Enzymatic production of soluble bioactive β-1,3-glucans from paramylon

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Doctor of Philosophy

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Abstract

Paramylon is a high-molecular weight unbranched β-1,3-glucan polymer produced by the flagellated microalga *Euglena gracilis*. Paramylon and other β-1,3-glucans have been reported to have antitumor and immunostimulating activities, making them ideal candidates as nutraceuticals or adjuvants. Their bioactivity can be increased by breaking them down to shorter-chain soluble saccharides by chemical or enzymatic methods. Here, chemical, physical and enzymatic approaches were examined as individual or combined strategies to facilitate the production of soluble bioactive compounds from paramylon.

A microwave method was developed for the pretreatment of paramylon granules, which otherwise are resistant to enzymatic hydrolysis. The granules were sourced commercially after attempts to produce paramylon in an open pond cultivation proved unsuccessful. The paramylon granules were hydrolysed with a selection of commercially-sourced enzymes and a dye-based assay was devised to assess the efficacy of the hydrolysis. The new microwave pretreatment resulted in improved enzyme accessibility and thereby substantially enhanced the hydrolysis of paramylon granules. The soluble β-1,3-glucans produced with this method were characterised by high-performance liquid chromatography (HPLC) and an immunological assay on mouse macrophages showed that they were bioactive.

The pathway for enzymatic degradation of paramylon in *E. gracilis* was also explored with *Escherichia coli* cells transformed with an *E. gracilis*-derived cDNA library. Recombinants were screened for the production of *E. gracilis* β-1,3-glucanases using an assay which combines fluorescence-activated cell sorting (FACS) with enzyme activity tests in microtiter plates. Both methods were based on the use of the fluorogenic β-glucan analogue fluorescein di-β-D-

glucopyranoside. However, the identification of new enzymes was prevented probably due to the suboptimal quality of the original cDNA library.

In a parallel approach, protein fractions obtained from *E. gracilis* were analysed using mass spectrometry and a recently published transcriptomic database. Four putative enzymes with a high amino acid sequence similarity to known β-1,3-glucanase sequences were identified based on their translated DNA sequences. One of the corresponding genes was introduced into *E. coli* and into *Saccharomyces cerevisiae* to produce a recombinant enzyme. Unfortunately, expression levels were too low for reliable enzyme activity assays and further characterisation.

Finally, various commercially-available and in-house enzyme preparations were shown to degrade paramylon on a dye-based plate assay. This new source of paramylon-degrading enzymes deserves further characterisation, which unfortunately was outside the time-frame of this project but could form the basis for promising future work.

Statement of originality

This work has not previously been submitted for a degree or diploma in any university.

To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Alexander Gissibl

August 2018

Acknowledgments

This thesis comprises three years of full-time research conducted in the Department of Molecular Sciences at Macquarie University from 2015 to 2018.

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I also acknowledge the international Macquarie University Research Excellence Scholarship (iMQRES) and the Australian Research Council Industrial Transformation Training Centre funding scheme (ARC ITTC) for supporting me financially over the course of this project, and also the Macquarie University Postgraduate Research Fund (PGRF) funding scheme for giving me the opportunity to travel abroad to present my research.

Abschließend bedanke ich mich ganz herzlich bei meiner Familie dafür, dass sie mich immer und in Allem unterstützt und mir den Weg, den ich bis heute gehe, ermöglicht hat.

Memberships, conferences, workshops, presentations and awards

Overview of memberships, conferences, workshops and presentations:

2017

- Member of the 'American Society for Microbiology' (ASM)
- Member of the 'Synthetic Biology Australasia' (SBA) Society
- 'ASM Microbe 2017' conference by the American Society for Microbiology in New Orleans, USA. Accompanying workshop 'Microbiology Career Choices'. **Poster presentation**.
- 'Synthetic Biology Australasia Conference' by the SBA Society in Sydney

2016

- 'Synthetic Biology Cutting Edge Symposium' conference by Commonwealth Scientific and Industrial Research Organisation (CSIRO) and the SBA Society in Canberra
- 'Advances in Biotechnology for Food and Medical Applications' conference by the Australian Research Council Industrial Transformation Training Centre (ARC ITTC) for Food Processing and the Institute of Biomedical Engineering and IT at the University of Sydney. **Poster presentation**.
- 'Integrated Molecular Science: a few good stories' seminar by the Biomolecular Frontiers Research Centre (BMFRC) and the ARC ITTC for Molecular Technology in the Food Industry (MTFI) in Sydney
- 'Annual Biofocus Research Conference' by the Biofocus Research Centre at Macquarie University. **2 minute talk and interactive discussion session**.

2015

- 'IP commercialisation seminar' by Davies Collison Cave in Sydney
- 'CBMS Seminar on Intellectual Property and Commercialisation of Research' at Macquarie University
- 'Inaugural Science Next Collaborative Forum' by Sigma-Aldrich Oceania in Sydney
- 'Tell us a story' seminar by the BMFRC and the ARC ITTC MTFI in Sydney
- Industrial training (fermentation technology) and workshop (commercialisation of research) by Agritechnology Pty Ltd in Orange, New South Wales
- 'BioFocus Research Conference' by the Biofocus Research Centre at Macquarie University

2014

- 'Microbial Formation of Biofuels and Platform Chemicals' conference by the Philipp University of Marburg in Marburg (Germany)

Overview of awards:

2016

- 'Macquarie University Postgraduate Research Fund' (PGRF) **travel grant**: **5,000 AUD**
- **1st prize for 2 minute talk and interactive discussion session** at the 'Annual Biofocus Research Conference': **100 AUD**

2015 - 2018

- 3-year ARC ITTC **stipend** (incl. top-up of approx. 5000 AUD per annum) and 3-year 'international Macquarie University Research Excellence Scholarship' (iMQRES) tuition fees only **scholarship**: approx. **200,000 AUD total**

List of publications

This thesis includes one peer-reviewed publication (publication 1, see Chapter 4) and two manuscripts prepared for submission (manuscripts 1 and 2, see Chapters 1 and 5, respectively).

1. Gissibl A, Sun A, Care A, Nevalainen H and Sunna A. Bioproducts from *Euglena gracilis* - Current and future applications. Prepared for submission to *Biotechnology for Biofuels*.

2. Gissibl A, Care A, Parker LM, Iqbal S, Hobba G, Nevalainen H and Sunna A (2018). Microwave pretreatment of paramylon enhances the enzymatic production of soluble β-1,3-glucans with immunostimulatory activity. *Carbohydrate Polymers* 196:339-347.

3. Gissibl A, Care A, Sun A, Hobba G, Nevalainen H and Sunna A. Development of screening strategies for the identification of paramylon-degrading enzymes. Prepare for submission to the *Journal of Microbiological Methods*.

List of abbreviations

A	absorbance
A_C	cut-off A
AD	atopic dermatitis
ARA	arachidonic acid
B	bound fraction
BC	buffer control
BLAST	basic local alignment search tool
BODIPY	boron-dipyrromethene
CAZy	carbohydrate active enzymes
CB	bound fraction of the control
CBM	carbohydrate-binding modules
CIEX	cation exchange chromatography
CU	unbound fraction of the control
DAPI	4',6-diamidino-2-phenylindole
D-GalUA	D-galacturonate
D-GalUA-P	D-galacturonate-1-P
DHA	docosahexaenoic acid
DMEM	Dulbecco's Modified Eagle's Medium
DMPH	2,3-dimethyl-5-phytyl-1,4-hydroquinone
DNS	dinitrosalicylic acid
DP	degree of polymerisation
DPA	docosapentaenoic acid
DSC	Differential scanning calorimetry
DW	dry weight

DγLA	dihomo-γ-linoleic acid
EDA	eicosadienoic acid
EgFAR	*E. gracilis* fatty acyl-CoA reductase
EgKAT	*E. gracilis* 3-ketoacyl-CoA thiolase
EgWS	*E. gracilis* wax synthase
ENDO	endo-β-1,3-glucanases
EPA	eicosapentaenoic acid
EXO	exo-β-1,3-glucanases
ExPASy	expert protein analysis system
F	protein fraction
FA	fatty acid
FACS	fluorescence-activated cell sorting
FAlcs	fatty alcohols
FDGlu	fluorescein di-β-D-glucopyranoside
FITC	fluorescein isothiocyanate
GH	glycoside hydrolase
GM	genetic modification
H	homogentisate
HPLC	high-performance liquid chromatography
HPP	4-hydroxyphenylpyruvate
HT	heterotrophic
IPTG	isopropyl β-D-thiogalactoside
LA	linoleic acid
L-Asc	L-ascorbate
LB	lysogeny broth
LBP	laminaribiose phosphorylase

LC ESI MS/MS	liquid chromatography electrospray ionisation tandem mass spectrometry
L-GalA	L-galactonate
L-Gal-L	L-galactono-1,4-lactone
M	marker
MP	membrane proteins
MPH	2-methyl-6-phytyl-1,4-hydroquinone
MT	mixotrophic
M_W	molecular weight
OG	oxidative glucanases
ORF	open reading frames
PAGE	polyacrylamide gel electrophoreses
PBS	phosphate buffered saline
PDP	phytyl diphosphate
P_i	inorganic phosphate
PT	photoautotrophic
PUFA	polyunsaturated fatty acid
RID	refractive index detector
ROS	reactive oxygen species
SBA	standard binding assay
SD	standard deviation
SDS	sodium dodecyl sulphate
SEC	size exclusion chromatography
SEM	scanning electron microscopy/standard error of the mean
SP	sucrose phosphorylase
Th	T helper cells
TNFα	tumour necrosis factor alpha

