LC-MS/MS). Duomenys užrašyti naudojant dvi skirtingas LC-MS/MS sistemas ir skirtingus duomenų užrašymo metodus. *Rheinheimera* sp. GKH ir *Cellulosimicrobium* sp. GKH baltymų frakcijų duomenys užrašyti naudojant nuo duomenų priklausomą užrašymo (*angl.* data-dependent acquisition (DDA)) metodą naudojant „LTQ Orbitrap XL“ masių spektrometrą (Thermo Fisher Scientific, Bremenas, Vokietija). Tuo tarpu *Paenibacillus* sp. GKG baltymų frakcijų duomenys užrašyti naudojant nuo duomenų nepriklausomą užrašymo (*angl.* data-independent acquisition (DIA)) metodą naudojant „Synapt G2“ masių spektrometrą (Thermo Fisher Scientific, Bremenas, Vokietija) (Waters Corporation, Milfordas, Masačusetsas, JAV). Gauti rezultatai buvo sulyginti su kiekvienam organizmui parengtomis duomenų bazėmis, parengtomis naudojantis teoriniais proteomais. Teorinių proteomų duomenys gauti iš Uniprot duomenų bazės (*Paenibacillus* sp. GKG, *Cellulosimicrobium* sp. GKH) arba transliuotos genomo sekos (*Rheinheimera* sp. GKH). dbCAN3 serveris buvo pritaikytas angliavandenius veikiančių fermentų atrankai iš bendrų identifikuotų baltymų sąrašų.

## 5.2. Rekombinantinių fermentų variantų gavimas

Identifikavus tikėtinus angliavandenius veikiančių fermentų genus, buvo susintetinti rekombinantiniai šių fermentų variantai. *Cellulosimicrobium* sp. GKH GH16 ir GH64 fermentai susintetinti naudojant sintetinius genus, įsigytus iš Twist Bioscience (San Franciskas, Kalifornija, JAV). *Rheinheimera* sp. GKH ir *Paenibacillus* sp. GKG fermentus koduojantys genai klonuoti naudojant pradmenis bei plazmidinius vektorius. Plazmidiniai vektoriai buvo konstruojami standartiniais molekulinės biologijos metodais, naudojantis komerciniais rinkiniais. Rekombinantiniai baltymai buvo susintetinti įvairiuose *E. coli* kamienuose: BL21(DE3) (Novagen, Darmstadt, Germanija), LOBSTR (Kerafast, Bostonas, Masačusetsas, JAV), soluBL21 (Amsbio, Miltonas, JK), SHuffle T7 (New England Biolabs, Ipsvičas, Masačusetsas, JAV), HMS174(DE3) (Novagen, Darmštatas, Vokietija) ir KRX (Promega, Viena, Austrija)) naudojant indukuojamas baltymų sintezės sistemas. Baltymų gryninimas atliktas metalų afininės, jonų mainų chromatografijos metodais. Buferinių tirpalų keitimas atliktas dializės arba dydžio išskyrimo chromatografijos metodais (*angl.* size exclusion chromatography (SEC)).

### 5.3. Fermentų charakterizavimas

Išgryninti fermentai charakterizuoti naudojant standartinius GH fermentų charakterizavimui naudojamus substratus (pvz. laminariną, pustulaną, mielių  $\beta$ -gliukaną) ir hidrolizės produktus analizuojant plonasluoksnės chromatografijos (TLC), dinitrosalicilinės rūgšties (DNS), efektyviosios skysčių chromatografijos-lūžio rodiklio (*angl.* high performance liquid chromatography (HPLC-RI)) metodais. Grynai komerciniai oligosacharidai (laminaribiozė, laminaritrioze, laminaritetrozė, laminaripentozė, laminariheksozė, gentiobiozė), naudoti kaip standartai. Fermentų charakterizavimo metu nustatytas fermentų substratinis specifiskumas, optimalų veikimą užtikrinančios sąlygos, specifinis aktyvumas, hidrolizės produktai.

### 5.4. Fermentų taikomųjų savybių įvertinimas

Taikomosios fermentų savybės YCW konversijoje į funkcionalius ir bioaktyvius oligosacharidus buvo įvertintos apskaičiuojant gautų oligosacharidų išeigą.  $\beta$ -Gliukanų kiekis YCW mėginyje nustatytas naudojant komercinį rinkinį „ $\beta$ -glucan assay kit (Yeast and Mushroom)“ (Megazyme, Viklou, Airija), sausų medžiagų kiekis nustatytas drėgmės analizatoriumi „MX-50“ (A&D, Tokijas, Japonija), o gautų oligosacharidų koncentracijos apskaičiuotos HPLC-RI kiekybinio nustatymo metodu. Eksperimentinius duomenis surinko Jurgita Kailiuvienė.

Fermentų pritaikymas mielių ląstelių lizei įvertintas matuojant optinio tankio OD<sub>600nm</sub> pokytį laike inkubacijos su lizuojančiais fermentais metu ir vertinant mikroskopijos vaizdus po inkubavimo su pavieniais GH16 ir GH64 fermentais, šių dviejų fermentų deriniu ir *Zymolyase* komerciniu mieles lizuojančiu fermentų mišiniu. *Saccharomyces cerevisiae* BY4741 kamienas (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*) naudotas lizavimo sąlygų nustatymui bei palyginimui. Eksperimentinius duomenis surinko dr. Ramunė Stanevičienė.

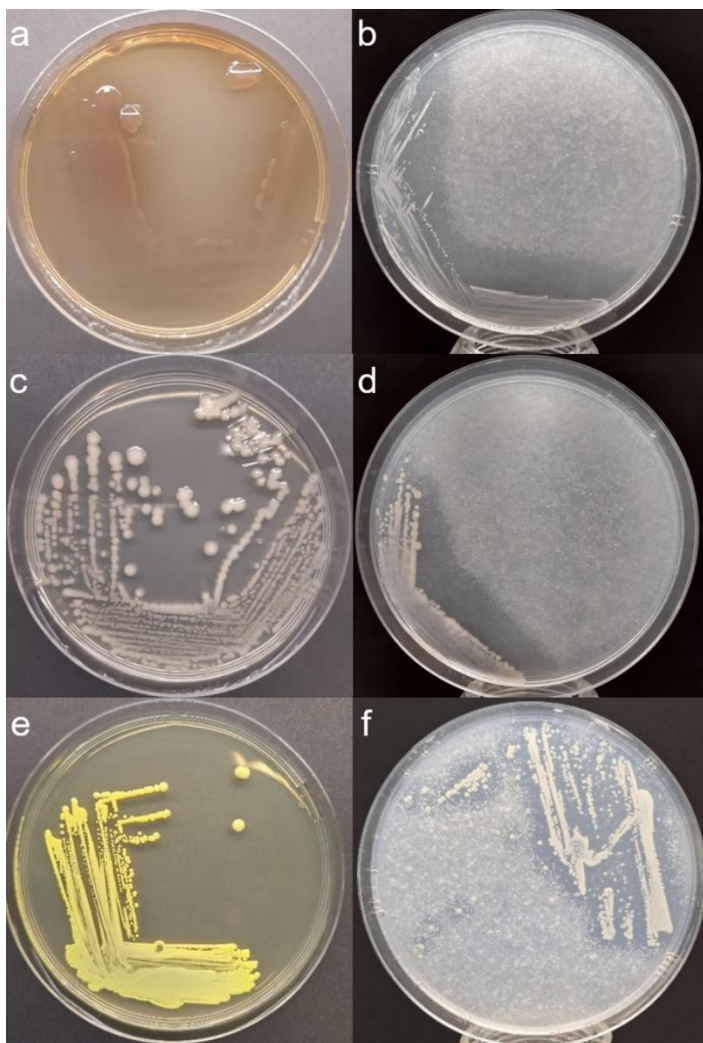
## 6. REZULTATAI

### 6.1. Mielių sienelės degraduojančių bakterijų izoliavimas

Fermentinė mielių sienelių (YCW) hidrolizė reikalinga šio darbo tikslui pasiekti – konvertuoti mielių sienelės į funkcinius bei bioaktyvius oligosaccharidus; taip pat suardyti gyvas mielių ląsteles. Šio tikslo įgyvendinimui reikalingi fermentai, galintys efektyviai hidrolizuoti mielių sienelių polisacharidus. Įvairūs dirvožemio ir vandens telkinių mėginiai buvo tiriami, siekiant juose aptikti mielių sienelės degraduojančias bakterijas, kurios mielių sienelės gebėtų naudoti kaip anglies šaltinį. Pirminių tyrimų metu buvo įvertintos dvi tipinės naujų fermentų paieškos strategijos: metagenominių bibliotekų konstravimas ir mielių sienelės degraduojančių bakterijų sekretuojamų baltymų frakcijų proteominė analizė. Pastaroji strategija parodė geresnius rezultatus ir buvo pritaikyta šiame darbe. Šios metodologijos taikymas leido identifikuoti *Paenibacillus* sp. GKG, *Rheinheimera* sp. GKH, ir *Cellulosimicrobium* sp. GKH bakterijas kaip perspektyviausius mielių sienelės skaidančius kandidatus.

Literatūros analizė parodė, kad *Cellulosimicrobium* genties bakterijos buvo tyrimo objektas daugelyje praeitų studijų, nukreiptų į mieles lizuojančius fermentų mišinius. Šių bakterijų sekretuojamų baltymų frakcija naudojama gaminant komercinį fermentinės mielių lizės preparatą *Zymolyase*. Atsižvelgus į ankstesnes studijas, buvo nuspręsta dėmesį sutelkti į gryną ir specifiskų GH aktyvumą. Ši kryptis priešinga ankstesnėse studijose taikytai kryptčiai, kur mielių lizei buvo taikomos įvairiais fermentiniais aktyvumais pasižyminčios sekretomų frakcijos. Tuo tarpu *Rheinheimera* ir *Paenibacillus* genčių bakterijos iki šiol nebuvo žinomos kaip mieles lizuojančių fermentų producentai. Taigi, konkretūs *Rheinheimera* sp. GKH ir *Paenibacillus* sp. GKG fermentai nebuvo pasirinkti iš anksto kaip prioritetiniai.

Trijų skirtingų genčių bakterijos, *Paenibacillus* sp. GKG, *Rheinheimera* sp. GKH ir *Cellulosimicrobium* sp. GKH, buvo izoliuotos iš vandens telkinio, dirvožemio ir mielių sienelių mėginių. Pirminė sienelės degraduojančių kolonijų atranka buvo vykdoma pagal skaidrių zonų atsiradimą aplink vieną ar keletą skirtingų kolonijų, augančių ant standžios mineralinės mitybinės terpės su mielių sienelėmis kaip anglies šaltiniu. Kamienai buvo išgryninti pakartotinai persėjant ir stebint savybę formuoti skaidrias hidrolizės zonas (2 pav.). Hidrolizės zonų atsiradimas rodo, kad vyko mielių sienelių hidrolizė – vandenyje netirpios mielių sienelės buvo konvertuojamos į vandenyje tirpius atskirus mažesnės molekulinės masės komponentus.



**2 paveikslas.** Izoliuotos *Rheinheimera* sp. GKU (a, b), *Paenibacillus* sp. GKG (c, d), *Cellulosimicrobium* sp. GKH (e, f) bakterijos. a, c, e – standi LB terpė, b, d, f – standi mineralinė terpė su mielių sienelėmis kaip anglies šaltiniu. Skaidrios hidrolizės zonos rodo YCW degraduojančių CAZymes fermentų aktyvumą.

Bakterijų klasifikacinė priklausomybė nustatyta remiantis 16S rRNR geno sekos analize. Izoliatas, išskirtas iš dirvožemio mėginio ir pavadintas *Rheinheimera* sp. GKU (2 pav. a, b), turėjo 98% 16S rRNR geno sekos sutaptį su *Rheinheimera pacifica* kamieno SJOD-R-25 16S rRNR geno seka (GenBank identifikacinis nr. MK955341). Izoliatas, išskirtas iš vandens telkinio mėginio ir pavadintas *Paenibacillus* sp. GKG (2 pav. c, d), turėjo 99% 16S rRNR geno sekos sutaptį su *Paenibacillus* sp. B01 16S rRNR geno seka (GenBank identifikacinis nr. CP045802). Izoliatas, išskirtas iš nesterilaus mielių sienelių mėginio ir pavadintas *Cellulosimicrobium* sp. GKH (2 pav. e,

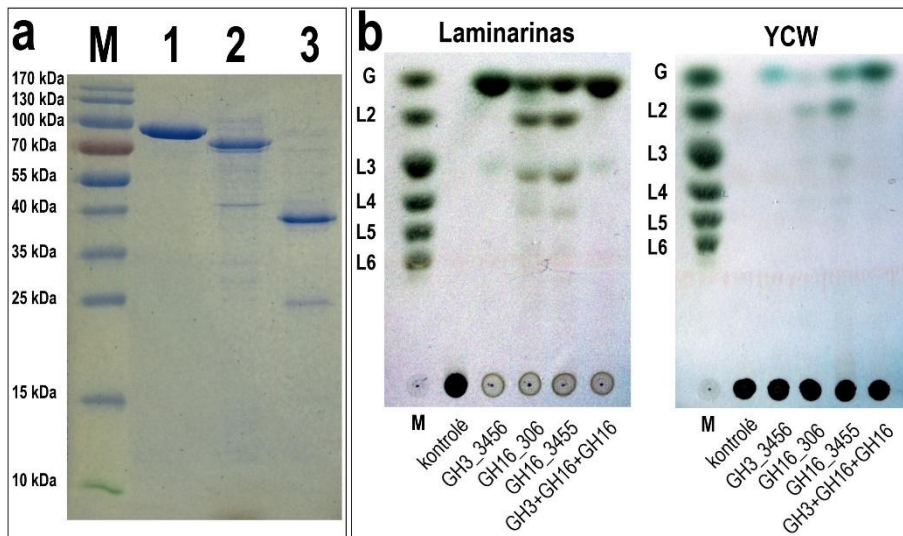
f), turėjo 100% 16S rRNR geno sekos sutaptį su *Cellulosimicrobium cellulans* kamieno MC29 (GenBank identifikacinis nr. ON629814.1) ir *Cellulosimicrobium funkei* kamieno NFZ10 (GenBank identifikacinis nr. MW391761.1) 16S rRNR genų sekomis.

## 6.2. Mielių sieneles degraduojančių GH charakterizavimas

Identifikavus mielių sieneles degraduojančias bakterijas buvo atlikta viduląstelių, periplazminių ir sekretuojamų baltymų frakcijų LC-MS/MS proteominė analizė. Iš gautų identifikuočių baltymų sąrašų buvo atrinktos glikozilhidrolazės (GH), kurių tikėtinas hidrolizuojamas substratas – įvairūs  $\beta$ -gliukanai. Fermentinis aktyvumas hidrolizuojant  $\beta$ -1,3/1,6-gliukanus buvo nustatytas septyniems fermentams: trims iš *Rheinheimera* sp. GKU, dviems iš *Paenibacillus* sp. GKG ir dviems iš *Cellulosimicrobium*. Toliau buvo susintetinti atrinktų GH rekombinantiniai variantai, gryninti metalų afininės chromatografijos ir jonų mainų chromatografijos metodais, o fermentinis aktyvumas nustatytas analizuojant polisacharidų hidrolizės produktus TLC, HPLC-RI metodais.