TYR	tyrosine
U	unbound fraction
UDP	uridine diphospho
UDP-D-GalUA	UDP-D-galacturonate
UDP-D-Glc	UDP-D-glucose
UDP-D-GlcUA	UDP-D-glucuronate
WE	wax ester
WT	wild type
α-T	α- tocopherol
β-1,3-GP	β-1,3-glucan phosphorylase
β-G	β-glucosidases
γ-T	γ-tocopherol
ρ_{DCW}	biomass concentration
ρ_{DPW}	paramylon concentrations

Chapter 1: Bioproducts from *Euglena gracilis* - Current and future applications

1.1. Introduction

Euglena gracilis is a freshwater microalga with a versatile metabolism capable of producing high-value compounds like proteins, vitamins, lipids and its storage polysaccharide paramylon, which is composed of pure unbranched β-1,3-glucan. The review contained within Chapter 1 provides a comprehensive overview of the current and potential bioproducts that can be obtained from *E. gracilis*, in particular those with applications in healthcare and biofuel production. The economic feasibility of these bioproducts is discussed, with a focus on the various cultivation techniques for the improvement of biomass and bioproduct yields. Accordingly, the latest advances concerning the industrial-scale cultivation of *E. gracilis*, its current limitations and its future potential are discussed.

The review in this chapter was prepared as a manuscript for submission to *Biotechnology for Biofuels*.

1.2. Manuscript 1

Bioproducts from *Euglena gracilis* - Current and future applications

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Keywords

Euglena gracilis, essential amino acids, ascorbate, α-tocopherol, polyunsaturated fatty acids, wax esters, paramylon, large-scale cultivation

Abstract

The versatile metabolism of the freshwater microalga *Euglena gracilis* is a vast source for products such as proteins, free amino acids, vitamin C (ascorbate), vitamin E (α-tocopherol), lipids (e.g. wax esters and polyunsaturated fatty acids) as well as the β-1,3-glucan paramylon. Compellingly, algal lipids have found applications in industry as potential biofuels and β-1,3-glucan in the health sector as nutraceuticals or immunostimulating agents. The yields of these products are mainly dependent on the cultivation mode (photoautotrophic vs. mixo-/heterotrophic, aerobic vs. anaerobic). Therefore, an understanding of the complex metabolism of *E. gracilis* is crucial for the optimisation of the product yields. In this review, the aforementioned bioproducts from *E. gracilis* are introduced and critically discussed in the context of large-scale cultivation, focussing on their current and future commercial relevance.

1. Background

E. gracilis is a flagellated unicellular microalga (Fig. 1), which is ubiquitous in most freshwater biotopes. This versatile microalgal species is capable of growing both autotrophically using sunlight and heterotrophically on a variety of substrates including sugars and ethanol [1-3]. In nature, it is able to tolerate a number of environmental stresses including the presence of heavy metals, acidic growing conditions and ionizing radiation. [4-6]. This physical endurance and versatile metabolism provide *E. gracilis* with an advantage in natural functions such as bioremediation of polluted water containing high levels of nitrogen, phosphates and organic carbon [7].

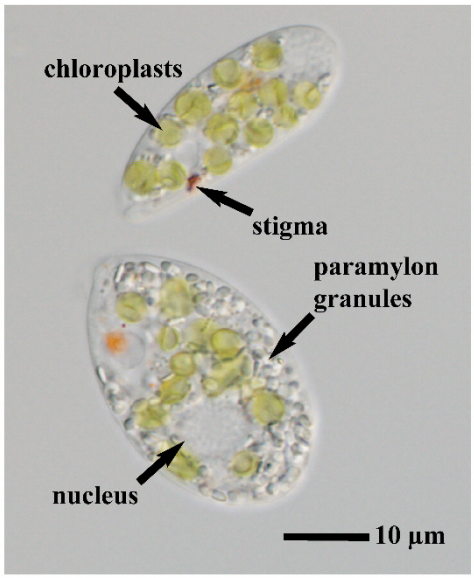

Fig. 1. Microscopic image of *E. gracilis* cells. Paramylon granules, chloroplasts, nucleus and a characteristic eyespot (stigma) are labelled after Buetow, 2001 [1]. The stigma plays a role in phototaxis [8]. Note that the flagella of the cells are not visible in this image.

E. gracilis is considered an attractive organism for several biotechnological applications. For examples, *E. gracilis* has a high nutritional value and it is an excellent source for vitamin C and E, amino acids and long-chain ω-3 polyunsaturated fatty acids (PUFAs) as a whole cell meal [2, 9, 10]. Lipids accumulated by *E. gracilis* under certain growth conditions have potential for biofuel production [11]. *Euglena* spp. can also accumulate large amounts of the reserve polysaccharide paramylon, reaching up to 95% of the cell mass [12]. Paramylon is a β-1,3-glucan storage polysaccharide synthesised and deposited as granules in the cytosol (Fig. 1) of a broad variety of euglenoids [12-14]. *E. gracilis* will quickly degrade and utilise these paramylon granules as a carbon source under carbon starvation [15]. Paramylon and other β-1,3-glucans are of special interest as bioproducts because of their reported immunostimulatory and antimicrobial bioactivities [12, 13, 16]. These compounds also have been shown to lower cholesterol levels and to exhibit antidiabetic, antihypoglycemic and hepatoprotective activities and have been used for the treatment of colorectal and gastric cancers [17-19].

Only few molecular biology methods have been developed for *E. gracilis* [20, 21]. Some efforts have been taken to establish a genetic transformation system for *E. gracilis* based on chloroplast transformations with gold particle bombardment [20, 21]. However, neither a molecular toolbox for the effective genetic manipulation nor the complete genome sequence data with gene annotations have been made available [20, 21]. The large size and complexity of the *E. gracilis* genome (presumably 2 Gbp with around 80% repetitive sequences) seems to be the main factors preventing the completion of the attempts to sequence the genome of *E. gracilis* [22]. Moreover, the genome contains the hypermodified Base J, a thymidine derivate also found in kinetoplastids thought to have regulatory functions (e.g. in gene silencing), which has been considered to be potentially detrimental to the sequencing process by inhibiting polymerase processivity [22, 23]. Due to the limited genome information available, transcriptomic and proteomic data have become a valuable source of insight into the complex metabolic pathways of *E. gracilis* and their regulation under different growth conditions [24-26]. Furthermore, a number of genes from *E. gracilis* have been expressed heterologously in other organisms such as *Arabidopsis thaliana*, *Escherichia coli*, *Saccharomyces cerevisiae* and insect cells for the purpose of their biochemical characterisation or to establish pathways for the production of compounds of interest. [27-30].

In this review, we will provide a comprehensive and critical overview of selected bioproducts produced by *E. gracilis* with special consideration to their industrial relevance. The current state of large-scale cultivation of *E. gracilis* is presented and discussed as well.

2. Nutrition

2.1. Dietary protein

There is a growing global demand for dietary protein with high nutritional value due to the increasing human population in concurrence with socio-economic changes [31]. The amino acids isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine are

essential for adults while arginine, cysteine, histidine and tyrosine are additionally essential for children [32-34].

Microalgae can accumulate large quantities of proteins intracellularly and as such represent an attractive alternative to more traditional sources of dietary protein like meat and fish. In addition, animal and *in vitro* studies have shown an excellent digestibility of protein derived from *E. gracilis* biomass [35]. The *E. gracilis* protein content can reach up to 0.7g/g of the biomass dry weight (DW) and is on a par with other popular microalgae food supplements, such as those produced from *Chlorella* spp. and *Arthrospira maxima* (better known as Spirulina) [31, 35, 36].

E. gracilis produces all 20 proteinogenic amino acids (including the essential ones) and in their free form they reach levels of up to 45.6 mg/g DW [2, 24, 37]. Initial transcriptomic studies indicated that amino acid synthesis pathways in *E. gracilis* are similar to those used by plants and bacteria, while an in-depth proteomic analysis recently revealed a unique pathway for arginine in *E. gracilis*, which is independent from the urea cycle commonly utilised by other eukaryotic organisms [24, 25].

The mode of cultivation has a major impact on the protein content of *E. gracilis*, especially when comparing photoautotrophic (PT) growth (i.e. utilisation of light as energy and carbon dioxide as carbon source) with mixotrophic (MT) and heterotrophic (HT) growth conditions (i.e. utilisation of an organic carbon and energy source with or without additional light energy, respectively). For example, the supplementation of HT growth medium with external nitrogen in the form of ammonium maximises protein production (0.7 g/g DW) in comparison with PT cultivation (0.47 g/g DW) [2, 38].

2.2. Vitamin C and E biosynthesis by E. gracilis

Vitamin C (ascorbate) is critical for human health and acts as antioxidant and as co-factor for several enzymes involved in essential biosynthesis pathways [39, 40]. The unique ascorbate synthesis pathway from *E. gracilis* (Fig. 2) has been elucidated using radiotracer experiments as well as transcriptomic and proteomic studies [24, 25, 41]. The only enzymes of the ascorbate synthesis pathway that have been biochemically characterised *in vitro* are the D-galacturonate reductase and the aldonolactonase (Fig. 2 enzymes 5 and 6, respectively).

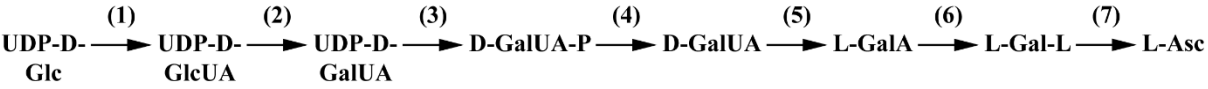

Fig. 2. Ascorbate synthesis pathway of *E. gracilis*. Not all substrates and reaction products are shown. Enzymes involved (numbered) and main substrates/products (abbreviated) are indicated: uridine diphospho (UDP)-glucose 6-dehydrogenase (1), UDP-glucuronate 4-epimerase (2), UDP-galacturonate pyrophosphatase (3), D-galacturonate 1-phosphate phosphatase (4), D-galacturonate reductase (5), aldonolactonase (6), L-galactonolactone dehydrogenase (7), UDP-D-glucose (UDP-D-Glc), UDP-D-glucuronate (UDP-D-GlcUA), UDP-D-galacturonate (UDP-D-GalUA), D-galacturonate-1-P (D-GalUA-P), D-galacturonate (D-GalUA), L-galactonate (L-GalA), L-galactono-1,4-lactone (L-Gal-L), L-ascorbate (L-Asc) [24, 25, 41].

PT cultivation induces the synthesis of ascorbate in *E. gracilis* as a mechanism to cope with reactive oxygen species (ROS) produced during photosynthesis (i.e. ROS scavenging) and the enzyme ascorbate peroxidase additionally reduces excess hydrogen peroxide to water under oxidation of ascorbate [24, 42, 43]. The typical ascorbate yield of a PT-cultivated *E. gracilis* culture is around 4 mg/g DW, which is corresponding to approximately 8 mg/L, whereas the yield is negligible for *E. gracilis* cultivated under HT conditions [24, 43]. The overall yield can be increased to 86.5 mg/L by batch-fed two-step cultivation (change from MT to PT growth) [10]. Ascorbate production by *E. gracilis* has not attracted much attention, probably because consumption of just 100 to 200 mL of juices from citrus fruits like oranges are enough to fulfil the daily recommended intake of adults (75 and 90 mg for females and males, respectively) [40, 44]. Nevertheless, ascorbate and other bioproducts like vitamin E remain an important component of a whole cell meal based on *E. gracilis* biomass for human consumption.

Both α- and γ-tocopherol are, amongst β- and δ-, the major forms of dietary vitamin E. The α-form is one of the most abundant lipophilic antioxidants and considered more important for mammalian physiology. Adverse health-effects, including neurological damage or anaemia, are associated with α-tocopherol deficiency [45]. However, the main dietary form is γ-tocopherol in countries where consumed vegetable oils derive predominantly from soy and corn (e.g. in the USA), leading to an insufficient intake of α-tocopherol [46-48].

E. gracilis produces almost exclusively the α-form of tocopherol and a key enzyme in its α-tocopherol synthesis pathway (Fig. 3) is the γ-tocopherol methyltransferase (Fig. 3, enzyme 6) [49, 50]. The enzyme converts γ- to α-tocopherol using *S*-adenosyl methionine as the methyl group donor and shows promiscuity towards β- and δ-tocopherol [49]. The pathway has been confirmed by transcriptomics and proteomics studies [24, 25].

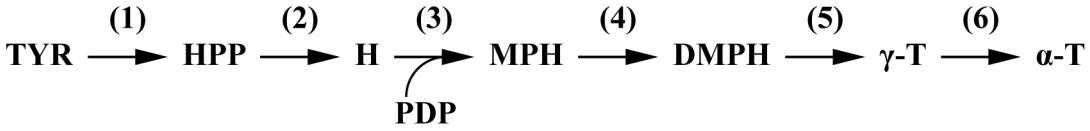

Fig. 3. The *E. gracilis* synthesis pathway of α-tocopherol. Not all substrates and reaction products are shown. Enzymes involved (numbered) and main substrates/products (abbreviated) are indicated: tyrosine aminotransferase (1), 4-hydroxyphenylpyruvate dioxygenase (2), homogentisate phytyltransferase (3), 2-methyl-6-phytyl-1,4-hydroquinone methyltransferase, tocopherol cyclase (5), γ-tocopherol methyltransferase (6), tyrosine (TYR), 4-hydroxyphenylpyruvate (HPP), homogentisate (H), phytyl diphosphate (PDP), 2-methyl-6-phytyl-1,4-hydroquinone (MPH), 2,3-dimethyl-5-phytyl-1,4-hydroquinone (DMPH), γ-tocopherol (γ-T), α-tocopherol (α-T) [24, 25, 49].

There are several factors influencing α-tocopherol levels in *E. gracilis* and cultivation in light (MT/PT) conditions or the addition of ethanol to the medium have been shown to increased α-tocopherol production. This increase in α-tocopherol could be a response to ROS generated in the chloroplasts or in the mitochondria. Yet, the influence of light was shown to be independent of the presence of chlorophyll (i.e. photosynthesis), suggesting that mitochondria may be primarily responsible for the regulation of α-tocopherol synthesis. It should be noted though that HT/MT cultivation reaches higher cell densities and the overall yield of α-tocopherol is up to 7 times higher

when compared to PT cultivation [24, 51-53]. Longer incubation times result in an overall increase of α-tocopherol yield. However, α-tocopherol per g DW decreases under MT/HT cultivation (without ethanol) over time [24, 54]. The technical set-up also plays a role, for example hydrodynamic stress caused by fast stirring with baffle plates was shown to be detrimental to final α-tocopherol yields [51]. So far, the highest total α-tocopherol levels of 44.2 mg/L culture (1.12 mg/g DW) were achieved with *E. gracilis* in HT fed-batch cultivation after 455 h in medium containing ethanol as carbon source [51]. Comparable levels of 40 mg/L culture (3.7 mg/g DW) were reached after a relatively short incubation time of 120 h using a bleached strain in HT batch cultivation with a medium supplemented with ethanol, glutamate and malate [2]. *E. gracilis* was thereby shown to be an industrially feasible source of α-tocopherol when compared to other sources like olive oil (0.0037 mg/g DW), which is considered to be a good source for the daily intake of α-tocopherol [55].

2.3. Polyunsaturated fatty acids (PUFAs) and their biosynthesis by E. gracilis

PUFAs of the ω-3 or ω-6 series are defined by their first double bond at the position between the third and fourth or fifth and sixth carbon counted from the methyl end, respectively, and are considered essential for the mammalian nutrition. In the Western world, major health conditions like cardiovascular and neurological diseases have been linked directly to a lack of long-chain ω-3 PUFAs in the dietary intake, especially of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [56, 57]. *E. gracilis* has a pathway for the synthesis of EPA and subsequently DHA [9]. The enzymes of the pathway have been characterised biochemically either by measuring their activity in *E. gracilis* cell extract as the genes and amino acid sequences of the Δ17-desaturase (Fig. 4, enzyme 4) and C20/22 elongase (Fig. 4, enzyme 5) enzymes are currently unknown or by activity assays with recombinantly-produced proteins [9, 28, 30, 58-60].

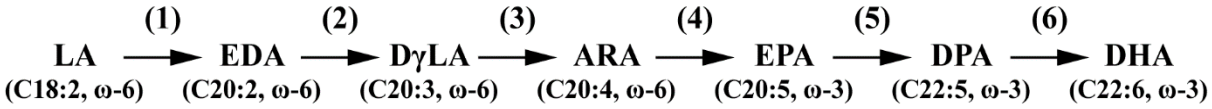

Fig 4. PUFA synthesis pathway of *E. gracilis*. Not all substrates and reaction products are shown. Enzymes involved (numbered) and main substrates/products (abbreviated) are indicated: Δ9-elongase (1), Δ8-desaturase (2), Δ5-desaturase (3), Δ17-desaturase (4), C20/22 elongase (5), Δ4-desaturase (6), linoleic acid (LA), eicosadienoic acid (EDA), dihomo-γ-linoleic acid (DγLA), arachidonic acid (ARA), docosapentaenoic acid (DPA) [9].

Levels of DHA in *E. gracilis* are reported at around 2% of the total fatty acid (FA) content and thereby negligible. These levels are apparently independent of cultivation conditions (e.g. PT vs. HT growth) and represent a good target for improvement by metabolic engineering approaches like adaptive laboratory evolution, a technique based on the selection of improved phenotypes after the application of a selective pressure [61-64]. In an alternative approach, single enzymes of the *E. gracilis* PUFA synthesis pathway have been incorporated into PUFA pathways of other organisms. For example, the Δ8-desaturase from *E. gracilis* pathway has been incorporated into *Arabidopsis thaliana* along with enzymes from other organisms for the production of EPA. In another report, the Δ4-desaturase from *E. gracilis* was expressed together with the Δ15-desaturase from *Caenorhabditis elegans* in mammalian cells, resulting in a shift of the intracellular FA profile towards more valuable EPA and DHA [28, 60].