Trys *Rheinheimera* sp. GKU fermentai, GH3\_3456, GH16\_306 ir GH16\_3455, buvo atrinkti kaip atsakingi už YCW konversiją į panaudojamą anglies šaltinį gliukozę. Natrio dodecilsulfato poliakrilamidinio gelio elektroforezė (NDS-PAAG) (3 pav. a) parodė, kad rekombinantinių fermentų nustatytos molekulinės masės ( $M_w$ ) atitiko teorines: GH3\_3456  $M_w = 89$  kDa, GH16\_306  $M_w = 75$  kDa, GH16\_3455  $M_w = 36$  kDa. Pagal skirtingų  $\beta$ -gliukanų hidrolizės produktų profilius buvo nustatyta, kad GH3\_3456 fermentas yra  $\beta$ -gliukozydazė, kuri gali hidrolizuoti tokius disacharidus kaip laminaribiozė ir gentiobiozė. GH16\_306 ir GH16\_3455 fermentai veikė kaip endo-1,3- $\beta$ -gliukanazės, ir hidrolizavo tiek linijinius (kurdlaną, pachymaną, miežių  $\beta$ -gliukaną), tiek šakotus (laminariną, mielių  $\beta$ -gliukaną ir YCW) polisacharidus. Esminis skirtumas tarp GH16\_306 ir GH16\_3455 endo-1,3- $\beta$ -gliukanazių yra tas, kad GH16\_3455 gebėjo dalinai hidrolizuoti laminaribiozę ir hidrolizavo YCW efektyviau nei GH16\_306. Naudojant šių trijų *Rheinheimera* sp. GKU fermentų derinį buvo galima konvertuoti laminariną ir YCW iki panaudojamo anglies šaltinio – gliukozės (3 pav. b). Laminarino hidrolizės efektyvumas buvo didesnis nei YCW. Labiausiai tikėtina, dėl to, kad YCW  $\beta$ -gliukanas yra sunkiau prieinamas fermentams dėl egzistuojančių tarppolimerinių ryšių tarp  $\beta$ -gliukanų ir kitų YCW polimerų (manoproteinų, chitino). Be to,  $\beta$ -1,3/1,6-gliukano grynumas YCW substrate yra mažesnis ir pats substratas mažiau tirpus vandenyje nei laminarinas. Gauti rezultatai dėl šių trijų fermentų vaidmens konvertuojant YCW į anglies šaltinį – gliukozę

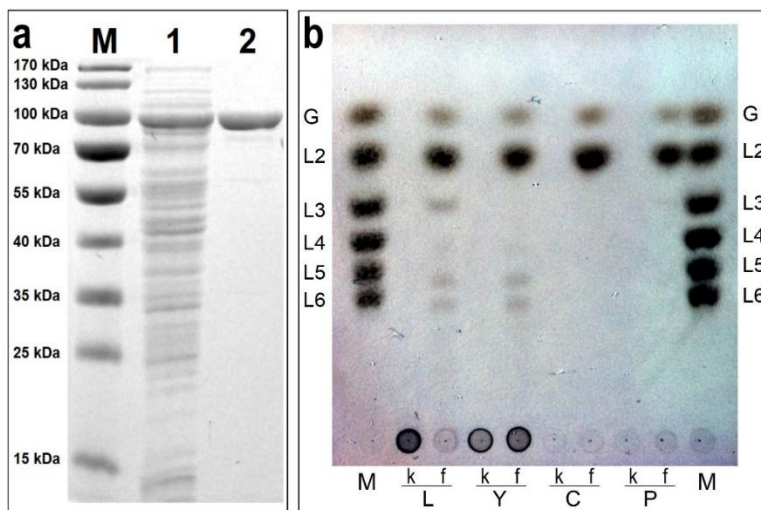
papildė proteomikos rezultatus. Proteomikos rezultatai rodė, kad GH3\_3456, GH16\_306 ir GH16\_3455 glikozidazės yra indukuojamos į mineralinę augimo terpę pridėjus YCW kaip anglies šaltinio.



**3 paveikslas.** *Rheinheimera* sp. GH rekombinantinių GH charakterizavimas. a – rekombinantinių GH molekulinės masės nustatymas NDS-PAAG metodu. M – molekulių masių standartas, 1 – GH3\_3456 fermentas, 2 – GH16\_306 fermentas, 3 – GH16\_3455 fermentas. b – laminarino ir YCW hidrolizės produktų profilis, nustatytas TLC metodu. M – standartų mišinys: G – gliukozė, L2 – laminaribiozė, L3 – laminaritrizozė, L4 – laminaritetrozė, L5 – laminaripentozė, L6 – laminariheksozė, kontrolė – substratai be fermentų poveikio, GH3\_3456, GH16\_306, GH16\_3455 – poveikis atskirais fermentais. GH3+GH16+GH16 – poveikis GH3\_3456, GH16\_306 ir GH16\_3455 fermentų deriniui.

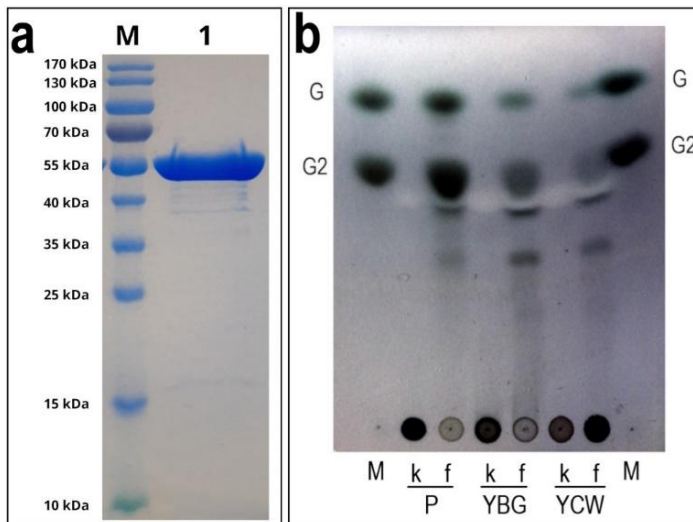
Du *Paenibacillus* sp. GKG fermentai, PsLam81A ir PsGly30A, buvo charakterizuoti kaip mielių  $\beta$ -gliukaną ir YCW į disacharidus konvertuojančios GH. NDS-PAAG analizė (4 pav. a) parodė, kad PsLam81A rekombinantinio fermento nustatyta molekulinė masė atitiko teorinę  $M_w = 95$  kDa. Pagal skirtingų  $\beta$ -gliukanų hidrolizės produktų profilius buvo nustatyta, kad PsLam81A fermentas yra endo-1,3- $\beta$ -gliukanazė. PsLam81A fermentas hidrolizavo laminariną, mielių  $\beta$ -gliukaną, kurdlaną ir pachymaną (4 pav. b). Visi keturi polisacharidai buvo hidrolizuojami iki laminaribiozės kaip pagrindinio hidrolizės produkto ir gliukozės kaip antro pagal gausumą hidrolizės produkto. Priklausomai nuo substrato struktūros susidarė įvairių šalutinių reakcijos produktų. Šalutinių produktų nesusidarė hidrolizuojant linijinius  $\beta$ -1,3-gliukanus, kurdlaną ir pachymaną. Hidrolizuojant  $\beta$ -1,3/1,6-gliukanus, laminariną ir mielių  $\beta$ -gliukaną, susidarė nedideli kiekiai junginių, pagal mobilumą, panašių į laminariheksozę. Kadangi šie produktai susidarė

tik hidrolizuojant  $\beta$ -1,3/1,6-gliukanus, tikėtina, kad tai mišrius  $\beta$ -1,3/1,6-ryšius turintys oligosacharidai.



**4 paveikslas.** *Paenibacillus* sp. GKG rekombinantinio fermento PsLam81A charakterizavimas. a – rekombinantinio fermento molekulinės masės nustatymas NDS-PAAG metodu. M – molekulinių masių standartas, 1 – PsLam81A fermentas beląsteliniam *E.coli* ekstrakte, 2 – PsLam81A glikozidazė po gryninimo. b – laminarino (L), mielių  $\beta$ -gliukano (Y), kurdano (C), ir pachymano (P) hidrolizės produktų profilis, nustatytas TLC metodu. M – standartų mišinys: G – gliukozė, L2 – laminaribiozė, L3 – laminaritriozė, L4 – laminaritetrozė, L5 – laminaripentozė, L6 – laminariheksozė, k – substratai be fermento poveikio, f – substratai po fermento poveikio.

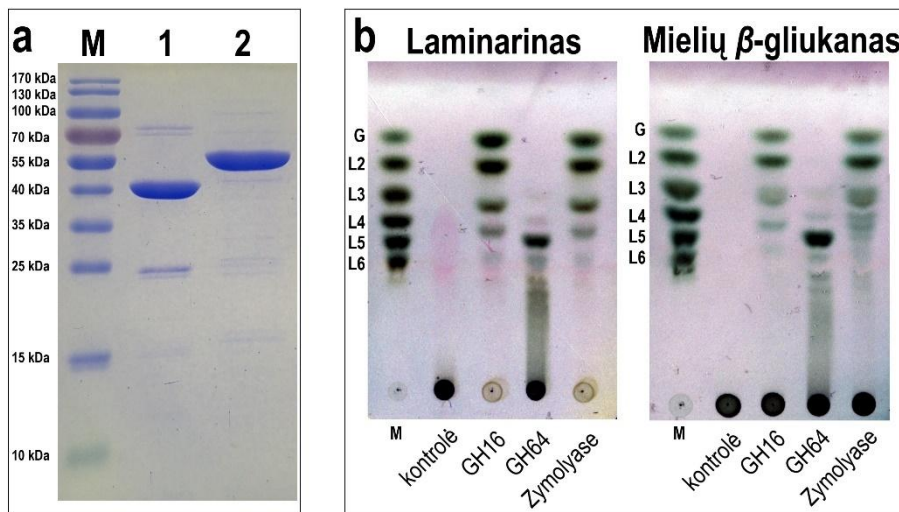
NDS-PAAG analizė (5 pav. a) parodė, kad PsGly30A rekombinantinio fermento nustatyta molekulinė masė atitiko teorinę  $M_w = 56$  kDa. Pagal skirtingų  $\beta$ -gliukanų hidrolizės produktų profilius buvo nustatyta, kad PsGly30A fermentas yra endo-1,6- $\beta$ -gliukanazė. Šis fermentas hidrolizavo pustulaną, mielių  $\beta$ -gliukaną ir YCW iki gentiobiozės kaip pagrindinio hidrolizės produkto ir gliukozės kaip antro pagal gausumą produkto (5 pav. b). PsGly30A efektyviausiai hidrolizavo pustulaną, kaip ir dauguma kitų ankstesnėse studijose charakterizuotų GH30 šeimos fermentų. Išskirtinė PsGly30A fermento savybė – didžiausias iki šiol pademonstruotas GH30\_3 šeimos fermento specifinis aktyvumas ( $1262 \pm 82$  U/mg) hidrolizuojant pustulaną. Technologiniu požiūriu mielių  $\beta$ -gliukanas ir YCW yra lengviau prieinami substratai nei pustulanas. PsGly30A sugebėjo atpalaiduoti gentiobiozė naudodant visus šiuos tris substratus (5 pav. b). Visų trijų substratų atveju hidrolizės produktų profiliai buvo panašūs. Svarbu paminėti, kad lyginant su pustulanu, gentiobiozės koncentracija mielių  $\beta$ -gliukano ir YCW hidrolizatuose yra mažesnė, tačiau  $\beta$ -gliukano ir YCW substratai yra lengviau prieinami pramoninėje skalėje.



**5 paveikslas.** *Paenibacillus* sp. GKG rekombinantinio fermento PsGly30A charakterizavimas. a – rekombinantinio fermento molekulinės masės nustatymas NDS–PAAG metodu. M – molekulių masių standartas, 1 – PsGly30A fermentas po gryninimo. b – pustulano (P), mielių  $\beta$ -gliukano (YBG) ir mielių sienelių (YCW) hidrolizės produktų profilis, nustatytas TLC metodu. M – standartų mišinys: G – gliukozė, G2 – gentiobiozė, k – substratai be fermento poveikio, f – substratai po fermento poveikio.

Atlikus sekretuojamų baltymų proteominę analizę du *Cellulosimicrobium* sp. GKH fermentai, GH16\_CELCE ir GH64\_CELCE buvo identifikuoti kaip potencialios mielių sienelių  $\beta$ -gliukaną hidrolizuojančios endo-1,3- $\beta$ -gliukanazės. Atlikus literatūros analizę buvo identifikuoti du jau charakterizuoti GH16\_LLG109 ir GH64\_21606 fermentai, kurie su identifikuotais *Cellulosimicrobium* sp. GKH fermentais GH16\_CELCE ir GH64\_CELCE turėjo 97% ir 100% sekų sutaptį, atitinkamai. Kadangi ankstesnių studijų metu apie GH16\_LLG109 ir GH64\_21606 fermentus jau buvo surinkta duomenų, tolesniam pritaikymui mielių lizei taip pat buvo pasirinkti ankstesnėse studijose charakterizuoti fermentai. Buvo susintetinti rekombinantiniai GH16\_LLG109 ir GH64\_21606 fermentai, kurie toliau tekste įvardijami tiesiog GH16 ir GH64. NDS-PAAG analizė (6 pav. a) parodė, kad rekombinantinių fermentų nustatytos molekulinės masės atitiko teorines: GH16  $M_w = 42$  kDa, GH64  $M_w = 56$  kDa. Prieš naudojant GH16 ir GH64 fermentus mielių lizavimui buvo nustatytas šių fermentų substratinis specifiškumas. Hidrolizės produktų profiliai, gauti naudojant GH16, GH64, komercinį mieles lizuojančių fermentų mišinį *Zymolyase* ir laminariną bei mielių  $\beta$ -gliukaną kaip substratus buvo palyginti (6 pav. b). GH16 fermentas atpalaidavo laminaribiozė ir gliukozę kaip pagrindinius laminarino ir mielių  $\beta$ -gliukano hidrolizės produktus. Tuo tarpu, GH64 fermentas atpalaidavo laminaripentozę kaip pagrindinį hidrolizės produktą naudojant laminariną ir

mielių  $\beta$ -gliukaną kaip substratus. Laminarino ir mielių  $\beta$ -gliukano hidrolizės produktų profilis naudojant *Zymolyase* komercinį fermentų mišinį buvo panašus į gautą naudojant GH16 fermentą. Gauti rezultatai atitiko komercinio fermentų mišinio specifikacijas, nurodančias, kad mišinyje yra laminaribiozės ir laminaripenozės generuojantys endo-1,3- $\beta$ -gliukanazės aktyvumai.



**6 paveikslas.** *Cellulosimicrobium* rekombinantinių fermentų charakterizavimas. a – rekombinantinių fermentų molekulinės masės nustatymas NDS–PAAG metodu. M – molekulinės masės standartas, 1 – GH16 fermentas, 2 – GH64 fermentas. b – laminarino ir mielių  $\beta$ -gliukano hidrolizės produktų profilis, nustatytas TLC metodu. M – standartų mišinys: G – gliukozė, L2 – laminaribiozė, L3 – laminaritriozė, L4 – laminaritetrozė, L5 – laminaripentozė, L6 – laminariheksozė, kontrolė – substratai be fermentų poveikio, GH16 – poveikis GH16 fermentu, GH64 – poveikis GH64 fermentu, *Zymolyase* – poveikis komerciniu mieles lizuojančiu fermentų mišiniu.

Apibendrinant, *Rheinheimera* sp. GKU fermentų charakterizavimas patvirtino šių fermentų vaidmenį konvertuojant mielių sieneles į lengvai panaudojamą anglies šaltinį – gliukozę. Deja, mielių sienelių konversija į laminaribiozės buvo neefektyvi ir šie fermentai oligosacharidų gavimui pasirodė neperspektyvūs. Geresnį efektyvumą sintetinant laminaribiozės pademonstravo *Paenibacillus* sp. GKG fermentas PsLam81A. *Paenibacillus* sp. GKG fermentas PsGly30A buvo vienintelis iš charakterizuotų fermentų, kuris galėjo konvertuoti mielių  $\beta$ -gliukaną į gentiobiozės. *Cellulosimicrobium* GH16 ir GH64 fermentų substratinis specifiskumas atitiko teorinį, todėl šie fermentai buvo atrinkti įvertinimui ardant gyvas mielių ląsteles. GH64 fermentas taip pat pasirinktas norint įvertinti tinkamumą mielių sienelių konversijai į laminaripentozės.