3. Biofuels and bioactive compounds

3.1 Wax esters and other lipids

Microalgae have been proposed as source of feedstock for the production of biofuels such as biodiesel because of the high amounts of lipids, especially FAs, accumulated during growth by some microalgal species [65]. However, FAs first need to be processed to wax esters (WEs) by transesterification with an alcohol to be used as biodiesel and the degree of saturation of the WEs is crucial for the quality of the biodiesel as saturated compounds have the more favourable properties (e.g. higher cetane number) [66]. In addition to biofuel production, there is high

industrial demand for WEs for their use as lubricants or as raw material for candles and cosmetics [67].

In *E. gracilis*, WE synthesis serves as an electron sink for ATP production through glycolysis during anaerobiosis. Accordingly, the cells synthesise FAs, fatty alcohols (FAlcs) and ultimately WEs in larger quantities from paramylon under oxygen-limiting conditions. The majority of the *E. gracilis*-derived lipids are suited for biodiesel production because they are saturated or have a low degree of unsaturation [68-71]. Central enzymatic steps of the pathway for WE formation are the conversion of FAs to their corresponding FAlcs by the *E. gracilis* fatty acyl-CoA reductase (EgFAR) followed by the esterification of fatty acyl-CoAs and FAlcs by the enzyme *E. gracilis* wax synthase (EgWS). The heterologous expression of these enzymes in the industrially-relevant organism *S. cerevisiae* has been shown to produce WEs of medium chain lengths from supplemented FAs, highlighting the potential of EgFAR and EgWS for the biotechnological production of biofuels [72, 73].

The lipid yield and the ratio of FAs/FAlcs to WEs have been shown to be strongly dependent on the cultivation conditions and the *E. gracilis* strain used [71]. Substantial research has been performed to improve the WE yields of *E. gracilis*. For example, a multi-step cultivation process has been developed aiming to maximise WE synthesis and it has been shown to increase the lipid content of *E. gracilis* cell mass by 7% compared to conventional cultivation. In the first step of this process, *E. gracilis* is cultivated under inexpensive PT conditions. Next, the cells are deprived of a nitrogen source to stimulate paramylon synthesis associated with nitrogen-deficiency [74, 75]. In the third step, WE synthesis from paramylon is induced by changing to anaerobic cultivation, followed by the extraction of the WEs with an organic solvent and column purification, resulting in the production of biofuel [75].

In another study, an *E. gracilis* mutant strain was generated by Fe-ion irradiation in an attempt to use selective breeding to increase lipid production. Intracellular lipids were stained with a boron-dipyrromethene dye ($BODIPY^{505/515}$) after mutagenesis and high-producers were isolated using fluorescence-activated cell sorting. This new strain was capable of accumulating lipids to up to 20% of its DW under hypoxic cultivation conditions, which represents a 40% increase in lipid content compared to the original strain [76]. The use of another cytometry approach was able to identify morphological and intracellular phenotypes associated with a high lipid content in cells *via* a high-throughput optofluidic image cytometer. This technique was capable of identifying lipid-overproducing mutants without the need for staining of the cells *via* potentially phenotype-altering dyes [77]. However, the described mutagenesis approach can only be seen as a proof-of-concept of potential technologies for the improvement of the WE production by *E. gracilis* as the natural isolate *E. gracilis* strain 1224-5/13 has been shown to accumulate a WE content of 57% of the DW when grown anaerobically (with the addition of an elongase inhibitor), which is almost three times the amount of the mutant strain [71, 76]. While other microalgae may achieve comparable WE/DW contents, with *Botryococcus braunii* producing up to 80% WE/DW, *E. gracilis* has the competitive advantage of a better WE/total lipid ratio of up to 80% compared to the total lipid production from other microalgae, which usually consist only of FAs [65, 71, 78].

There is a preference for the use of short-chain esters over long-chain esters in the industrial production of diesel and kerosene because short-chain esters provide better cold flow properties and oxidative stability [79]. Reportedly, the composition of WEs produced by *E. gracilis* was successfully shifted to short-chain esters using RNAi to meet this industrial preference. Three condensing enzymes involved in FA synthesis, the *E. gracilis* 3-ketoacyl-CoA thiolase (EgKAT) isozymes EgKAT 1, EgKAT 2 and EgKAT 3, were targeted and silenced, leading to an increased production of short-chain WEs. An artificial biodiesel inferred from the lipid composition before and after silencing indicated an improvement of cold flow properties [11]. Improvements to the

lipid production of *E. gracilis* as described above in combination with optimised cultivation and processing could soon push biofuel production from *E. gracilis* closer to industrial feasibility.

3.2. Paramylon

3.2.1. Production

Paramylon is the water-insoluble storage polysaccharide of *E. gracilis*, which consists of β-1,3-linked glucose subunits and has an estimated molecular weight of around 100-500 kDa (Fig. 5 A). Paramylon molecules are arranged in an intermolecular triple helix forming microfibrils, which in turn make up fibres. Rectangular and wedge-shaped segments consisting of these fibres are arranged to form the granules (Fig. 5 B and C), which can be synthesised by *E. gracilis* in different shapes like ellipses or rods (Fig. 5 C). The granules are around 1-6 µm long and show an unusual high degree of crystallinity, setting them apart from other carbohydrate storage products of plants and algae. [14, 17, 80-82].

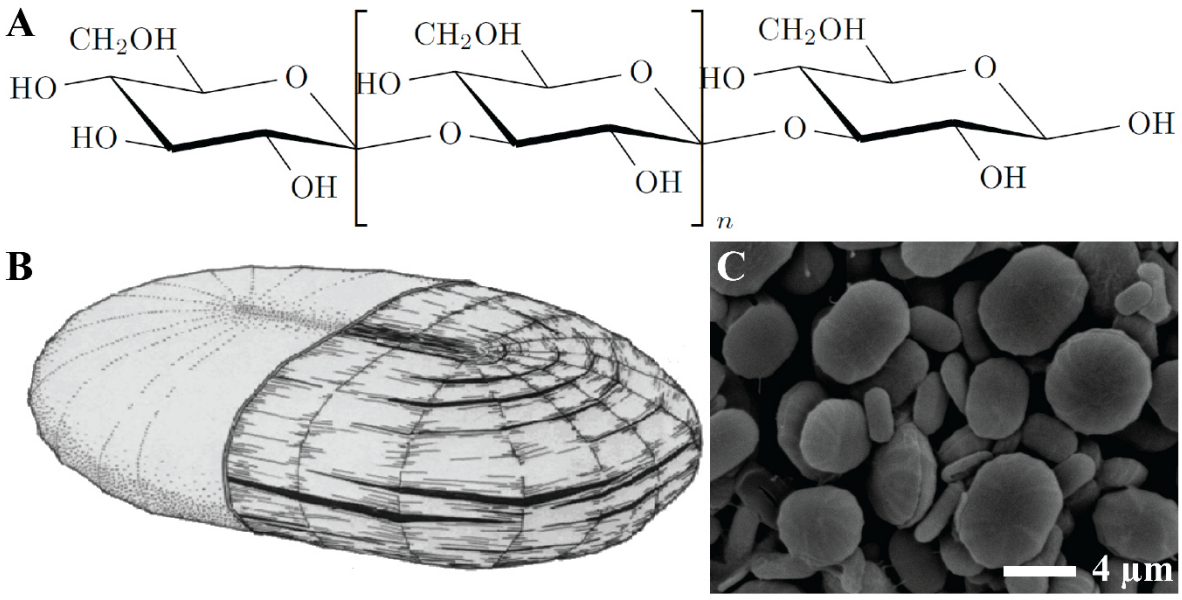

Fig. 5. Paramylom. A: molecular structure of a paramylon (i.e. β-1,3-glucan chain; ~700 ≤ *n* ≥ ~3000). B: schematics of a paramylon granule (note that the biomembrane of the granule is only partly shown), from Kiss *et al.*, Structure of the euglenoid storage carbohydrate, paramylon, *American Journal of Botany* © 1987 by John Wiley Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc [83]. C: microscopic image of paramylon granules (ellipses and rods) without biomembrane.

E. gracilis accumulates paramylon under PT, MT and HT growth [54]. Cultivation under strict HT conditions leads to increased paramylon levels during the exponential growth phase, while light has been shown to be detrimental to the accumulation and conservation of paramylon under MT growth, probably because the metabolic switch to paramylon degradation is under the influence of a photoreceptor [12, 13]. One of the highest paramylon yields (16 g/L) reported in the literature so far was obtained in a repeated-batch cultivation under HT conditions under dark conditions using a medium supplemented with potato liquor, vitamins and a high concentration of glucose (30 g/L) [84]. Figure 6 shows an example of *E. gracilis* cultivated for several days under different growth conditions and their influence on the final paramylon yield [54].

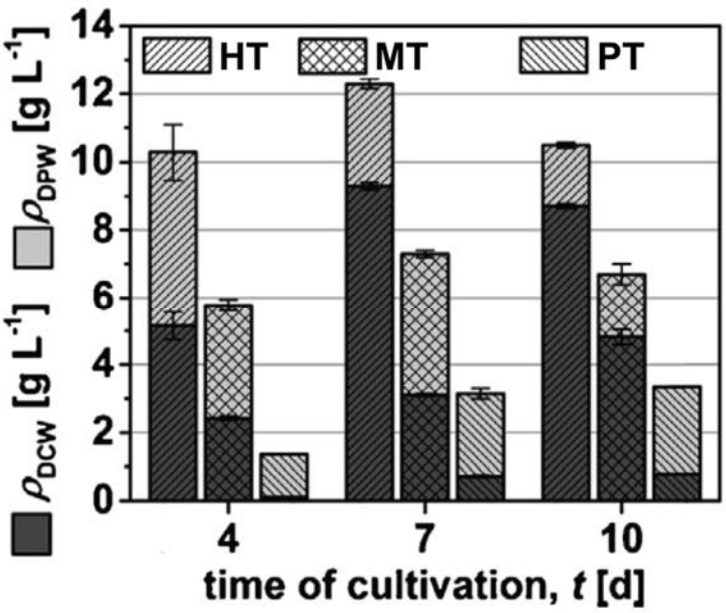

Fig. 6. Cultivation of *E. gracilis* under HT, MT and PT conditions over 10 d. The biomass concentration (ρ_{DCW}) and the paramylon concentration (ρ_{DPW}) of a culture (i.e. adding up to the DW) at each time point are indicated. The error bars represent the standard deviation (n = 3). Reprinted (slightly modified) from the *Journal of Biotechnology* 215:72-79, Grimm *et al.*, Applicability of *Euglena gracilis* for biorefineries demonstrated by the production of α-tocopherol and paramylon followed by anaerobic digestion © 2015 with permission from Elsevier [54].

3.2.2. Industrial applications

Various industrial applications have been postulated for paramylon including their use as substrates for thermoplasticisation by introduction of acyl groups with different alkyl chain length as an alternative to petroleum-based resins [85]. Another example is the production of self-

assembling β-1,3-glucan nanofibres derived from paramylon [86]. The nanofibres can be surface-modified with functional groups (e.g. carboxylic acid) and could serve as stimuli-responsive polymers or drug delivery systems [87].

Microalgal biomass is considered a third-generation biofuel, not only because of its potentially high lipid content but also because of the high amounts of complex carbohydrates (e.g. cellulose) that can be produced by many algal species [88]. These complex carbohydrates can be hydrolysed chemically or enzymatically into their constituent monosaccharide units (e.g. glucose) for subsequent fermentation to bioethanol [89, 90]. Enzymatic hydrolysis is preferable because harsh chemical conditions can interfere with downstream fermentation processes [91]. The same hydrolysis and conversion process could be applied to paramylon. However, paramylon granules were shown to be recalcitrant to enzymatic degradation and it is very likely that a consortium of different enzymes is required for the efficient degradation of this polysaccharide [92]. So far, only two enzymes of the degradation pathway of paramylon have been characterised, an endo-β-1,3-glucanase and a β- laminaribiose phosphorylase (LBP)/1,3-glucan phosphorylase (β-1,3-GP) [29, 93, 94]. A β-1,3-GP isozyme was recombinantly-produced in *E. coli* and characterised recently by Kuhaudomlarp *et al.*, resulting in the creation of a new glycoside hydrolase (GH) family, namely GH149 [29, 93-95]. So far, this is the only characterised enzyme in this new family and the only eukaryotic representative among 87 inferred sequences from bacterial origin [29, 93, 94]. A putative pathway for the complete hydrolysis of the polysaccharide paramylon to its glucose subunits is shown in Figure 7. Enzymes from other organisms, mostly from the fungi *Trichoderma* spp. and *Aspergillus* spp., have been explored as alternative sources for paramylon-degrading enzymes. For example, a fractionation of enzymes secreted by *Trichoderma harzianum* Rifai PAMB-86 led to the enrichment of β-1,3-glucanases able to degrade paramylon to some degree, yielding mostly glucose [92, 96].

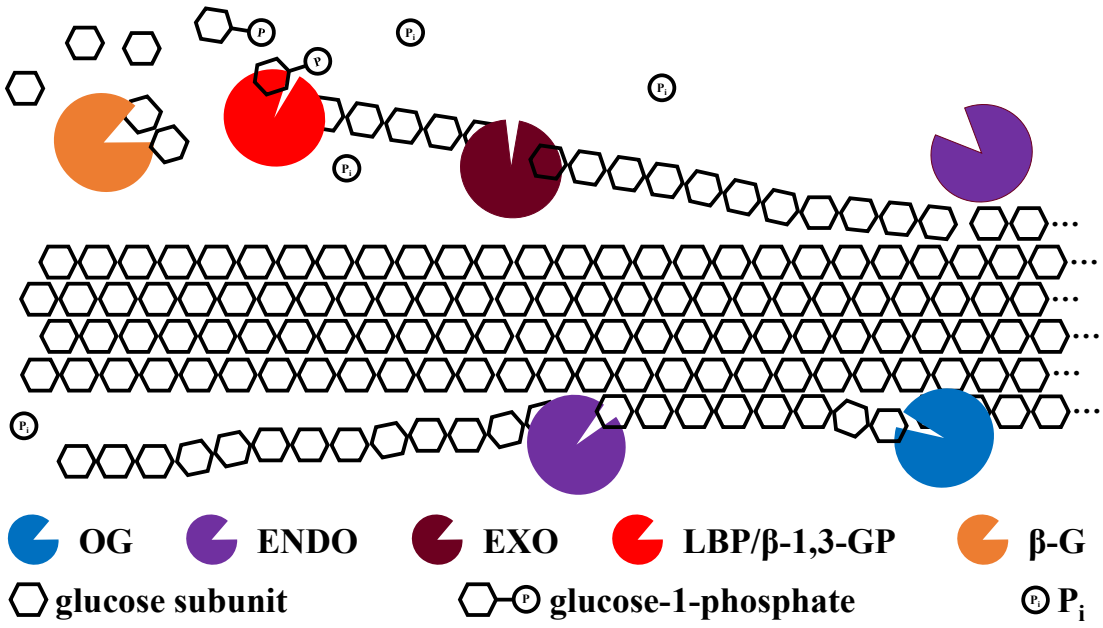

Fig. 7. Putative paramylon degradation pathway in *E. gracilis*. Enzymes involved, substrates and products are indicated. Oxidative glucanases (OG): cleavage of crystalline paramylon to make it accessible for other enzymes, possibly similar to oxidative cleavage of cellulose. [97]. Hydrolytic endo-β-1,3-glucanases (ENDO): random cleavage of the polysaccharide chain [29]. Hydrolytic exo-β-1,3-glucanases (EXO) and β-glucosidases (β-G): oligoglucans and single glucose units are cleaved off, respectively, at the ends of accessible and freed polysaccharide chains, comparable to the cellulolytic enzymes from *Trichoderma reesei* [98-100]. LBP/β-1,3-GP: cleaving of laminaribiose/laminarioligosaccharides into glucose/laminarioliosaccharides and glucose-1-phosphate under consumption of free inorganic phosphate (P_i) [93, 101].

Biogas and bio-oil also can be produced from microalgal carbohydrates by thermochemical conversion processes like gasification at high temperatures or pyrolysis in the absence of oxygen, respectively [102]. Another way to produce biogas consisting mostly of methane from microalgal biomass is through anaerobic digestion by a consortium of microorganisms. *E. gracilis* cell mass has been shown to be a suitable feedstock for production of biogas by anaerobic digestion, producing around 650 or 800 mL biogas/g DW, depending on the cultivation condition (PT or HT, respectively) [54]. These yields were higher than those from microalgae such as *Chaetomorpha litorea* (80 mL/g DW), *Chlamydomonas reinhardtii* (590 mL/g DW), *Durvillaea Antarctica* (180 mL/g DW), *Macrocystis pyrifera* (180 mL/g DW) and *Scenedesmus obliquus* (290 mL/g DW) [102].

Considering the commercial viability of the algal bioproducts, the lower market prices for microalgae-derived biofuel commodities are not making them an economically feasible target for

production at this time. On the other hand, microalgal products for food, health and personal care are generating a profit margin at 50-100 times greater than that of biofuels, so it may be a viable strategy to produce the high-value products with the biofuel commodities as a co-product [103].

3.2.3. Bioactivity and related applications

3.2.3.1. Health benefits

There are several health benefits associated with β-glucans including immunostimulatory and antioxidant effects [17]. They also act as dietary fibre and have been shown to lower blood cholesterol levels [104]. Mouse and rat studies as well as experiments with different mammalian cell lines have been performed to determine the potential impact of the β-1,3-glucan paramylon on human health . For example, mice fed with 2% (w/w) paramylon and challenged with the (human) influenza virus A/PR/8/34 (H1N1) showed higher survival rates and cytokine levels (IFN-γ IL-1β, IL-6, IL-10 and IL-12) compared to the control group in concurrence with lower virus titres, suggesting that paramylon served as an effective regulator of the immune response providing protection against the virus [105]. In a similar study, mice were fed paramylon at lower than 1% (w/w) of their diet and challenged with a potentially lethal dose of *E. coli*. The survival rate of paramylon-fed mice and of the control group were 70% and 0%, respectively, and the immune response (antibody titres, IL-2 production, natural killer cell cytotoxicity, and phagocytosis activity) was significantly increased in the group fed with paramylon. The performance of paramylon in this study was as effective as or more effective than two commercially-available β-glucan products for animal feed derived from yeast [106].