### 6.3. GH pritaikymas oligosacharidų gavimui

*Rheinheimera* sp. GKU, *Paenibacillus* sp. GKG ir *Cellulosimicrobium* sp. GKH sekretuojamų ir mielių sienelių indukuojamų baltymų frakcijų proteominė analizė leido identifikuoti septynis fermentus, kurie konvertavo mielių sieneles ir mielių  $\beta$ -gliukanus į keturis skirtingus oligosacharidus ir cukrus. Iš septynių GH trys buvo atrinktos pritaikymui konvertuoti YCW į tris funkcionalius ir bioaktyvius oligosacharidus.

Endo-1,3- $\beta$ -gliukanazė GH64 buvo vienintelis fermentas hidrolizuojantis mielių sieneles iki bioaktyvaus oligosacharido – laminaripentozės. Kitas fermentas, vienintelis pasižymėjęs endo-1,6- $\beta$ -gliukanaziniu aktyvumu buvo PsGly30A fermentas. Šis fermentas YCW hidrolizavo iki skonį gerinančio disacharido – gentiobiozės. Keturi skirtingi fermentai, PsLam81A, GH16, GH16\_306 ir GH16\_3455, YCW hidrolizavo iki prebiotinėmis savybėmis pasižyminčio disacharido – laminaribiozės. PsLam81A fermentas buvo pasirinktas kaip perspektyviausias kandidatas, kadangi sugebėjo ištirpinti didžiausią mielių  $\beta$ -gliukano ir YCW masės dalį. Be to, šio fermento hidrolizės produktų profilis pasižymėjo didžiausiu laminaribiozės:gliukozės santykiu. Kiti trys fermentai, GH16, GH16\_306, and GH16\_3455, pasirodė blogiau pagal abu paminėtus kriterijus ir taikomiesiems tyrimams panaudoti nebuvo.  $\beta$ -Gliukozidazė GH3\_3456 taikomiesiems tyrimams pasirinkta nebuvo, kadangi jos pagrindinis mielių sienelių hidrolizės produktas buvo cukrus su mažesniu funkcionalumo ir bioaktyvumo potencialu – gliukozė.

Tolesniame žingsnyje YCW fermentiniuose hidrolizatuose kiekybiškai įvertintos kiekvieno iš atrinktų oligosacharidų koncentracijos. Pagal gautus rezultatus apskaičiuotos YCW esančių  $\beta$ -gliukanų konversijos išeigos į kiekvieną iš atrinktų oligosacharidų.

Laminaripentozės, laminaribiozės ir gentiobiozės koncentracijos mielių sienelių hidrolizatuose buvo nustatytos HPLC-RI metodu. Laminaribiozė gauta po poveikio GH64 fermentu, laminaribiozė po poveikio PsLam81A fermentu, o gentiobiozė po poveikio PsGly30A fermentu. Skaičiavimai atlikti remiantis nustatytomis oligosacharidų koncentracijomis, literatūroje nurodytomis  $\beta$ -1,3-gliukano (50–55%) ir  $\beta$ -1,6-gliukano (5–10%) proporcijomis mielių sienelėje. Taip pat eksperimentiškai nustatytais bendru  $\beta$ -gliukanų kiekiu ( $35.7 \pm 0.3\%$  w/w) ir sausų medžiagų kiekiu ( $91.2 \pm 0.1\%$ ,  $n=2$ ) mielių sienelių mėginyje. Apskaičiuotos konversijos išeigos pateiktos 1 lentelėje. Pagrindinis veiksnys, ribojantis konversijos išeigą į linijinius oligosacharidus yra tarp mielių sienelių polimerų esantys ryšiai ir  $\beta$ -1,3/1,6-gliukanų šakotumas.

**1 lentelė.**  $\beta$ -gliukanų, esančių mielių sienelėje fermentinės konversijos į oligosacharidus išeiga. Rezultatai pateikti kaip (vidurkis $\pm$ standartinis nuokrypis, n=2). Eksperimentinius duomenis surinko Jurgita Kailiuvienė.

| Oligosacharidas | Konversijos išeiga, % |
|-----------------|-----------------------|
| Laminaribiozė   | 78.1 $\pm$ 1.7        |
| Gentiobiozė     | 72.5 $\pm$ 1.1        |
| Laminaripentozė | 51.4 $\pm$ 0.4        |

Apibendrinant, konversijos išeigos atrodo perspektyviai. Nuo 51 iki 78%  $\beta$ -1,3/1,6-gliukanų esančių žemos vertės pramoninės skalės šalutiniame produkte YCW gali būti konvertuoti į bioaktyvius ir funkcionalius oligosacharidus su polimerizacijos laipsniu (DP) nuo 2 iki 5 naudojant aplinkai draugišką fermentinės hidrolizės metodą.

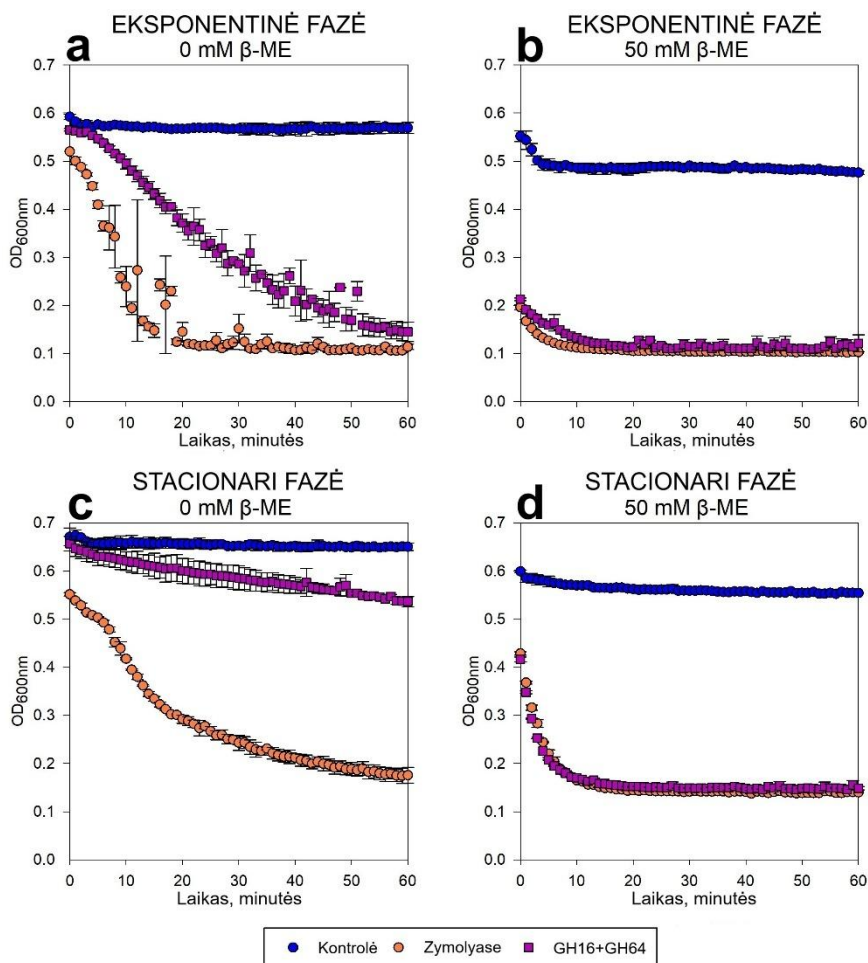
#### 6.4. GH pritaikymas mielių ląstelių lizei

Šiame darbe siekiama nustatyti sąlygas, kurios leistų mielių ląstelių lizę pasiekti naudojant tik endo-1,3- $\beta$ -gliukanazes be proteazių ar redukuojančio agento  $\beta$ -ME priedo. Pagrindinis grynų endo-1,3- $\beta$ -gliukanazių naudojimo privalumas tas, kad jos suardo YCW hidrolizuodamos  $\beta$ -1,3-gliukanus ir palikdamos kitus YCW bei viduląstelinius komponentus nepaveiktus. Viduląsteliniai baltymai ypač svarbūs, kadangi jie gali būti paveikti proteolizės, jei lizuojantys fermentų mišiniai, pvz. *Zymolyase*, sudėtyje turi proteazių. Jei lizuojančių fermentų mišiniai neturi proteazių, redukuojantys agentai paprastai naudojami vietoj jų. Proteazių ir redukuojančių agentų paskirtis – išardyti sienelių manoproteinų tinklą. Deja, nespecifinis proteazių ir redukuojančių agentų poveikis gali neigiamai paveikti ir viduląstelių baltymų savybes.

Įdomu, kad ankstesnės studijos tyrė grynų GH16 ir GH64 endo-1,3- $\beta$ -gliukanazių poveikį gyvoms mielių ląstelėms tik atskirai ir nenaudojo šių fermentų derinio. Dalyje ankstesnių studijų gebėjimas lizuoti gyvas mielių ląsteles netirtas visiškai, apsiribojant fermentų gebėjimu hidrolizuoti mielių sienelės. Atsižvelgus į šią nišą buvo nuspręsta susintetinti rekombinantinius GH16 ir GH64 fermentų variantus bei palyginti jų lizuojantį aktyvumą naudojant šiuos fermentus atskirai ir derinyje.

Mielių *Saccharomyces cerevisiae* kamienas BY4741 buvo naudojamas lizavimo efektyvumo įvertinimui. Pirmiausia buvo patikrinta, ar grynų lizuojančių endo-1,3- $\beta$ -gliukanazių derinys gali lizuoti mielių ląsteles, surinktas eksponentinėje ir stacionarioje augimo stadijose. Rezultatai parodė, kad naudojant 50 mM  $\beta$ -ME priedą (7 pav. b, d) lizavimo efektyvumo

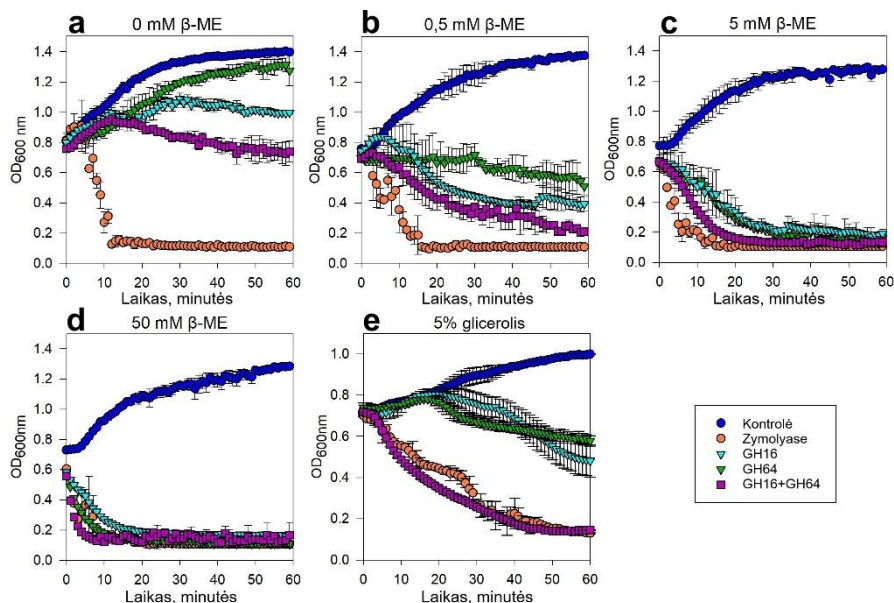
skirtumas tarp *Zymolyase* bei GH16 ir GH64 derinio buvo mažesnis, nei tuo atveju kai 50 mM  $\beta$ -ME priedas nebuvo naudojamas (7 pav. a, c). Kadangi grynintų GH16 ir GH64 fermentų derinys nesant  $\beta$ -ME priedo sulizavo didžiąją dalį ląstelių tik joms esant surinktomis eksponentinėje augimo fazėje (7 pav. a), tolesni eksperimentai buvo atliekami su eksponentinėje fazėje surinktomis ląstelėmis. Beje, svarbu paminėti, kad tais atvejais kai  $\beta$ -ME priedo naudojimas yra priimtinas, GH16 ir GH64 fermentų derinys efektyvumu nenusileidžia *Zymolyase* mišiniui ir gali jį pakeisti net stacionarioje fazėje surinktų ląstelių lizės atveju.



**7 paveikslas.** OD<sub>600nm</sub> pokytis eksponentinės ir stacionarios fazės *S. cerevisiae* BY4741 ląstelių lizės metu. a – ląstelės surinktos eksponentinėje augimo fazėje ir lizuotos be  $\beta$ -ME priedo, b – ląstelės surinktos eksponentinėje augimo fazėje ir lizuotos su  $\beta$ -ME priedu, c – ląstelės surinktos stacionarioje augimo fazėje ir lizuotos be  $\beta$ -ME priedo, d – ląstelės surinktos stacionarioje augimo fazėje ir lizuotos su  $\beta$ -ME. Įvertinti du fermentų preparatai: *Zymolyase* bei GH16 ir GH64 derinys. Rezultatai pateikti kaip dviejų matavimų vidurkis su standartinio nuokrypio vertėmis paklaidose. Eksperimentinius duomenis surinko dr. Ramunė Stanevičienė.

Kadangi buvo pastebėtas reikšmingas GH16 ir GH64 fermentų derinio lizavimo efektyvumo skirtumas nenaudojant ir naudojant  $\beta$ -ME priedą, osmosinio šoko poveikis naudojant glicerolį buvo išbandytas kaip alternatyvus lizės efektyvumą skatinantis priedas. 5% koncentracijos glicerolio tirpalas buvo nustatytas kaip efektyviausias.

Planuojant tolesnius eksperimentus buvo atsižvelgta į tai, kad *Cellulosimicrobium* sp. GKH sekretuojamų baltymų frakcijoje proteominės analizės metodu buvo aptikti ir GH16, ir GH64 fermentai. Lizavimo efektyvumas buvo nustatytas ir jau tirtam abiejų fermentų deriniui, ir pavieniams GH16 ir GH64 fermentams. Norint praplėsti tyrimo apimtį buvo įvertinta skirtingų  $\beta$ -ME koncentracijų (0,5–50 mM) įtaka lizavimo efektyvumui. Skirtingos  $\beta$ -ME ir 5% glicerolio priedų koncentracijos buvo įvertintos su visais tirtais fermentais: GH16, GH64, GH16 bei GH64 derinys ir *Zymolyase* (8 pav.).



**8 paveikslas.** OD<sub>600nm</sub> pokytis *Saccharomyces cerevisiae* ląstelių lizės metu. Skirtingi priedai naudoti lizavimo efektyvumui pagerinti. a – nenaudotas nei  $\beta$ -ME, nei osmosinis šokas 5% gliceroliu, b–d – buvo naudotos 0,5–50 mM  $\beta$ -ME koncentracijos, e – buvo naudotas osmosinis šokas 5% gliceroliu. Rezultatai pateikti kaip dviejų (a–d) arba trijų (e) matavimų vidurkis su standartinio nuokrypio vertėmis paklaidose. Eksperimentinius duomenis surinko dr. Ramunė Stanevičienė.

GH16 ir GH64 fermentų derinys pasižymėjo didžiausiu lizavimo efektyvumu tarp rekombinantinių GH variantų. Ši kombinacija pilnai sulizavo ląsteles su visomis naudotomis priedų koncentracijomis ir efektyvumu po 60 min. lizės prilygo *Zymolyase* komerciniam mišiniui. Svarbiausia, kad 5%

glicerolio priedas užtikrino pilną lizę (8 pav. e) ir sugebėjo pakeisti  $\beta$ -ME ir *Zymolyase* preparate esančias proteazes. Be to, pilna mielių lizė buvo pasiekta ir naudojant 0,5 mM  $\beta$ -ME koncentraciją su GH16 ir GH64 deriniu. Šis rezultatas parodo, kad ne visuomet ankstesniuose tyrimuose nurodomos iki 100 mM  $\beta$ -ME koncentracijos yra reikalingos, kai lizuojamos eksponentinės fazės ląstelės su dviejų lizuojančių fermentų, GH16 ir GH64, deriniu.