There are effects attributed to paramylon beyond the immune response to pathogens. For example, mice treated with 2,4,6-trinitrochlorobenzene, which would usually induced atopic dermatitis (AD)-like skin lesions, were fed with paramylon at 1% (w/w) of their diet. Paramylon inhibited the development of AD-like skin lesions, reduced cytokine (IFN-γ, IL-4, IL-12 and IL-18) levels

and dermatitis scores. Paramylon may be a suppressant of the T helper cells (Th) type 1 and type 2 responses and could be used as a potential therapy for AD [107]. Another indicator that paramylon has regulatory effects on Th cells was a study on a collagen-induced arthritis mouse model for rheumatoid arthritis, where 2% (w/w) paramylon in the diet relieved arthritis symptoms and decreased cytokine (IFN-γ, IL-6 and IL-17) levels. A possible involvement of Th type 17 cells was suggested by the authors [108]. Furthermore, paramylon can also act as a potent antioxidant protecting mice from acute hepatic injury induced by CCl_4 treatment [109].

Paramylon also has been shown to reduce the risk of cancer formation. For example, preneoplastic aberrant crypt foci (i.e. marker for colon cancer risk) were induced in the colon of mice by 1,2-dimethylhydrazine treatment [110, 111]. Subsequent inclusion of 2% (w/w) paramylon in the diet reduced the development of colon cancer by 50%. The mechanisms responsible for reduction are still unclear but might be related to an effect of paramylon on the gut microbiome [111].

The research on the health benefits of paramylon has focussed mainly on applications to treat human conditions but it has been suggested that paramylon could also be beneficial to livestock and food fish health. Studies undertaken with porcine leucocytes, rainbow trout and red drum have shown that paramylon incubation or feeding of paramylon as part of the normal diet, results in immunostimulatory and/or -regulatory effects on the cells and on the animals [112-114]. However, more studies would be needed to elucidate the nature of the observed effects and to confirm actual benefits for the animals (e.g. increased survival rates).

3.2.3.2. Activation of paramylon

Chemical derivatives of paramylon (activated paramylon) were shown to exhibit augmented or novel bioactivities. For example, the antimicrobial activity of paramylon was enhanced chemically by introducing charged groups (e.g. 2-hydroxy-3-trimethylammoniopropyl,

N,N-diethylaminoethyl and N,N-dimethylaminoethyl groups) and sulphated paramylon was shown to exhibit anti-HIV activity when the substitution ratio of the sulphate groups was increased to over 4% [80, 115]. Choi *et al.* showed that paramylon conjugated with hyaluronic acid promotes wound healing in rats to a higher degree than native paramylon. As a result, corneal epithelial cell migration was increased and the acute inflammatory reaction caused by corneal alkali burns *in vivo* was supressed by paramylon (native and activated) [116]. There are also strong indications that pretreatment of paramylon with sodium hydroxide enhances the stimulation of leucocytes, probably because the β-1,3-glucan triple-helices of paramylon are disrupted in the process and the apparently more bioactive single helices are thereby exposed [117]. Accordingly, alkalised paramylon was shown to increase proinflammatory factors (COX-2, IL-6, NO, TNF-α and translocation of NF-κB) in human lymphomonocytes at a higher rate than the commercial β-glucan product MacroGuard derived from *S. cerevisiae* treated similarly [16].

A so far neglected approach for the activation of paramylon would be its hydrolysis to soluble shorter-chain β-1,3-glucans, as these soluble compounds might show an increased blood plasma availability and a stronger or different immune response compared to an insoluble preparation, thereby maximising the bioactive potential of paramylon [118]. The *in vitro* synthesis of soluble β-1,3-glucans could be an alternative to the hydrolysis approach. However, the current chemical synthesis of oligosaccharides is still laborious and product yields are very low despite some advances in the field, whereas enzymatic synthesis has been considered as a technically viable option [119, 120]. Towards this end, a β-1,3-glycosyl transferase (paramylon synthase) complex was purified from *E. gracilis* and characterised. The enzyme produced β-1,3-glucan from UDP-D-glucose. However, the chain lengths of the products were not determined [82]. An alternative way of producing short-chain β-1,3-glucans is to reverse the equilibrium reaction of the LBP/β-1,3-GP as described in Figure 7, which is extending β-1,3-glucans from glucose by addition of glucose-1-phosphate to the reaction [93, 121]. The addition of other enzymes including sucrose

phosphorylase (SP) to keep P_i levels constant has been shown to make the synthesis reaction more feasible [120]. In a further development of this system, the reusability of LBP/β-1,3-GP was successfully demonstrated by its immobilisation *via* enzyme carriers and the cross-linking of enzyme aggregates. However, the product (laminaribiose) yield of the immobilisation approach in combination with the SP enzyme was just 20% [122].

3.2.3.3. Commercial potential

Despite substantial evidence for the health-enhancing bioactivities of paramylon and paramylon-derived compounds as outlined above, we are not aware of any clinical studies confirming these claims. By definition, paramylon and related compounds can only be considered 'nutraceuticals' and not 'pharmaceuticals', which has been restricting them to be used only as food supplements [123]. Currently, the health-enhancing properties of *E. gracilis* are being exploited by several new or already established companies in Japan and the United States, selling whole cell meal or paramylon for human consumption [103].

4. Large-scale cultivation

4.1. From laboratory- to industrial-scale cultivation

A prerequisite for the industrial application of microalgae is the availability of a large-scale cultivation system to produce an adequate amount of biomass [78]. The results of laboratory-scale experiments assessing *E. gracilis* biomass yields from PT, MT and HT cultivations can be used as an indicator for a potential scale-up of the processes. HT cultivation with glucose as carbon source in shake flasks usually reaches DW yields of around 12 g/L culture, whereas the repeated-batch cultivation supplemented with potato liquor and glucose performed by Šantek *et al.* (see above) in a bioreactor with 5 L working volume was shown to reach DW concentrations around 23 g/L [24, 54, 84]. One of the highest *E. gracilis* biomass yields of around 48 g DW/L was obtained in a fed-batch HT cultivation strictly in the dark, using a jar fermenter (2 L working volume) with glucose

concentrations kept constantly above 1 g/L [51]. However, other microalgal species, including *Chlorella vulgaris*, *Crypthecodinium cohnii* and *Galdieria sulphuraria*, have been shown under HT cultivation to reach almost twice the DW of *E. gracilis* [124]. Accordingly, the current *E. gracilis* HT cultivation process should be revised to improve its efficiency. MT cultures were shown to fall short of the yields of HT cultures in comparative experiments by around 30%, although it has been suggested that PT and HT metabolism could complement each other under MT conditions [24, 125]. PT cultivation produces approximately 20 times less biomass (2-3 g DW/L culture) compared to HT and MT cultivation. Attempts to enhance *E. gracilis* biomass yields under PT conditions, for example with the use of a photobioreactor, did not result in a significant improvement [24, 38, 54]. Nevertheless, these biomass yields are comparable to other microalgae grown under PT conditions and the Japan-based company euglena Co., Ltd. has implemented an open pond system for PT outdoor cultivation for the commercialisation of *E. gracilis* products [126, 127]. The ponds are around 30 m in diameter, 20 cm deep and stirred for aeration (Fig. 8) [127].

Fig. 8. Open pond cultivation of *E. gracilis* by euglena Co., Ltd. on Ishigaki Island (Japan). Reprinted (slightly modified) by permission from Suzuki: Springer Nature, *Euglena: Biochemistry, cell and molecular biology*, Suzuki, Large-scale cultivation of *Euglena* © 2017 [127].

Open ponds are low-tech solutions and potentially cheaper when compared to technically more demanding systems like raceway tanks and thin-layer cascades, which in turn promise higher yields (Fig. 9) [126]. Experiments by euglena Co., Ltd. with a raceway in a laboratory set-up

mimicking local conditions resulted in yields close to calculated theoretical values. However, the actual yields outdoors turned out to be much lower. The factors that are responsible for the discrepancy have not been reported. There are several prerequisites for outdoor cultivation, including the availability of at least 3000 ha of usable land area, 3 h of average sunlight per day, 500 mm annual rainfall and a tropical/dry/temperate climate, yet it is expected that this system would be easily implementable in countries outside of Japan, for example Australia, Brazil, Malaysia, the USA, Thailand and Vietnam [127].

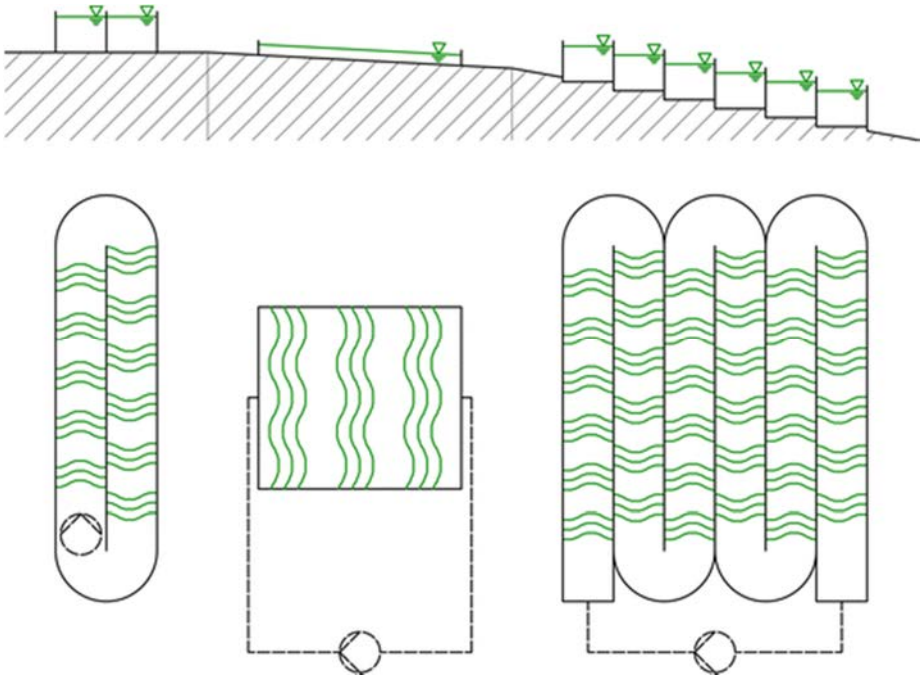

Fig. 9. Schematic drawings of common outdoor systems for the PT cultivation of microalgae (top: central cross section; bottom: plan view). Left: raceway tank (designed for level ground). Centre: thin-layer cascade (for slopes of ca. 1-3%). Right: sloped channel (for slopes of ca. 1-10 % and higher). Dashed lines pumps and pumping circuits. The wavy lines are pointing in the flow direction. Vertical scale exaggerated. Reprinted by permission from Apel and Weuster-Botz: Springer Nature, *Bioprocess and Biosystems Engineering*, Apel and Weuster-Botz, Engineering solutions for open microalgae mass cultivation and realistic indoor simulation of outdoor environments © 2015 [126]

4.2. Challenges and possible solutions

Reports on industrial-scale HT cultivation of *E. gracilis* have been only conceptual so far. For example, Levine *et al.* have proposed a multi-stage process where smaller bioreactors generate the inocula for the bigger stages (up to 1000,000 L) with the aim to increase biomass yields to 120 g/L

or higher [106]. This biomass yield would be comparable to or higher than those obtained with other microalgae grown under HT conditions [124]. However, there is no further information if the proposed system was ever built and tested. It is likely that certain disadvantages of HT cultivation prevented its realisation, such as the cost of the medium containing an organic carbon source like glucose [3]. The proposed medium would likely have to be sterilised and the cultivation system kept closed to avoid fast-growing contamination, increasing the overall cost of the process [127]. The use of wastewater or nutrition-rich waste products from the food industry like potato liquor (see above) or dairy effluent could lower the costs by replacing or supplementing the HT growth medium with the additional benefit of bioremediating these effluents [7, 84, 128, 129].

PT cultivation on the other hand has already been shown to be a feasible alternative to HT cultivation as described above. However, it is restricted by low yields, even after technical optimisation of the cultivation process. An alternative solution to this problem could be the genetic modification (GM) of *E. gracilis* to improve biomass and/or bioproduct yields. For example, expression of the *Synechococcus elongatus* PCC 7942 fructose-1,6-/sedoheptulose-1,7-bisphosphatase gene in *E. gracilis* chloroplasts was shown to enhance the photosynthetic activity significantly as well as the paramylon yield (almost by 2-fold), the WE yield under anaerobic conditions (around 100-fold) and the biomass yield (around 2-fold) [21]. However, local legal regulations could prevent (outdoor) cultivation of GM *E. gracilis* strains [130]. Alternatively, conventional mutagenesis could be used to generate 'natural' mutant strains with desired traits, a process which is generally not considered GM. Several reports have described the successful mutagenesis (e.g. by irradiation) of *E. gracilis* [76, 127, 131].

Another potential challenge for the economically-feasible industrial production of *E. gracilis* biomass is vitamin auxotrophy. Vitamin-dependence is common amongst microalgae but hardly discussed in the context of the scale-up of *E. gracilis* cultivation [132]. *E. gracilis* shows absolute

requirement for vitamins B_1 (thiamine) and B_{12} (cobalamin), so complex *E. gracilis* media usually contain at least 50 ng/L and 10 ng/L, respectively, of these vitamins as lower concentrations would hamper the cell growth [24, 133-135]. Accordingly, the supplementation of both vitamins, produced either by chemical synthesis (thiamine) or extracted from bacterial sources (cobalamin), would be a factor in a scaled-up *E. gracilis* cultivation and contributing to the overall costs of the process [136, 137]. As a potentially cheaper alternative to direct supplementation, co-culturing of *E. gracilis* with bacteria producing thiamine and cobalamin or the GM of *E. gracilis* to introduce foreign bacterial pathways for these vitamins could eliminate or reduce the need for their external supplementation [138]. Further research is needed to investigate these alternative approaches but in the meantime, vitamin-rich food waste like thiamine-fortified bread could be explored as a source of vitamins [139].

5. Conclusions

The biotechnological application of *E. gracilis* has been made possible through the extensive research on its versatile metabolism. There is an emerging market for *E. gracilis*-derived products, which is reflected in the recent founding of new companies specialised in the cultivation and commercialisation of *E. gracilis* as food supplements. PT cultivation of *E. gracilis* is currently favoured, probably due to lower cost and relative ease of set-up, but HT cultivation for higher yields of cell mass and paramylon could soon become reality. The nutritious whole cell meal and the nutraceutical paramylon are currently the only *E. gracilis* products that are commercially viable, while biofuel production from *E. gracilis* cell mass is in contrast not yet feasible. However, carbohydrate and lipid yields in *E. gracilis* can be improved by new cultivation technologies and metabolic engineering approaches, possibly providing an affordable alternative to fossil fuels in the near future.

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1.3. Aim of the study

The overarching aim of the present study was to facilitate the enzymatic hydrolysis of the recalcitrant substrate paramylon to soluble β-1,3-glucans for nutritional, biomedical or biotechnological applications.

More specific objectives of this study included:

1. To explore the cultivation of *E. gracilis* in an open pond setting as a low-cost option for the production of paramylon for subsequent experiments.
2. To investigate the use of microwave technology as a pretreatment step for paramylon and to develop an assay for the assessment of the efficacy of the pretreatment.
3. To combine physical (microwave) and enzymatic approaches to facilitate the production of soluble bioactive compounds from paramylon.
4. To assess the bioactive (i.e. immunostimulatory) effects of the obtained paramylon hydrolysis products.
5. To develop new strategies for the screening and identification of paramylon-degrading enzymes.

This thesis features work reported in one publication and two manuscripts which were prepared for submission to peer-reviewed scientific journals, including a review article (first manuscript) featured in **Chapter 1**.

Chapter 2 summarises the materials and methods used in this work with a reference to the chapter where the detailed description of each method can be found.

Chapter 3 covers the findings of the outdoor cultivation experiments.

The development of a microwave pretreatment method for paramylon and the enzymatic production of bioactive compounds are reported in **Chapter 4** (published article).

Chapter 5 (second manuscript) outlines strategies for the identification of enzymes able to degrade paramylon and presents specific new methods for this task.

Finally, a conclusive summary and future research directions are provided in **Chapter 6**.

Chapter 2:
Materials and methods

Materials and methods used in this work are summarised in Table 2.1. Detailed descriptions for each method can be found in the corresponding chapter.

Table 2.1. A list of the materials and methods used during this work.

Materials and methods	**Refer to Chapter**
E. gracilis strains, cultivation media and cultivation conditions	3,5
Determination of *E. gracilis* dry weight and paramylon content	3,5
Determination of pH	3,5
Determination of light intensity	3
Biofilm assay	3
Statistical analysis	3, 4 ,5
Differential scanning calorimetry (DSC) of paramylon	4
Microwave pretreatment of paramylon	4
Congo Red dye binding assay	4
Scanning electron microscopy (SEM) of paramylon	4
Enzymatic hydrolysis of paramylon	4
Clean-up of enzymatic hydrolysis products	4
Direct acid hydrolysis of paramylon	4
Carbohydrate content quantification	4

Table 2.1. Continued.

Materials and methods	**Refer to Chapter**
High performance liquid chromatography (HPLC)	4
Cell culture	4
Immunofluorescence, microscopy and image analysis	4
Harvest and cell lysis of *E. gracilis*	5
Preparation of an *E. gracilis* cDNA expression library	5
Flow cytometry screening/ fluorescence-activated cell sorting (FACS)	5
Protein analysis	5
Preparation of dispersed paramylon	5
Reducing sugar assay	5
Separation of proteins by liquid chromatography	5
Standard binding assay (SBA)	5
Isolation of paramylon granules membrane	5
Database searches	5
DNA sequence design, cloning and heterologous expression	5
Congo Red dye plate assay	5

Chapter 3:
Assessment of open pond cultivation and biofilm formation of *Euglena gracilis*

Scaling up the *E. gracilis* cultivation process from the laboratory to full industrial scale is a prerequisite for the commercial production of *E. gracilis* cell mass and paramylon. While challenging, large-scale outdoor cultivation of *E. gracilis* has been established by a Japan-based company (http://www.euglena.jp) as a more economical alternative to the cultivation in closed bioreactors. Open pond (outdoor) cultivation of two strains, *E. gracilis* Z (wild type) and *E. gracilis* var. *saccharophila* (sugar-loving variant) was attempted and assessed in this chapter in regards to biomass and paramylon production. The purpose was to establish whether there is potential for paramylon to be produced in technically simple open pond cultivation as feedstock for the enzymatic production of high-value soluble β-1,3-glucans. This is a non-publishable chapter as it only shows preliminary experiments. This PhD project was part of the ARC Training Centre for Molecular Technology in the Food Industry and a preliminary open pond cultivation investigation was directly requested by the industry partner Agritechnology Pty Ltd. The industry partner wanted to test the use of open pond cultivation without aeration and stirring as a low-tech and low-cost set up for cultivation of *Euglena gracilis*.