Pilnos lizės kriterijus buvo atrinktas remiantis ankstesnės studijos rezultatais – OD<sub>600nm</sub> sumažėjimas 70% procentų buvo prilygintas pilnai mielių lizei, remiantis mikroskopijos vaizdų analize. Mikroskopijos vaizduose nebuvo stebimos nepažeistos mielių ląstelės. Šiame darbe mikroskopijos vaizdų analizė taip pat buvo atlikta ir rezultatai gauti tiek su 50 mM  $\beta$ -ME, tiek su 5% gliceroliu parodė panašų efektyvumą ir praktiškai nebematomą nepažeistų mielių ląstelių kiekį.

## 7. IŠVADOS

1. *Rheinheimera* sp. GKU, *Paenibacillus* sp. GKG ir *Cellulosmicrobium* sp. GKH bakterijos degraduoja mielių sienelės į vandenyje tirpius junginius, kurie gali būti panaudojami kaip anglies šaltinis.
2. *Rheinheimera* sp. GKU, *Paenibacillus* sp. GKG ir *Cellulosmicrobium* sp. GKH sekretuoja angliavandenius veikiančius fermentus, daugiausia endo-1,3- $\beta$ -gliukanazes, kurių pakanka mielių sienelių konversijai į DP 2–5 oligosacharidus ir gliukozę.
3. *Rheinheimera* sp. GKU sekretuoja GH fermentus, kurie mielių sienelių degradacijoje atlieka skirtingus vaidmenis – GH16\_3455 ir GH16\_306 generuoja oligosacharidus, tuo tarpu GH3\_3456 hidrolizuoja oligosacharidus iki gliukozės.
4. PsLam81A, PsGly30A and GH64 gali konvertuoti nuo 51% iki 78% mielių sienelės  $\beta$ -gliukanų į funkcionalius ir bioaktyvius produktus – laminaribiozę (78%), gentiobiozę (72%) ir laminaripentozę (51%), atitinkamai.
5. Kombinuotas GH16 ir GH64 endo-1,3- $\beta$ -gliukanazių veikimas bei poveikis gliceroliu leidžia gyvas *Saccharomyces cerevisiae* BY4741 mielių ląsteles lizuoti be proteazių ir redukuojančių agentų priedo.

## APPENDICES

The appendices 1–17 are presented on the following pages.

**Appendix 1.** Oligonucleotide primers used in this study.

| <b>Primer name</b>        | <b>Primer Sequence 5'→3'</b>                |
|---------------------------|---------------------------------------------|
| Fw W <sub>oo1</sub>       | AGAGTTTGATCMTGGCTC                          |
| Rv W <sub>oo2</sub>       | GNTACCTTGTACGACTT                           |
| Fw pJET1.2                | CGACTCACTATAGGAGAGCGGC                      |
| Rv pJET1.2                | AAGAACATCGATTTCATGGCAG                      |
| Fw pLATE31_PsLam81A       | AGAAGGAGATATAACTATGAACAACGAATCGCCTG         |
| Fw pLATE31_PsLam81AΔCBM56 | AGAAGGAGATATAACTATGAACAACGAATCGCCTG         |
| Fw PsLam81A-mid           | GTACGCCTACTCGCATGTGACG                      |
| Rv PsLam81A-mid           | GTACTGCGTCAGGTAGAGC                         |
| Rv pLATE31_PsLam81A       | GTGGTGGTGATGGTATGGCCATGCGTATACGTATAATGC     |
| Rv pLATE31_PsLam81AΔCBM56 | GTGGTGGTGATGGTATGGCCATTGCTGCCGCCCGC         |
| Fw pLATE31_PsGly30A       | AGAAGGAGATATAACTATGTCCGACTGGAAGAAAAAAC      |
| Rv pLATE31_PsGly30A       | GTGGTGGTGATGGTGATGGCCCGAGCGGTTGCCGTAC       |
| Fw pLATE31_GH3_3456       | AGAAGGAGATATAACTATGTGCGCCAAAGCCCCAAC        |
| Rv pLATE31_GH3_3456       | GTGGTGGTGATGGTGATGGCCGTCTCCGCTGCAGGAAAGAG   |
| Fw pLATE31_GH16_306       | AGAAGGAGATATAACTATGTGTGGCGGAGGTGCC          |
| Rv pLATE31_GH16_306       | GTGGTGGTGATGGTGATGGCCGGGTGCTTAAAGGTAACGTTG  |
| GH16_306 del2             | CATCACCATCACCCACGGCTAATG                    |
| GH16_306 del3             | GCATCTTCAGGGATTGTGC                         |
| GH16_306 del4             | CATAGTTATATCTCCTCTG                         |
| GH16_306 del5N            | GGGTAATCCAGCGTAATTTCACG                     |
| GH16_306 del6N            | GAGCTGTGTTATTGTCTG                          |
| Fw pLATE31_GH16_3455      | AGAAGGAGATATAACTATGTGTAGCACCACAGTGCCAC      |
| Rv pLATE31_GH16_3455      | GTGGTGGTGATGGTGATGGCCAGGTTGCTGGCGCTGATATACC |

**Appendix 2.** Substrates used in this study.

| <b>Substrate</b>           | <b>Glycosidic Bond</b>                                                                | <b>Reference</b> | <b>Source</b>         |
|----------------------------|---------------------------------------------------------------------------------------|------------------|-----------------------|
| Laminaribiose              | $\beta$ -1,3                                                                          | O-LAM2           | Megazyme              |
| Laminaritriose             | $\beta$ -1,3                                                                          | O-LAM3           | Megazyme              |
| Laminaritetraose           | $\beta$ -1,3                                                                          | O-LAM4           | Megazyme              |
| Laminaripentaose           | $\beta$ -1,3                                                                          | O-LAM5           | Megazyme              |
| Laminarihexaose            | $\beta$ -1,3                                                                          | O-LAM6           | Megazyme              |
| Gentiobiose                | $\beta$ -1,6                                                                          | G-2450           | BioSynth              |
| Laminarin                  | $\beta$ -1,3; $\beta$ -1,6                                                            | L9634            | Sigma-Aldrich         |
| Carboxymethyl cellulose    | $\beta$ -1,4                                                                          | 419281           | Sigma-Aldrich         |
| Microcrystalline cellulose | $\beta$ -1,4                                                                          | 435236           | Sigma-Aldrich         |
| Curdlan                    | $\beta$ -1,3                                                                          | P-CURDL          | Megazyme              |
| Pachyman                   | $\beta$ -1,3                                                                          | P-PACHY          | Megazyme              |
| Pustulan                   | $\beta$ -1,6                                                                          | YPI5423          | Carbosynth            |
| Barley $\beta$ -glucan     | $\beta$ -1,3; $\beta$ -1,4                                                            | P-BGBL           | Megazyme              |
| Yeast $\beta$ -glucan      | $\beta$ -1,3; $\beta$ -1,6                                                            | P-BGYST          | Megazyme              |
| YCW                        | $\alpha$ -1,2; $\alpha$ -1,3; $\alpha$ -1,6; $\beta$ -1,3; $\beta$ -1,4; $\beta$ -1,6 | –                | (Plakys et al., 2022) |
| $\beta$ -1,2-glucan        | $\beta$ -1,2                                                                          | P-BGLUI2         | Megazyme              |
| pNP- $\alpha$ -glucoside   | –                                                                                     | 30716            | Serva                 |
| pNP- $\beta$ -glucoside    | –                                                                                     | 30720            | Serva                 |
| pNPG2                      | $\beta$ -1,6                                                                          | –                | (Plakys et al., 2024) |

**Appendix 3.** List of *Paenibacillus* sp. GKG CAZymes, manually filtered from proteomics data.

| <b>Accession</b> | <b>Description</b>                                                 | <b>Score</b> | <b>Sequence coverage (%)</b> |
|------------------|--------------------------------------------------------------------|--------------|------------------------------|
| A0A5Q2NY17       | Family 16 glycosylhydrolase OS= <i>Paenibacillus</i> sp. B01       | 777          | 18                           |
| A0A2N5N8G8       | Endo-1 4-beta-xylanase A OS= <i>Paenibacillus pasadenensis</i>     | 777          | 17                           |
| A0A218NL06       | Chitin-binding protein OS= <i>Paenibacillus pasadenensis</i>       | 536          | 28                           |
| A0A5Q2NWL3       | Chitin-binding protein OS= <i>Paenibacillus</i> sp. B01            | 536          | 28                           |
| A0A2N5ND26       | Chitin binding protein OS= <i>Paenibacillus pasadenensis</i>       | 536          | 28                           |
| A0A343F0L1       | Chitinase OS= <i>Paenibacillus pasadenensis</i>                    | 451          | 19                           |
| A0A5Q2NX09       | Chitinase OS= <i>Paenibacillus</i> sp. B01                         | 451          | 20                           |
| A0A2N5N0S9       | Chitinase OS= <i>Paenibacillus pasadenensis</i>                    | 451          | 19                           |
| A0A6H2GTK2       | Chitinase OS= <i>Paenibacillus</i> sp. UniB2                       | 421          | 17                           |
| A0A6H2H2T3       | Chitin-binding protein OS= <i>Paenibacillus</i> sp. UniB2          | 343          | 9                            |
| A0A5Q2NJK9       | Glycoside hydrolase family 81 OS= <i>Paenibacillus</i> sp. B01     | 255          | 29                           |
| A0A2N5N1W0       | Chitin binding protein OS= <i>Paenibacillus pasadenensis</i>       | 210          | 21                           |
| A0A5Q2NS91       | Glucan endo-1 6-beta-glucosidase OS= <i>Paenibacillus</i> sp. B01  | 182          | 23                           |
| A0A2N5N256       | Glycosyl hydrolase family 30 OS= <i>Paenibacillus pasadenensis</i> | 182          | 23                           |
| A0A6H2H3I3       | Glycoside hydrolase family 81 OS= <i>Paenibacillus</i> sp. UniB2   | 142          | 9                            |
| A0A2N5N8U3       | Endo-1 4-beta-xylanase A OS= <i>Paenibacillus pasadenensis</i>     | 162          | 25                           |
| A0A5Q2NJJ9       | Family 16 glycosylhydrolase OS= <i>Paenibacillus</i> sp. B01       | 132          | 16                           |
| A0A2N5N0U5       | Beta-glucanase OS= <i>Paenibacillus pasadenensis</i>               | 132          | 16                           |
| H6CEV7           | Beta-1 6-glucanase OS= <i>Paenibacillus</i> sp. Aloe-11            | 128          | 16                           |
| A0A2N9YZ34       | Endoglucanase OS= <i>Paenibacillus polymyxa</i>                    | 122          | 14                           |
| A0A222WSD2       | Beta-1 6-glucanase OS= <i>Paenibacillus kribbensis</i>             | 120          | 14                           |
| A0A5Q2NPP4       | Chitinase OS= <i>Paenibacillus</i> sp. B01                         | 95           | 13                           |
| A0A343F0L2       | Chitinase OS= <i>Paenibacillus pasadenensis</i>                    | 95           | 13                           |
| A0A2N5NAZ7       | Chitinase OS= <i>Paenibacillus pasadenensis</i>                    | 95           | 13                           |

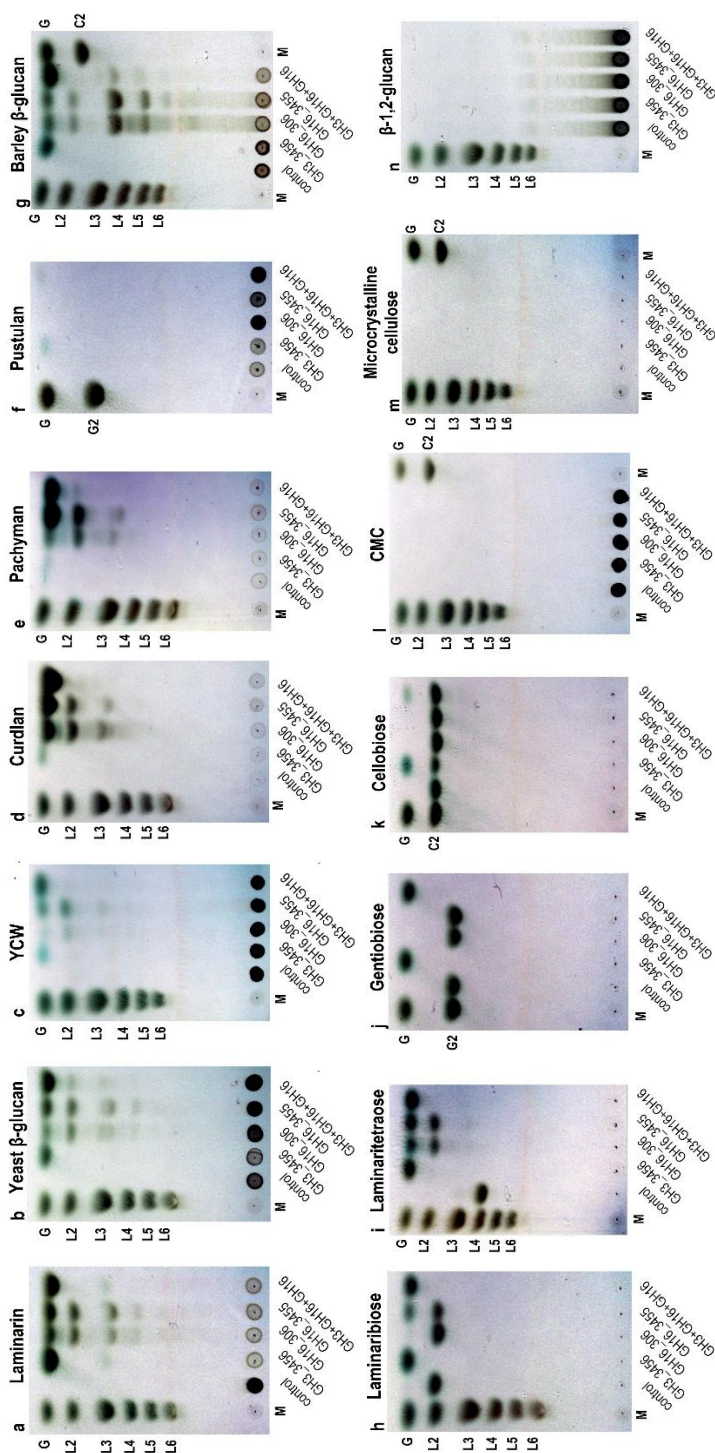


**Appendix 5.** List of *Rheinheimera* sp. GKU CAZymes, extracted from proteomics data using the dbCAN3 tool.

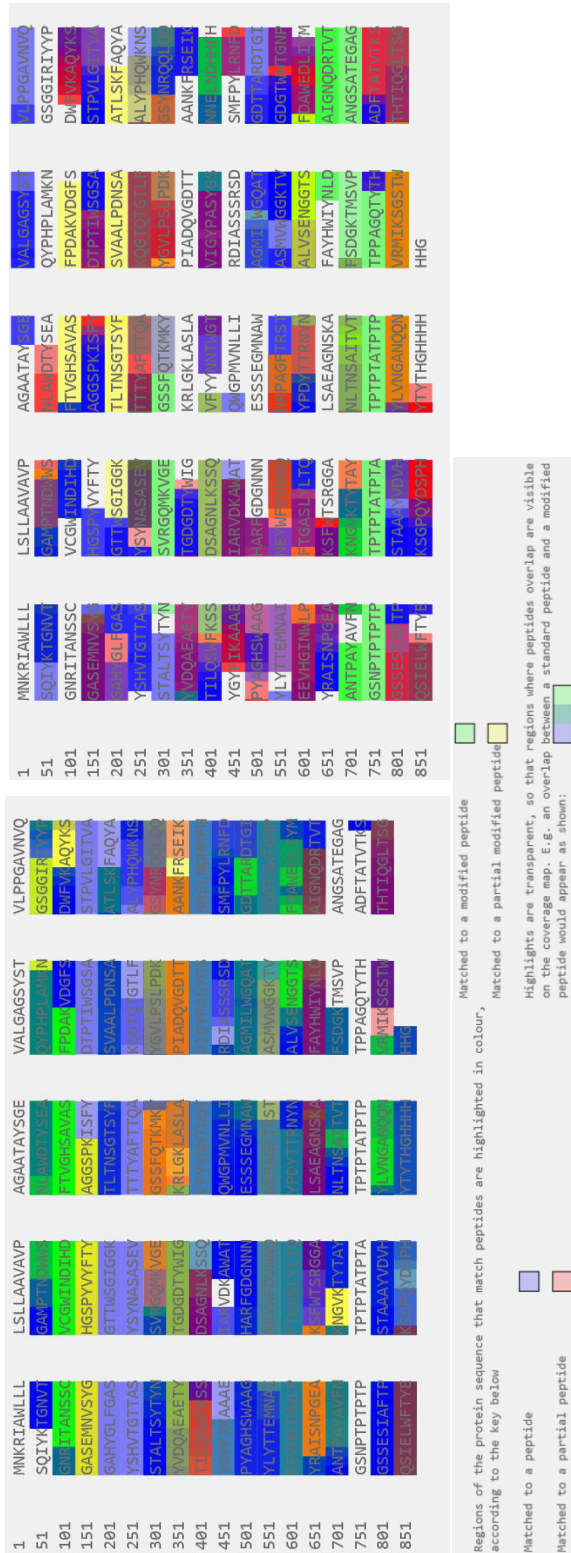
| Protein description                                       | Samples      |              |                |                 |                  |                   | CAZyme domain identified |
|-----------------------------------------------------------|--------------|--------------|----------------|-----------------|------------------|-------------------|--------------------------|
|                                                           | Secreted_YCW | Secreted_Glc | Periplasmic_CW | Periplasmic_Glc | Intracellular_CW | Intracellular_Glc |                          |
| GKU 000110 DUF1343 domain-containing protein              |              |              |                |                 |                  | x                 | GH171                    |
| GKU 000114 beta-N-acetylhexosaminidase                    |              |              |                |                 |                  | x                 | GH3                      |
| GKU 000124 discoidin domain-containing protein            | x            |              |                |                 |                  |                   | CBM32+CBM32+CBM32+CBM32  |
| <b>GKU 000306 family 16 glycosylhydrolase</b>             | <b>x</b>     |              | <b>x</b>       |                 | <b>x</b>         |                   | <b>GH16</b>              |
| GKU 000527 glycoside hydrolase family 88 protein          |              |              |                |                 |                  | x                 | GH105                    |
| GKU 000696 GMC family oxidoreductase                      |              |              | x              |                 |                  |                   | AA3                      |
| GKU 001178 catalase/peroxidase HPI                        | x            |              |                |                 |                  |                   | AA2                      |
| GKU 001586 endo-1 4-beta-xylanase                         |              |              |                |                 |                  | x                 | GH10                     |
| GKU 003084 di-heme oxidoreductase family protein          |              |              | <b>x</b>       |                 |                  |                   | GH16+CBM56               |
| GKU 003206 hypothetical protein                           |              | x            |                |                 |                  |                   | PL1                      |
| GKU 003226 alpha-amylase family protein                   |              | x            |                |                 |                  |                   | GH13                     |
| <b>GKU 003455 glycoside hydrolase family 16 protein</b>   | <b>x</b>     |              |                |                 |                  | <b>x</b>          | <b>GH16</b>              |
| <b>GKU 003456 glycoside hydrolase family 3</b>            |              |              | <b>x</b>       |                 |                  | <b>x</b>          | <b>GH3</b>               |
| GKU 003651 transglycosylase SLT domain-containing protein |              |              |                |                 | <b>x</b>         |                   | GH23                     |
| GKU 003709 pectate lyase                                  |              | x            |                |                 |                  |                   | PL1                      |

**Appendix 6.** CAZymes of the GH16 and GH64 families filtered from the proteomic analysis results of *Cellulosimicrobium* sp. GKH proteins secreted into solid (a) or liquid (b) medium supplemented with YCW as a carbon source.

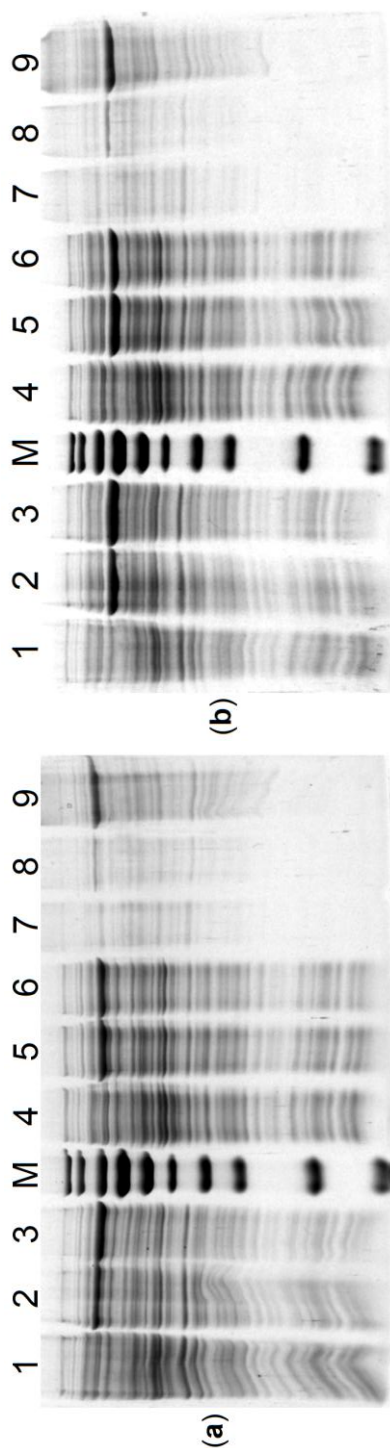
| Accession number        | Description                 | CAZyme domain identified | -10lgP | Sequence coverage (%) |
|-------------------------|-----------------------------|--------------------------|--------|-----------------------|
| <i>(a) solid media</i>  |                             |                          |        |                       |
| A0AAV5P1C8              | Glucan 1 3-beta-glucosidase | GH64+CBM13               | 156.26 | 6                     |
| A0AAV5P7L0              | Hydrolase                   | GH16_3+CBM13             | 181.66 | 11                    |
| <i>(b) liquid media</i> |                             |                          |        |                       |
| A0AAV5P1C8              | Glucan 1 3-beta-glucosidase | GH64+CBM13               | 319.82 | 35                    |
| A0AAV5P7L0              | Hydrolase                   | GH16_3+CBM13             | 228.55 | 40                    |



**Appendix 7.** The ability of GHs from *Rheinheimera* sp. GKU to produce glucose from various glucans. a – laminarin; b – yeast  $\beta$ -glucan, c – YCW, d – curdian, e – pachyman, f – pustulan, g – barley  $\beta$ -glucan, h – laminaribiose, i – laminaritetraose, j – gentiobiose, k – cellobiose, l – carboxymethyl cellulose (CMC), m – standard mixture of C2 – cellobiose, G – glucose, G2 – gentiobiose, L2 – laminaribiose, L3 – laminaritetraose, L4 – laminaripentaose, L5 – laminaripentaose, L6 – laminarihexaose. Control – substrate with no enzymes. GH3\_3456, GH16\_306, GH16\_3455 – represent hydrolysis products of separate GHs. GH3+GH16+GH16 – represents synergistic action of GH3\_3456, GH16\_306, and GH16\_3455.



**Appendix 8.** The graphical representation of the PsLam81A sequence coverage. The results were obtained after tryptic (left side – 86% coverage) and chymotryptic (right side – 78% coverage) digestion.



**Appendix 9.** SDS-PAGE analysis of recombinant PsLam81A and PsLam81A $\Delta$ CBM56 proteins induced at different temperatures. *E. coli* strain BL21(DE3) cells expressing (a) PsLam81A and (b) PsLam81A $\Delta$ CBM56 were induced at 20 °C and 30 °C for 24 hours. Lane M – Molecular mass marker (10–170 kDa, Thermo Fisher Scientific). Lane 1 – cell lysate induced at 30 °C (without target gene plasmid (wp)), lane 2 – cell lysate induced at 20 °C (plasmid with target gene present (p)), 3 – lysate of cells induced at 30 °C (p), 4 – soluble protein fraction\* of cell lysate induced at 30 °C (wp), 5 – soluble protein fraction of cell lysate induced at 20 °C (p), 6 – soluble protein fraction of cell lysate induced at 30 °C (p), 7 – 50 $\times$  concentrated culture medium proteins\*\* separated from cells induced at 30 °C (wp), 8 – 50 $\times$  concentrated culture medium proteins separated from cells induced at 20 °C (p), 9 – 50 $\times$  concentrated culture medium proteins separated from cells induced at 30 °C (p).  
\*soluble protein fraction was obtained by centrifugation of cell lysate at 16,000 $\times$  g at 4 °C for 20 minutes.  
\*\*proteins were concentrated by incubating the culture medium for 10 minutes in a boiling water bath and collecting precipitated proteins by centrifugation at 16,000 $\times$  g for 20 minutes.

**Appendix 10.** FASTA sequences of recombinant proteins characterised in this study.

>GH3\_3456  
MCGGGAETHSDFDNVDVTQPVTDWQLVWSDDFDGA TISSTKWNYEINCSGGNNKQC YTDSSDNAFIKDGM LNIV ALPAAE GA EK  
PYTSTRLTTHKADFKYGRFEIRAKLPYQGGSWPAFWMPDTSVYGGWPRSGEIDILEAVNLKVVGEDGVAESNVHGT LHYGREWP  
NNA SSGKSYQLPDGVNPADDFHTYAIEWQEGERWYVDGYLYATQRQSVVRYNANGEAVGLAHRGWFAEYFNIASGELETHWDAA  
PFDQEFYLILNLA VGGNWPENVNNLGIDASAFADGQH FQIDYIRVYECATDPVTGKG CETVRGGYDSPEDGLVTGLAPIPTASSGIVQS  
LTIFDGT PNP NWAAWDCCGGTTPALVQDVQRGEVFEFSVGATPTVNGFTSRAEFVPAPGVASPF DASGITEIGYIRFDMQVVSAPGNSA  
APWIFKVESVNGTPVELELTASMEAAAPVTGQWQTYTFPLQMLADAGLDVA AIDVVMVFP AWDQGN GAVYRLDNVEITLDYPELV L  
FAEEANSGWPLWDCCGGSVPTEEMDDAEHGTVAQFSIGATPTVMGFSTRSLHGGSDVEFDASLLTNGGVVQFEMK VVSAPANA AAD  
WLFKLEANGSGDGAVELSLADSV EQTAPVTGQWQTYTFNLADIAATGFDVSAIDVLMVFP A WGQGE GAVYRMDNVTFKQP GHHHH  
HHG

>GH16\_306  
MCGGGAETHSDFDNVDVTQPVTDWQLVWSDDFDGA TISSTKWNYEINCSGGNNKQC YTDSSDNAFIKDGM LNIV ALPAAE GA EK  
PYTSTRLTTHKADFKYGRFEIRAKLPYQGGSWPAFWMPDTSVYGGWPRSGEIDILEAVNLKVVGEDGVAESNVHGT LHYGREWP  
NNA SSGKSYQLPDGVNPADDFHTYAIEWQEGERWYVDGYLYATQRQSVVRYNANGEAVGLAHRGWFAEYFNIASGELETHWDAA  
PFDQEFYLILNLA VGGNWPENVNNLGIDASAFADGQH FQIDYIRVYECATDPVTGKG CETVRGGYDSPEDGLVTGLAPIPTASSGIVQS  
LTIFDGT PNP NWAAWDCCGGTTPALVQDVQRGEVFEFSVGATPTVNGFTSRAEFVPAPGVASPF DASGITEIGYIRFDMQVVSAPGNSA  
APWIFKVESVNGTPVELELTASMEAAAPVTGQWQTYTFPLQMLADAGLDVA AIDVVMVFP AWDQGN GAVYRLDNVEITLDYPELV L  
FAEEANSGWPLWDCCGGSVPTEEMDDAEHGTVAQFSIGATPTVMGFSTRSLHGGSDVEFDASLLTNGGVVQFEMK VVSAPANA AAD  
WLFKLEANGSGDGAVELSLADSV EQTAPVTGQWQTYTFNLADIAATGFDVSAIDVLMVFP A WGQGE GAVYRMDNVTFKQP GHHHH  
HHG

>GH16\_306\_CBM\_CBM  
MASSGIVQSLTIFDGT PNP NWAAWDCCGGTTPALVQDVQRGEVFEFSVGATPTVNGFTSRAEFVPAPGVASPF DASGITEIGYIRFDMQ  
VVSAPGNSAAPWIFKVESVNGTPVELELTASMEAAAPVTGQWQTYTFPLQMLADAGLDVA AIDVVMVFP AWDQGN GAVYRLDNVEI  
TLDYPELVLF AEEANSGWPLWDCCGGSVPTEEMDDAEHGTVAQFSIGATPTVMGFSTRSLHGGSDVEFDASLLTNGGVVQFEMK VVS  
APANA AADWLFKLEANGSGDGAVELSLADSV EQTAPVTGQWQTYTFNLADIAATGFDVSAIDVLMVFP A WGQGE GAVYRMDNVTF  
KQPGHHHHHHG

**Appendix 10** (continued). FASTA sequences of recombinant proteins characterised in this study.

```
>GH16_306_CBM_1
MASSGIVQSLTIFDGTNPNNWAAWDCCGGTTPALVQDVQRGEVFEFSVGATPTVNGFTSRAEFVPAPGVASPF DASGITEIGYIRFDMQ
VVSAPGNSAAPWIFKVESVNGTPVELELTASMEAAAPVTGQWQTYTFPLQMLADAGLDVAIDVVMVFPAWDQGN GAVYRLDNVEI
TLDYPHHHHHHG

>GH16_306_CBM_2
MELVLF AE EANS GWPLWDCCGGSVPT EEMDDAEHGTVAQFSIGATPTVMGFSTRSLHGGSD E VFDASLLTNGGVVQFEMKVVVSAPA
NAAADWLFKLEANGSGDGAVELSLADSV EQTAPVTGQWQTYTFNLADIAATGFDVSAIDVLMVFPAWGQGE GAVYRMDN VTFKQP
GHHHHHHHG

>GH16_3455
MCSTTVPEPDEQSV AQHQPD AID S ADWQLVWQDEFDSNSIDQSKWSEFQNCWGGGNDEQQCYTDRPENAYV DNGV L N I V A R K E T F
TGPALHEDAPDFSPHQRKTLPYTSARLRKSGKGDWLYGRFEIRAKLPTGQGSWPAIWMLPTDWQYGPWPLSGEIDIMEAVNLGTVSD
EPSALADEPERRIHGTLHYGELWPDNVHSGQSYKL PDGQSPADDFHTY AIEWQPGEIRWYVDDVL YATQK SSEWHTGA VTLK T A L P R
HAFDRPFHLLNLAVGGNWAASVNETGIDPSAYPQSLQIDYVRVYQRQQPGHHHHHHHG

>PsLam81A
MNKRIA W L L L L L L L A A V A P A G A A T A Y S G E V A L G A G S Y S T V L P P G A V N V Q S Q I Y K T G N V T G A M P T N D W W S N L A W D T Y S E A Q Y P H P
L A M K N G S G G I R I Y P G N R I T A N S S C V C G W I N D I H D F T V G H S A V A S F P D A K V D G F S D W F V K A Q Y K S G A S E M N V S Y G H G S P Y V Y F T Y A G
G S P K I S F Y D T P T I W S G S A S T P V L G I T V A G A H Y G L F G A S G T T W S G I G G K T L T N S G T S Y F S V A A L P D N S A A T L S K F A Q Y A Y S H V T G T T A S Y S
Y N A S A S E V T T Y A F T T Q A K Q G T Q T G T L F A L Y P H Q W K N S S T A L T S Y T Y N S V R G Q M K V G E G S S F Q T K M K Y Y G V L P S L P D K G S Y N R Q Q L
Q Q Y V D Q A E A E T Y T G D G D T Y W I G K R L G K L A S L A P I A D Q V G D T T A A N K F R S E I K T I L Q S W F K S D S A G N L K S S V F Y Y N N T W T G T V I G Y P
A S Y G S N N E L N D H H F H Y G Y F I K A A A E I A R V D K A W A T Q W G P M V N L I R D I A S S R S D S M F P Y L R N F D P Y A G H S W A A G H A R F G D G N N N E
S S S E G M N A W A G M I L W G Q A T G D T T A R D T G I Y L Y T T E M N A I N E Y W F D V N N Q N R P A G F T R S T A S M V W G G K T V G D G T W W T G N P E E V H G
I N W L P F T G A S L Y L T Q Y P D Y T T R N Y N A L V S E N G G T S F D A W E D L I Y M Y R A I N S P G E A K S F W T S R G G A L S A E A G N S K A F A Y H W I Y N L D A I G
N Q D R T V T A N T P A Y A V F N K N G V K Y T A Y N L T N S A I T Y T F S D G K T M S V P A N G S A T E G A G G S N P T P T P T A T P T A T P T P T P
P A G Q Y T H A D F T A T V T K S G S S E I A F T P S T A A A Y V D V H Y L V N G A N Q N V R M I K S G S T W T H T I Q G L T S G Q S I E L W F T Y E K S G P Q Y D S P H
Y T Y T H G H H H H H H G
```

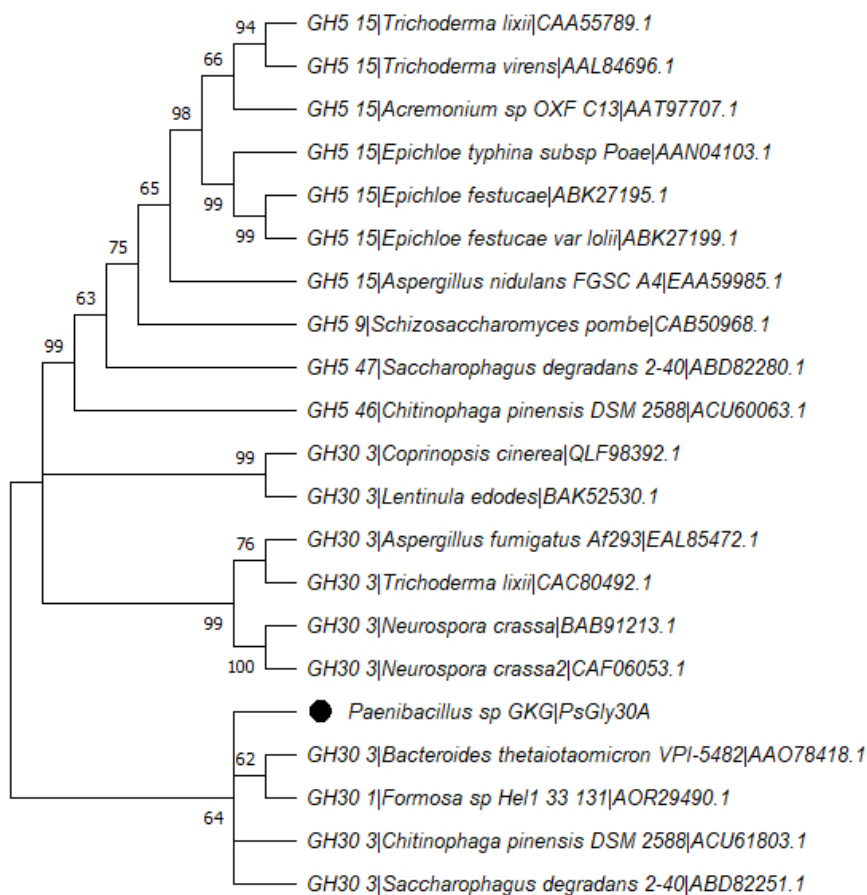
**Appendix 10** (continued). FASTA sequences of recombinant proteins characterised in this study.

>PsLam81AΔCBM56  
 MNKRIALLLLSLLAAVAVPAGAATAYSGEVALGAGSYSTVLPPGAVNVQSQIYKTNVTGAMPTNDWWSNLAWDTYSEAQYPHP  
 LAMKNGSGGIRIYYPGNRITANSSCVCGWINDIHDFTVGHSAVASFPDAKVDGFSDWVFKAQYKSGASEMNVSYGHGSPYVYFTYAG  
 GSPKISFYDTPTIWSGSASTPVLGITVAGAHYGLFGASGTTWSGIGKLTLSNGTSYFSVAALPDNSAATLSKFAQYAYSHV/TGTTASYS  
 YNASASEVTTTYAFTTQAKQGTQTGTLFALYPHQWKNSSALTSTYTNSVRGQMKVGESSFTKMKYYGVLPSPDKGSYNRQQL  
 QQYVDQAEAEITYGDGDTYWIGKRLGKLASLAPIADQVGDTTAANKFRSEIKTILQSWFKSSDSAGNLKSSQVFFYYNNTWGTVIGYP  
 ASYGSNNELNDHHFHYGYFIKAAAEIARVDKAWATQWGPMVNLLIRDIASSRSSDSMFPLYLRNFDPYAGHSWAAAGHARFGDGNNE  
 SSSEGMAWAGMILWGOATGDTTARDTGILYLTTEMNAINEYWFVDVNNQNRPAFTTRSTASMVWGGKTVGDGTWWTGNPEEVHG  
 INWLPFTGASLYLTQYPDYTTRNYNALVSENGGTSFDAWEDLIYMYRAISNPGEAKSFWTSRGGALSAEAGNSKAFAYHWIYNLDAIG  
 NQDRTVTANTPAYAVFNKNGVKITYTAYNLTNSAITVTFSDGKTMSVPANGSATEGAGGSNGHHHHHHH  
 >PsGly30A  
 MSDWKKKTAVALAAALVSAGGTYSAPPAKAAAGEQVEVWISTDPNSEPAVGLSPSARLTRIGDKSFSTAGSADFTISVDENTTFQQ  
 MDGFGVSMTDSSAWLMNYKLDANKRAEVMERLFGQTGIGLSVLRQPIGSSDFAWSAYTYADTANDTALNQFSIGRDLPIYIVPMVKQ  
 AIAKNPNIKVFGSPWSAPAWMKYSNTLNGGKLRAEHYGTANYFKKYIQAYQAQGIPIYAVTVQNEPLYEPTHYPSMGMTQDEIGFI  
 GDYLGPTLRGAGINTKIIAFDHNFLDWSFPNTVINSLKSSGKGGYIDGSFHHYDSGDGSTMQLRNNHPDKGIWFTEGGFGNWNDPQ  
 NGTSQGFDMMMNEFINITRNWSKTHILWNAALDQKDGPELLAANNNTKGMITIRNSDNRNDAPENVVTYHKQYYLLGHFSKFVVPGA  
 YRVQSNVTGSEIKSVAFRNPDGSKVVVAYNSSSSARNVKVQWGSQSFNVSVPGKSAMTYKWYGNRSGHHHHHHH  
 >GH16  
 MDLARHRSLTPRPTGRRPRARRRLASALVAALTAALAAALAVTVAA TSAAPGDLLWSDEFDGAAGSAPNPAVWNHETGAHW  
 GNAELQNYTASRANSALDQGQNLVITARREGDGSYTSARMTTQGYQPQYQRIQIPRGQGIWPAFVWMLGGSPGTPWPSSGEIDI  
 MENVGFEPHRVHGTVHGPYSGDSGITGMYPHPQGW/SFADTEHTFAVDWKPEGITWFDGQQQFHRVTRASVGANA W/FDQPFLLIL  
 NVAVGGQWPGYPDGTTQLPQQMKVDYVRVYDNGSGSSSPGNPGTGLPTGTGAVRAANGM/CVDVPWADPTDGNPVQIVTCSGNA  
 QTWTRGSDGTVALGKCLDVRDGTSTRGAAVQVWTCNGTGAQKWAYDAGSKALRNPQSGLCCLDATGGAPLRDQRLQTWTCNGT  
 TAQQWTLLEHHHHHHH

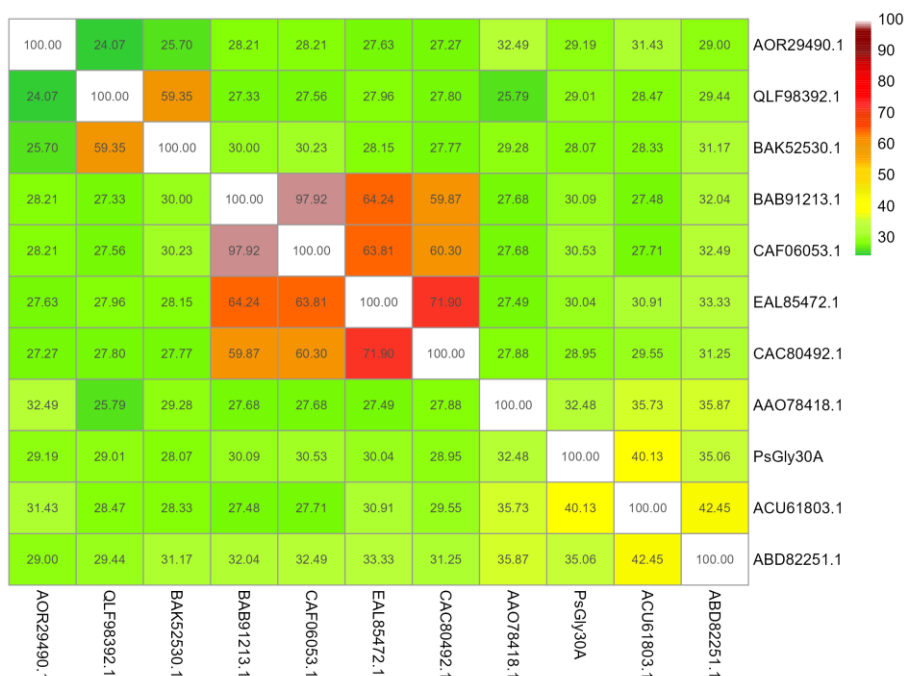
**Appendix 10** (continued). FASTA sequences of recombinant proteins characterised in this study.

>GH64

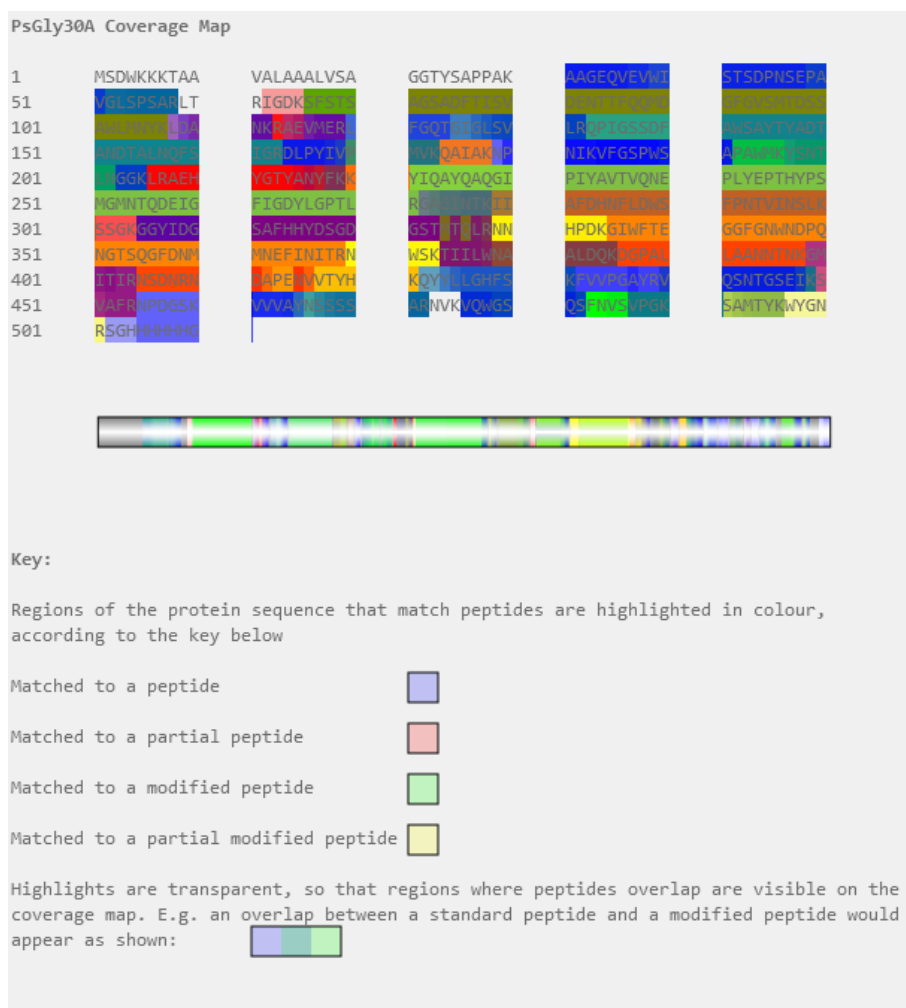
```
MPHDKNSSRRAWAALCAAVLAVSGALVGVAAPASAVPATIPLTITNDSGRGPIYLYVLGERDGVAGWADAGGTFHPWPGGVGPVP
VPAPDASIA GPGPGQSVTIRLPKLSGRVYYSYGQKMTFQIVLDGRLVQPAVQNDSDPNRNILFNWTEYTLNDGGLWINSTQVDHWSAP
YQVG VQRADGGQVLSTGMLKPNGYEAFYTALEGAGWGGLVQRAPDGSRLRALNPSHGIDVGKISSASIDSYYVTEVWNSYRTRDMVVVT
PFSHEPGTQFRGRVDDGWFRFRSGSGQEVAAFKKPDASSVYGCHKDLQAPNDHVVGPIARTLCAALVRTTALTNPNPQPDANSAGFYQ
DARTNVYAKLAHQQMANGKAYAFADFDDVGAHESLVHDGNPQAAYIKLDPFTGTATPLNGGGSTEQPGTPGGLPAGTGALRIGSTLCL
DVPWADPTDTNQVLATCSGNAQQWTRGTDGTVRALGKCLDVARSGTADGTAVWIYTCNGTGAQKWYDSTATKALRNPQSGKC
LDAQGGAPLRDGGKVQLWTCNQTEAQRWTLLEHHHHHH
```



**Appendix 11.** Phylogenetic analysis of PsGly30A (●) and other  $\beta$ -1,6-glycosidases with endo-1,6- $\beta$ -glucanase (EC 3.2.1.75) and  $\beta$ -1,6-glucosidase (EC 3.2.1.-) activities. The tree is based on protein sequence alignments of sequence regions identified as GH5 and GH30 family domains. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model (Jones et al., 1992). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test 1000 replicates) are shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. This analysis involved 21 amino acid sequences. There were a total of 558 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).



**Appendix 12.** Identity matrix for full-length amino acid sequences of GH30 family  $\beta$ -1,6-glycosidases. Amino acid sequences of characterised GH30  $\beta$ -1,6-glycosidases ( $\beta$ -1,6-glucosidase (EC 3.2.1.-) and endo-1,6- $\beta$ -glucanase (EC 3.2.1.75)) were obtained from the NCBI database (<https://www.ncbi.nlm.nih.gov/protein>), and the amino acid sequence of PsGly30A was translated from the sequenced *Paenibacillus* sp. GKG gene sequence (GenBank accession no. OR933702). Sequence alignment and identity comparison were performed using the Clustal Omega web server (<https://www.ebi.ac.uk/jdispatcher/msa/clustalo>).



**Appendix 13.** Graphical representation of the PsGly30A sequence coverage (92%) obtained after tryptic digestion. In-gel protein digestion and Data-independent LC-MS proteomics were performed as described earlier (Plakys et al., 2024).



GH30\_3|B.thetaiotaomicron\_VPI-5482|AAO78418.1

$\eta 6$   $\alpha 11$   $\alpha 12$   $\beta 14$   $\beta 15$   
 350 360 370 380

GH30\_3 B.thetaiotaomicron\_VPI-5482|AAO78418.1 GTWNSGRDLKSLRLMEDEEVA..... LGTINNNWCKGVIVNNLML  
 GH30\_3 P.sp\_GKG|Psgly30A GNWNDPQNGTSGQFDNMNNEF..... INITRNWSTIILNNAL  
 GH30\_3 B.cellulosilyticus\_WH2|ALJ59776.1 GTWNNHGLDLTKTLLSNMESTIG..... IGTINNNYCSAVTINNNLMT  
 GH30\_3 C.pinensia\_DSM\_2588|ACU61803.1 SVGGSFSGDLNWHHTQNVV..... IGSINNWSKTIVLNNLIS  
 GH30\_3 S.degradans\_2-40|ABD82251.1 GANSNFWGDVAVHVVNLII..... VGATRNWCKTIVLNNLAA  
 GH30\_3 A.fumigatus\_Af293|EAL85472.1 PASG.....AWHQAADF..... MGPLQNWASGVAAVNLGT  
 GH30\_3 C.cinerea|QLF98392.1 TYGSDWSDIKWYMDNLW..... IGSVERFSTGLMMNLAL  
 GH30\_3 L.edodes|BAK52530.1 SLGSDWWSIDIKWYMDNIF..... IGAISYSGASTGLMMNLAL  
 GH30\_3 N.crassa|BAB91213.1 SARQP.....TPWNWAASFT..... MGPLQNWASGVAAVNLGT  
 GH30\_3 T.lixi|CAC80492.1 PSTG.....AWNQAASF..... MGPLQNWASGVAAVNLGT  
 GH30\_1 F.sp\_Hell\_33\_131|AOR29490.1 DSEIPKQDDKWKYWSKEATDWGFDWREQEKYLLHKPYAVNRYARDI LGLNNWVDGVWVNNMLV

GH30\_3|B.thetaiotaomicron\_VPI-5482|AAO78418.1

$\beta 16$   $\beta 17$   $\beta 18$   $\alpha 13$   
 390 400 410 420

GH30\_3 B.thetaiotaomicron\_VPI-5482|AAO78418.1 DNDRG.....PNRE..GGCQT..CYGAVDINNND.....YKTIIRNNSHVITIAHLSSVIV  
 GH30\_3 P.sp\_GKG|Psgly30A DQKDG.....PALL..AANNT..NKGMITIRNSDNRNDAPENVVTVYHKKYVLLGHFVKFV  
 GH30\_3 B.cellulosilyticus\_WH2|ALJ59776.1 DTFDNGSPISNNGGQPNRP..GGCQT..CFGAVDINNND.....LKTIVNNNSHYMICHLISAVV  
 GH30\_3 C.pinensia\_DSM\_2588|ACU61803.1 DSQGG.....PHTP..GGSTV..SMGAVDIDGGN.....IVRVVGVYIIGHVSKFV  
 GH30\_3 S.degradans\_2-40|ABD82251.1 DSNLO.....PHTL..GGCDA..CLGALTIDGGN.....VKNRNVYVIAHAHAKHV  
 GH30\_3 A.fumigatus\_Af293|EAL85472.1 NAQDG.....PHLS..TGCCAT..CGLVLTIN..NG..GYTLNTAVYMAAQFVKFM  
 GH30\_3 C.cinerea|QLF98392.1 DGAGN.....PKLPGTITSCGGPGGRPLVTINNNDGS.....YTFNQEFGVMAAQSKAV  
 GH30\_3 L.edodes|BAK52530.1 DNGN.....PFLPGSDSCGG.GGRGIVGINSDDGT.....YSVNQEFGVMAAQSKAT  
 GH30\_3 N.crassa|BAB91213.1 DNGN.....PFLPGSDSCGG.GGRGIVGINSDDGT.....YSVNQEFGVMAAQSKAT  
 GH30\_3 T.lixi|CAC80492.1 TAQDS.....PHLTGSDACDK..CTGLVMDTAAG.....TYNLRGDVMMAQFVKFM  
 GH30\_1 F.sp\_Hell\_33\_131|AOR29490.1 DRQGG.....PHLS..SGGCGT..CTGLVLTIN..NG..QYTFQVGVMAAQFVKFM

GH30\_3|B.thetaiotaomicron\_VPI-5482|AAO78418.1

$\beta 19$   $\beta 20$   $\beta 21$   $\beta 22$   
 430 440 450 460 470

GH30\_3 B.thetaiotaomicron\_VPI-5482|AAO78418.1 KPR...GAVRTATTG...YTDNGITCSFEMTD.....GTIAFVLI...NNNEKSKKITVSD..  
 GH30\_3 P.sp\_GKG|Psgly30A RPR...GAVRVQSN...TGSEIKSVNRPD.....GSKVAYNSSSARNYKQWG.  
 GH30\_3 B.cellulosilyticus\_WH2|ALJ59776.1 RP...GAVRIGSN...NSGNIQTTFRNSD.....GSAFVLI...NNKSKKITVAPD  
 GH30\_3 C.pinensia\_DSM\_2588|ACU61803.1 PP...GAVRIGSN...NSGNIQTTFRNSD.....GSKVLI...NNNGGKNSVSKVWG.  
 GH30\_3 S.degradans\_2-40|ABD82251.1 PP...GAVRIGSN...NSGNIQTTFRNSD.....GSKVLI...NNNGGKNSVSKVWG.  
 GH30\_3 A.fumigatus\_Af293|EAL85472.1 PP...GAVRIGSN...NSGNIQTTFRNSD.....GSKVLI...NNNGGKNSVSKVWG.  
 GH30\_3 C.cinerea|QLF98392.1 PP...GAVRIGSN...NSGNIQTTFRNSD.....GSKVLI...NNNGGKNSVSKVWG.  
 GH30\_3 L.edodes|BAK52530.1 PP...GAVRIGSN...NSGNIQTTFRNSD.....GSKVLI...NNNGGKNSVSKVWG.  
 GH30\_3 N.crassa|BAB91213.1 PP...GAVRIGSN...NSGNIQTTFRNSD.....GSKVLI...NNNGGKNSVSKVWG.  
 GH30\_3 T.lixi|CAC80492.1 PP...GAVRIGSN...NSGNIQTTFRNSD.....GSKVLI...NNNGGKNSVSKVWG.  
 GH30\_1 F.sp\_Hell\_33\_131|AOR29490.1 PP...GAVRIGSN...NSGNIQTTFRNSD.....GSKVLI...NNNGGKNSVSKVWG.

GH30\_3|B.thetaiotaomicron\_VPI-5482|AAO78418.1

$\beta 23$   $\beta 24$   
 480 490

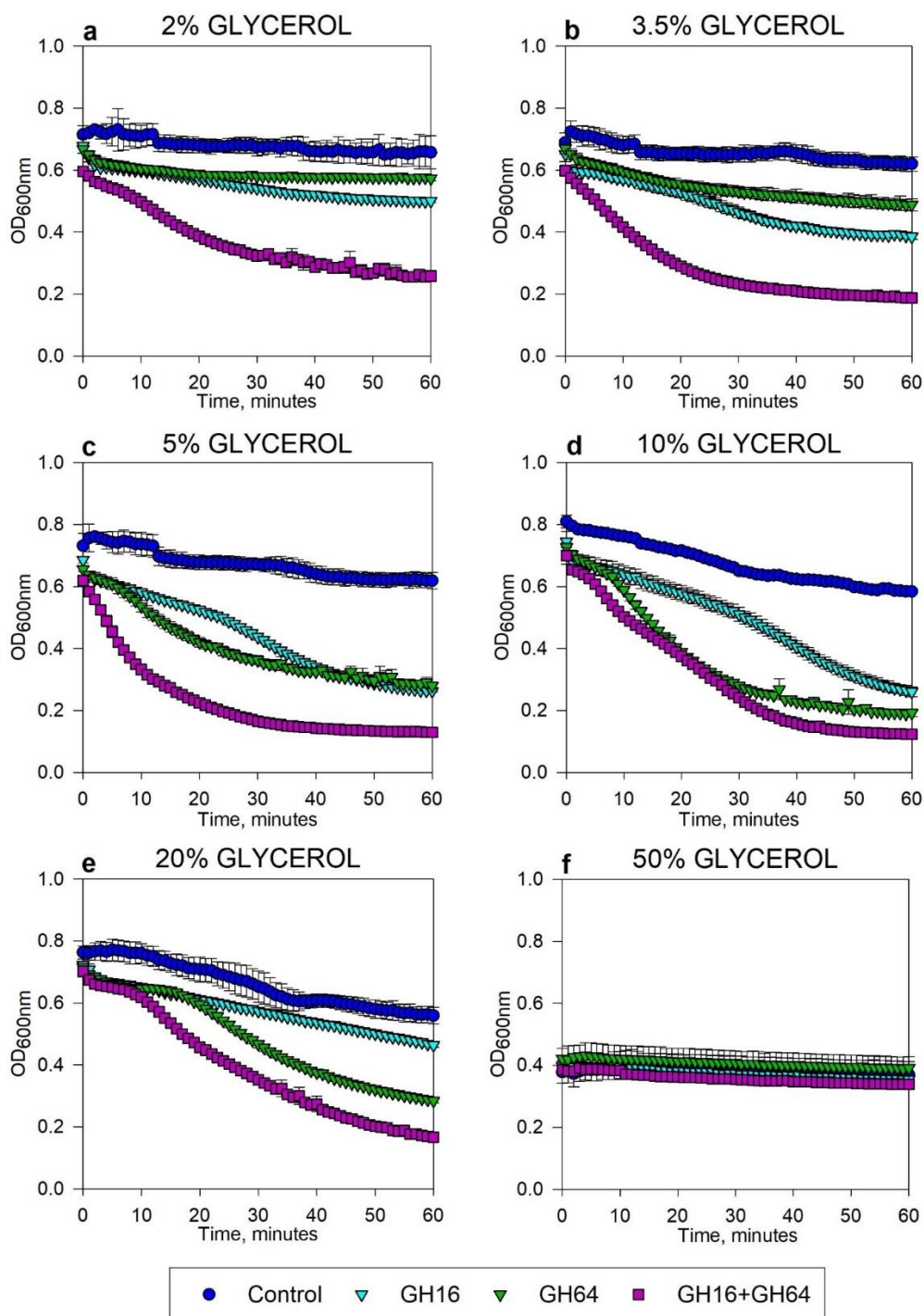
GH30\_3 B.thetaiotaomicron\_VPI-5482|AAO78418.1 ..GQRHFA.YDVPKGSITSYRWAKSK.....  
 GH30\_3 P.sp\_GKG|Psgly30A ..SQSFNVSVPGKSAITYKWKYGNRS.....  
 GH30\_3 B.cellulosilyticus\_WH2|ALJ59776.1 DAGKKHSASCTLPQRSVNSPKFKW.....  
 GH30\_3 C.pinensia\_DSM\_2588|ACU61803.1 ..SQSFNVSVPGKSAITYKWKYGNRS.....  
 GH30\_3 S.degradans\_2-40|ABD82251.1 ..RQKPAYSMFPAQSIVTIVLID.....  
 GH30\_3 A.fumigatus\_Af293|EAL85472.1 ..SQKWSQNPQPSQSVITVWVLSA.....  
 GH30\_3 C.cinerea|QLF98392.1 TIEFRGMQATYVFPVGVITLWVFASTISLNSINAESYALYSETDQKQPLHFPFQ  
 GH30\_3 L.edodes|BAK52530.1 ..SCEVWSGLIVYRNSVVTWVLPAAAGA.....  
 GH30\_3 N.crassa|BAB91213.1 ..SQGEWSGNVPTNSVITWVLPVAV.....  
 GH30\_3 T.lixi|CAC80492.1 ..ESSTIIAIPQQAIPQIVLFPN.....

GH30\_3|B.thetaiotaomicron\_VPI-5482|AAO78418.1

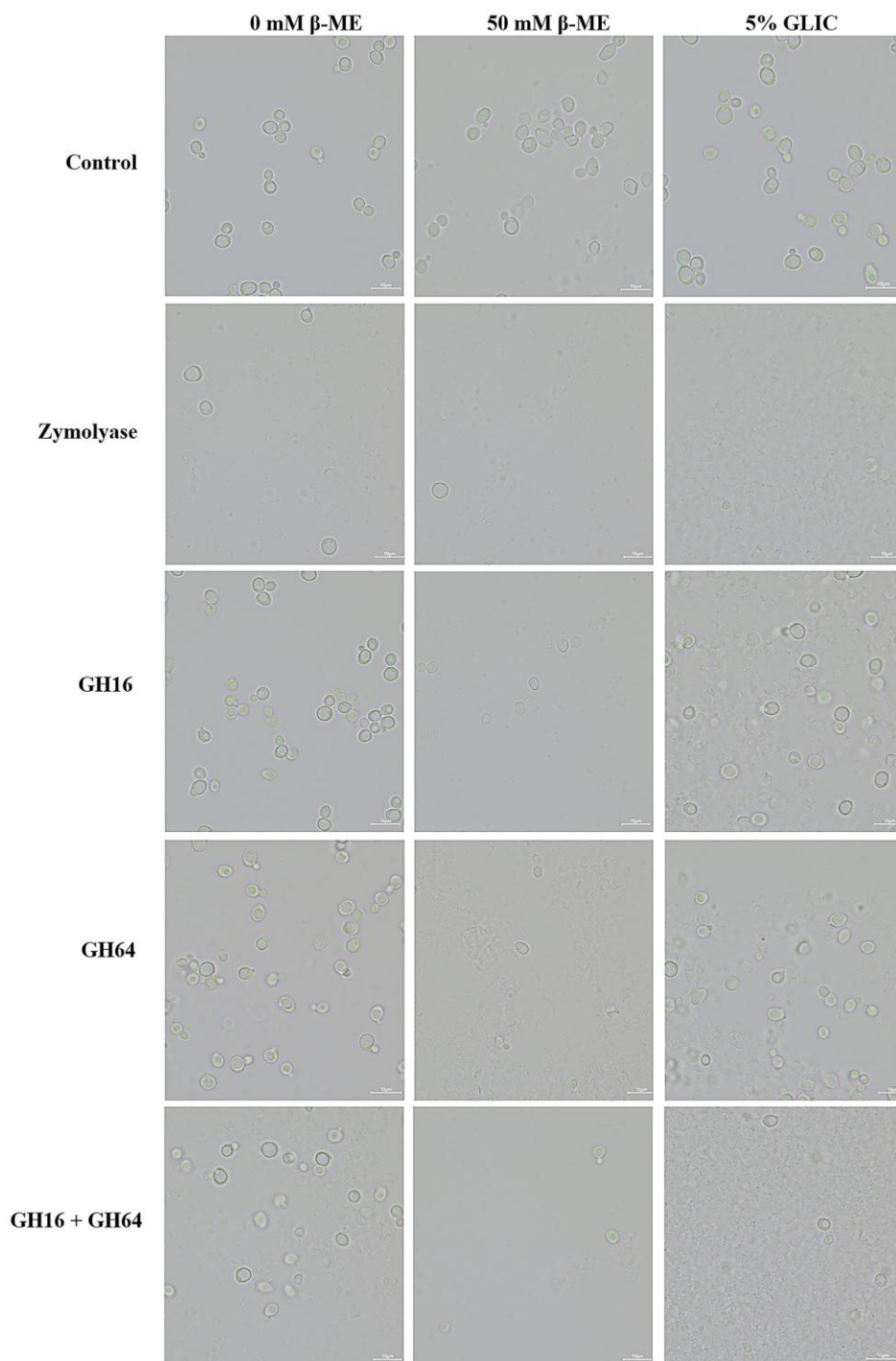
GH30\_3 B.thetaiotaomicron\_VPI-5482|AAO78418.1  
 GH30\_3 P.sp\_GKG|Psgly30A  
 GH30\_3 B.cellulosilyticus\_WH2|ALJ59776.1  
 GH30\_3 C.pinensia\_DSM\_2588|ACU61803.1  
 GH30\_3 S.degradans\_2-40|ABD82251.1  
 GH30\_3 A.fumigatus\_Af293|EAL85472.1  
 GH30\_3 C.cinerea|QLF98392.1  
 GH30\_3 L.edodes|BAK52530.1  
 GH30\_3 N.crassa|BAB91213.1  
 GH30\_3 T.lixi|CAC80492.1  
 GH30\_1 F.sp\_Hell\_33\_131|AOR29490.1

GH30\_3|B.thetaiotaomicron\_VPI-5482|AAO78418.1  
 GH30\_3 B.thetaiotaomicron\_VPI-5482|AAO78418.1  
 GH30\_3 P.sp\_GKG|Psgly30A  
 GH30\_3 B.cellulosilyticus\_WH2|ALJ59776.1  
 GH30\_3 C.pinensia\_DSM\_2588|ACU61803.1  
 GH30\_3 S.degradans\_2-40|ABD82251.1  
 GH30\_3 A.fumigatus\_Af293|EAL85472.1  
 GH30\_3 C.cinerea|QLF98392.1  
 GH30\_3 L.edodes|BAK52530.1  
 GH30\_3 N.crassa|BAB91213.1  
 GH30\_3 T.lixi|CAC80492.1  
 GH30\_1 F.sp\_Hell\_33\_131|AOR29490.1

## Appendix 14. (continued)



**Appendix 15.** OD<sub>600nm</sub> dynamics during lysis of *S. cerevisiae* BY4741 cells incubated in lysis buffers containing different concentrations of glycerol solutions: a – 2% glycerol, b – 3.5% glycerol, c – 5% glycerol, d – 10% glycerol, e – 20% glycerol, f – 50% glycerol. Measurements were performed in triplicate and the results were plotted as an average. Error bars represent SD.



**Appendix 16.** Microscopy analysis of yeast cell lysis after 60 minutes of incubation. The first column represents conditions when neither  $\beta$ -ME nor 5% glycerol was used, the second shows the results when  $\beta$ -ME was used as an additive to enhance lysis, and the third shows results when osmotic shock was induced using 5% glycerol as an additive. The rows represent the enzymatic preparations used. Dr. Ramunė Stanevičienė recorded experimental data.

**Appendix 17.** Comparison of the lysis capability of *Zymolyase*, GH16, GH64, and GH16+GH64 enzymatic preparations on 16 different yeast strains.

| Yeast strains                             | Enzymes and additives used |                    |                  |                   |              |                  |                    |                  |                   |              |                  |                    |                  |                   |                  |                   |
|-------------------------------------------|----------------------------|--------------------|------------------|-------------------|--------------|------------------|--------------------|------------------|-------------------|--------------|------------------|--------------------|------------------|-------------------|------------------|-------------------|
|                                           | <i>Zymolyase</i>           |                    |                  |                   | GH16+GH64    |                  |                    |                  | GH16              |              |                  |                    | GH64             |                   |                  |                   |
|                                           | 0 mM $\beta$ -ME           | 0.5 mM $\beta$ -ME | 5 mM $\beta$ -ME | 50 mM $\beta$ -ME | 5 % glycerol | 0 mM $\beta$ -ME | 0.5 mM $\beta$ -ME | 5 mM $\beta$ -ME | 50 mM $\beta$ -ME | 5 % glycerol | 0 mM $\beta$ -ME | 0.5 mM $\beta$ -ME | 5 mM $\beta$ -ME | 50 mM $\beta$ -ME | 5 mM $\beta$ -ME | 50 mM $\beta$ -ME |
| <i>Aureobasidium pullulans</i> PC-6-30.2N |                            |                    |                  |                   |              |                  |                    |                  |                   |              |                  |                    |                  |                   |                  |                   |
| <i>Aureobasidium pullulans</i> SB-18-2    |                            |                    |                  |                   |              |                  |                    |                  |                   |              |                  |                    |                  |                   |                  |                   |
| <i>Candida albicans</i> VM-3-52.1         |                            |                    |                  |                   |              |                  |                    |                  |                   |              |                  |                    |                  |                   |                  |                   |
| <i>Candida kruisii</i> SV-5-43.1          |                            |                    |                  |                   |              |                  |                    |                  |                   |              |                  |                    |                  |                   |                  |                   |
| <i>Hanseniaspora osmophila</i> RUC-7-30.1 |                            |                    |                  |                   |              |                  |                    |                  |                   |              |                  |                    |                  |                   |                  |                   |
| <i>Hanseniaspora uvarum</i> PC-5-12       |                            |                    |                  |                   |              |                  |                    |                  |                   |              |                  |                    |                  |                   |                  |                   |
| <i>Kazachstania aerobia</i> SN-3-57       |                            |                    |                  |                   |              |                  |                    |                  |                   |              |                  |                    |                  |                   |                  |                   |
| <i>Kazachstania africana</i> FV-1-43      |                            |                    |                  |                   |              |                  |                    |                  |                   |              |                  |                    |                  |                   |                  |                   |
| <i>Kazachstania africana</i> FV-1-43      |                            |                    |                  |                   |              |                  |                    |                  |                   |              |                  |                    |                  |                   |                  |                   |
| <i>Lachancea kluyveri</i> AM-5-38.3       |                            |                    |                  |                   |              |                  |                    |                  |                   |              |                  |                    |                  |                   |                  |                   |
| <i>Lachancea thermotolerans</i> PC-6-21.1 |                            |                    |                  |                   |              |                  |                    |                  |                   |              |                  |                    |                  |                   |                  |                   |
| <i>Lachancea thermotolerans</i> PC-6-21.1 |                            |                    |                  |                   |              |                  |                    |                  |                   |              |                  |                    |                  |                   |                  |                   |
| <i>Meischnikowia fructicola</i> PC-6-12.1 |                            |                    |                  |                   |              |                  |                    |                  |                   |              |                  |                    |                  |                   |                  |                   |
| <i>Meischnikowia pulcherrima</i> PA-5-47  |                            |                    |                  |                   |              |                  |                    |                  |                   |              |                  |                    |                  |                   |                  |                   |
| <i>Pichia anomala</i> PC-5-24             |                            |                    |                  |                   |              |                  |                    |                  |                   |              |                  |                    |                  |                   |                  |                   |
| <i>Pichia fermentans</i> PC-4-19.1        |                            |                    |                  |                   |              |                  |                    |                  |                   |              |                  |                    |                  |                   |                  |                   |
| <i>Saccharomyces cerevisiae</i> BY4741    |                            |                    |                  |                   |              |                  |                    |                  |                   |              |                  |                    |                  |                   |                  |                   |
| <i>Saccharomyces paradoxus</i> PA-6-10.1  |                            |                    |                  |                   |              |                  |                    |                  |                   |              |                  |                    |                  |                   |                  |                   |

Lysis efficiency: – good (OD decreased by >70%), – average (OD decreased between 50% and 70%), – weak (OD decreased between 10% and 50%), – none (OD did not decrease by more than 10% or increased).

## ACKNOWLEDGEMENTS

I am deeply grateful to my supervisor, Prof. Dr. Rolandas Meškys, for the trust and the opportunity to join his group. His continuous guidance and support made this journey an exciting and rewarding experience.

I would like to express my gratitude to the consultant of this thesis, Dr. Edita Mažonienė, for her initial encouragement and open-minded attitude that made this PhD thesis possible. I am grateful to all my colleagues at Roquette who provided advice and encouragement. Special thanks to Jurgita Kailiuvienė for the experimental data.

I appreciate the insightful comments, suggestions and questions of thesis reviewers and defence panel members Prof. Dr. Saulius Serva and Dr. Dalius Ratautas. Also, I am thankful to defence panel members Prof. Dr. Renata Gudiukaitė, Dr. Inga Matijošytė, and Dr. Ilma Tapio for their time and interest in this work.

Many thanks to all colleagues of the Department of Molecular Microbiology and Biotechnology for their generous advice. Especially, for Dr. Renata Gasparavičiūtė for her experienced eye in selection of the most promising microorganisms, Dr. Justas Vaitekūnas for limitless answers to my “What would you do?” questions, Dr. Rasa Rutkienė for shared expertise in protein purification, and Dr. Rūta Stanislauskienė for stepping in to assist with the experimental part when needed. Also, I would like to thank technician Nijolė Uždavinienė for the support in laboratory preparations, therefore, ensuring the high pace of my investigation. Special thanks to fellow PhD students for all the entertaining moments and activities.

I would like to thank Dr. Aleksandras Konovalovas for *Rheinheimera* sp. GKU genome sequence determination and analysis.

I am also thankful to Prof. Dr. Elena Servienė and Dr. Ramunė Stanevičienė for making wide-spectrum yeast lysis experiments possible.

Finally, I would like to express my sincere gratitude to my family for their unwavering support since the beginning of my scientific journey, and to my friends for the encouragement and companionship during challenging periods.

## LIST OF PUBLICATIONS

**Plakys, G.,** Gasparavičiūtė, R., Vaitekūnas, J., Rutkienė, R., & Meškys, R. (2022). Characterization of *Paenibacillus* sp. GKG Endo- $\beta$ -1, 3-Glucanase, a Member of Family 81 Glycoside Hydrolases. *Microorganisms*, 10(10): 1930. DOI: 10.3390/microorganisms10101930

**Author contribution** – planning and performing of FASP sample preparation and LC-MS/MS proteomics, synthesis and purification of recombinant proteins, enzymatic activity assay, determination of substrate specificity assay experiments, and preparation of the manuscript.

**Plakys, G.,** Urbelienė, N., Urbelis, G., Vaitekūnas, J., Labanauskas, L., Mažonienė, E., & Meškys, R. (2024). Conversion of  $\beta$ -1,6-Glucans to Gentiobiose using an endo- $\beta$ -1,6-Glucanase PsGly30A from *Paenibacillus* sp. GKG. *ChemBioChem*, e202400010. DOI: 10.1002/cbic.202400010

**Author contribution** – planning and performing cloning, synthesis and purification of recombinant protein, in-gel digestion and LC-MS/MS identification of PsGly30A protein, enzymatic activity assay, determination of substrate specificity assay experiments, and preparation of the manuscript.

## CONFERENCE PRESENTATIONS

**Plakys G.**, Gasparavičiūtė R., Vaitekūnas J., Rutkienė R., Meškys R. Application of bacterial hydrolases for the production of (oligo)saccharides from yeast cell walls. In: 47th FEBS Congress, Tours, France, July 8–12th, 2023. P-03.1-19.

**Plakys G.**, Vaitekūnas J., Gasparavičiūtė R., Urbelis G., Urbelienė N., Rutkienė R., Labanauskas L., Mažonienė E., Meškys R. Conversion of yeast *Saccharomyces cerevisiae*  $\beta$ -1,3/1,6-glucans to functional disaccharides, laminaribiose and gentiobiose, using endo-glucanases PsLam81A and PsGly30A. In: 11th International Congress on Biocatalysis, Hamburg, Germany, August 25–29th, 2024. P3-40

## CURRICULUM VITAE

Gediminas Plakys

Vilnius University Life Sciences Centre

Saulėtekio av. 7, LT-10257 Vilnius, Lithuania

E-mail: gediminas.plakys@bchi.stud.vu.lt

### EDUCATION

|           |                                                                       |
|-----------|-----------------------------------------------------------------------|
| 2021–2025 | Vilnius University,<br>PhD student in Chemical Engineering            |
| 2017–2020 | Vilnius Gediminas Technical University,<br>Master of Bioengineering   |
| 2013–2017 | Vilnius Gediminas Technical University,<br>Bachelor of Bioengineering |

### INDUSTRY EXPERIENCE

|              |                                                  |
|--------------|--------------------------------------------------|
| 2026–present | Roquette, Lithuania<br>Senior Research Scientist |
| 2017–2025    | Roquette, Lithuania<br>Researcher                |

### SCIENTIFIC EXPERIENCE

|                 |                                                       |
|-----------------|-------------------------------------------------------|
| 2024/09–2025/09 | Vilnius University<br>Junior Scientist                |
| 2019/03–2019/09 | Kaunas University of Technology<br>Project Specialist |

## PUBLISHED SCIENTIFIC RESEARCH

1. **Plakys, G.**, Urbelienė, N., Urbelis, G., Vaitekūnas, J., Labanauskas, L., Mažonienė, E., Meškys, R., 2024. Conversion of  $\beta$ -1,6-Glucans to Gentiobiose using an endo- $\beta$ -1,6-Glucanase PsGly30A from *Paenibacillus* sp. GKG. *ChemBioChem* 25, e202400010. <https://doi.org/10.1002/cbic.202400010>
2. Preitakaitė, V., Barasa, P., Aučynaitė, A., **Plakys, G.**, Koplūnaitė, M., Zubavičiūtė, S., Meškys, R., 2023. Bacterial amidohydrolases and modified 5-fluorocytidine compounds: Novel enzyme-prodrug pairs. *PLoS ONE* 18, e0294696. <https://doi.org/10.1371/journal.pone.0294696>
3. **Plakys, G.**, Gasparavičiūtė, R., Vaitekūnas, J., Rutkienė, R., Meškys, R., 2022. Characterization of *Paenibacillus* sp. GKG Endo- $\beta$ -1, 3-Glucanase, a Member of Family 81 Glycoside Hydrolases. *Microorganisms* 10, 1930. <https://doi.org/10.3390/microorganisms10101930>
4. Budreviciute, A., Damiati, S., Sabir, D.K., Onder, K., Schuller-Goetzburg, P., **Plakys, G.**, Katileviciute, A., Khoja, S., Kodzius, R., 2020. Management and Prevention Strategies for Non-communicable Diseases (NCDs) and Their Risk Factors. *Front. Public Health* 8, 574111. <https://doi.org/10.3389/fpubh.2020.574111>
5. Katileviciute, A., **Plakys, G.**, Budreviciute, A., Onder, K., Damiati, S., Kodzius, R., 2019. A Sight to Wheat Bran: High Value-Added Products. *Biomolecules* 9, 887. <https://doi.org/10.3390/biom9120887>

## NOTES

## NOTES

## NOTES

## NOTES

**Presentation of PhD studies' results.**

Vilniaus universiteto leidykla  
Saulėtekio al. 9, III rūmai, LT-10222 Vilnius  
El. p. [info@leidykla.vu.lt](mailto:info@leidykla.vu.lt), [www.leidykla.vu.lt](http://www.leidykla.vu.lt)  
[bookshop.vu.lt](http://bookshop.vu.lt), [journals.vu.lt](http://journals.vu.lt)  
Tiražas 15 egz.