3.1. Introduction

E. gracilis is a uniflagellated microalga that is ubiquitous in most freshwater biotopes [1]. It is free-living, motile and phototactic but also capable of producing biofilms. *Euglena* spp. have a natural role in the bioremediation of polluted water, where it removes nitrogen, organic carbon and phosphates [2-4]. *E. gracilis* has a mixotrophic (MT) metabolism in which the photoautotrophic (PT) and the heterotrophic (HT) metabolism complement each other. This metabolic flexibility provides *E. gracilis* with an advantage over other organisms with more limited metabolic capabilities [5]. In addition, *E. gracilis* is able to endure a number of environmental stresses including ionizing radiation and acidic growth conditions [6, 7]. The resilience and metabolic flexibility of *E. gracilis* makes this organism a perfect candidate for outdoor cultivation. Outdoor cultivation of microalgae in open systems, especially if sunlight can

be used as energy source, is considered to be more favourable from an economical perspective as opposed to the cultivation in closed bioreactors. There are technically sophisticated outdoor cultivation systems including thin-layer cascades with a constant flow but also low-tech solutions such as open pond cultivation without agitation [8].

The formation of biofilms during the cultivation of microalgae in bioreactors has been considered as having either a detrimental effect to optimal growth (e.g. because of unwanted shading effects) or a desired effect (e.g. for enhanced biomass accumulation). Thus, biofilm formation is an important factor to consider when it comes to commercial cultivation of microalgae [9, 10]. However, little is known about the natural prevalence of *E. gracilis* to form biofilms besides that it exhibits biofilm formation as a natural reaction to copper-stress [2].

E. gracilis cells as a whole are an excellent source for vitamin E and amino acids [11]. The cells also produce a storage polysaccharide unique to *Euglena* spp. called paramylon, which can make up to 95% of the cell mass. Paramylon is a β-1,3-glucan which has been claimed to have immunostimulatory and other health-enhancing properties [12, 13]. The biomass of *E. gracilis* with its high carbohydrate content is also an ideal feedstock for the production of third-generation biofuels [14]. Furthermore, *E. gracilis* produces lipids under anaerobic conditions that represent a viable source for making biodiesel [15].

The *E. gracilis* strain var. *saccharophila* excels at accumulating paramylon compared to the wild type (WT) strain Z when cultivated under HT growth conditions [16, 17]. Therefore, *E. gracilis* var. *saccharophila* could be a potential candidate for the production of paramylon on an industrial scale. Companies worldwide like Algeon in the United States or the Japan-based euglena Co., Ltd have recognised the commercial potential of *E. gracilis* and have turned whole cell preparations

and paramylon into consumer products. Euglena Co., Ltd is already cultivating *E. gracilis* on a commercial scale in an open pond outdoor cultivation system [18, 19].

Preliminary cultivation experiments were performed with *E. gracilis* under PT and MT growth to assess the viability of open pond cultivation (without agitation) and to explore the biofilm formation for PT and MT growth under laboratory and open pond conditions. The cultivation of *E. gracilis* in open ponds was expected to facilitate the production of paramylon under low-cost conditions for the experiments presented in the following chapters and to provide initial data as the first step towards a commercial production of *E. gracilis* using this cultivation system.

3.2. Materials and methods

3.2.1. Strains

E. gracilis Z (WT, strain 25 in the original publication) was obtained from Southern Biological Pty Ltd (Australia) [16]. *E. gracilis* var. *saccharophila* (UTEX 752) was obtained from the University of Texas Culture Collection (Austin, USA).

3.2.2. Chemicals and cultivation media

Chemicals and growth media components were purchased from Sigma-Aldrich (St. Louis, USA). The cultivation medium for PT growth was prepared following a modified protocol (https://utex.org/products/euglena-medium). It contained 0.1% (w/v) sodium acetate, 0.1% (w/v) peptone, 0.2% (w/v) tryptone, 0.2% (w/v) yeast extract, and 0.001% (w/v) calcium chloride dihydrate; the pH was adjusted to 3.5 before autoclaving. The basal medium for MT growth was prepared following a protocol described previously [11]. The basal medium pH 3.5 (adjusted with HCl) consisted of 0.02% (w/v) $CaCO_3$, 0.02 (w/v) KH_2PO_4, 0.04 (w/v) $(NH_4)_2HPO_4$, 0.181 (w/v) NH_4Cl, 0.05 (w/v) $MgSO_4$, 1% (w/v) yeast extract, 0.01 (v/v) Mineral Stock B and 0.02 (v/v) Mineral Stock A, all dissolved in 925 mL of ultrapure

water. Mineral Stock A consisted of 44 g/L $ZnSO_4 \cdot 7H_2O$, 30.4 g/L $MnSO_4 \cdot H_2O$, 10 g/L $Na_2MoO_4 \cdot 2H_2O$ and 0.8 g/L $CoCl_2 \cdot 6H_2O$. Mineral Stock B consisted of 0.78 g/L $CuSO_4.5H_2O$ and 0.57 g/L H_3BO_3. The autoclaved basal medium was then supplemented with 75 mL of sterile 20% (w/v) glucose. The MT medium was completed after cooling to ambient temperature by adding vitamins B_1 and B_{12} at final concentrations of 10 mg/L and 0.5 mg/L, respectively.

3.2.3. Open pond cultivation

Open pond cultivation was performed at the end of May at Macquarie University (Sydney, Australia). Each strain (*E. gracilis* WT and *E. gracilis* var. *saccharophila*) was cultivated as inocula before starting the growth experiments in medium for MT growth until the cell density plateaued. A volume of 450 mL of each medium in a rectangular vessel (tub) with a volume of 1 L was inoculated in triplicate with 50 mL of a culture of each *E. gracilis* strain for MT and PT cultivation (operational depth: less than 6 cm). The cultures were placed in a greenhouse kept a constant temperature of 23°C, receiving approximately 10 h of direct sunlight daily. The light intensity was reduced to around 60% by the wall material of the greenhouse. Samples of ca. 15 mL each were collected daily at around 12:00 noon.

3.2.4. Determination of dry weight, paramylon content, pH and light intensity

The dry weight of an *E. gracilis* culture was determined by sampling 3 mL in triplicate daily (see section 3.2.3.). The cells were centrifuged at 5000×g for 10 min at 4°C and the supernatants were discarded. The pellets were washed in ultrapure H_2O by centrifugation (5000×g for 10 min, the supernatants were discarded) transferred to pre-weighed aluminium dishes and dried at 70°C for 2 d before weighing (in g, with an accuracy of 4 digits after the decimal point).

The paramylon content of *E. gracilis* cultures was determined after isolating the paramylon granules from a sample following an established protocol (in short, by boiling cell pellets in 1% SDS) [11] in combination with an UV spectrometry assay [20]. In short, the total carbohydrate content was determined directly *via* UV spectroscopy after hydrolysis of the paramylon pellets dissolved in 1 M NaOH, using H_2SO_4. Samples were transferred in duplicate to a microtiter plate and absorbance values were measured at 315 nm. The values of solvent blanks were deducted. A calibration curve was prepared similarly using different concentrations of glucose and the total carbohydrate/paramylon content was expressed as glucose equivalents.

The pH of a sample was determined using a Eutech Instruments pH 510 pH meter (Thermo Fisher Scientific, Waltham, USA). The light intensity was recorded using a LI-250A light meter (LI-COR Biosciences, Lincoln, USA) with 3 individual readings at the time of sampling.

3.2.5. Biofilm assay

A protocol for the procedure [21] and the evaluation [22] of a (destructive) biofilm assay (originally for bacteria) was adapted and modified for the cultivation of *E. gracilis* (*E. gracilis* growth medium was used). In short, 200 µL of MT and PT cultures were transferred in triplicate into 96 well microtiter plates. The plates were incubated at 23°C for 3 d, either with lid under laboratory conditions, or uncovered under open pond conditions in the greenhouse. Wells containing 200 µL of the respective medium served as blank samples (controls). Biofilms were dried at 60°C for 1h and then stained for 15 min with 200 µl each of a 0.4% (w/v) crystal violet solution, washed with running ultrapure water, dried for 15 min at 60°C and the absorbance (A) of the subsequently solubilised stain (solvent: 200 µl each of a 7:3 ethanol-acetone solution) was measured at 570 nm to categorise possible biofilm formation. The degree of biofilm formation was determined as follows: $A \leq A_C$ = no biofilm formation, $A_C < A \leq (2 \times A_C)$ = weak biofilm formation, $(2 \times A_C) < A \leq (4 \times A_C)$ = moderate biofilm formation, $(4 \times A_C) < A$ = strong biofilm

formation. The cut-off A (A_C) was defined as three standard deviations above the mean A of the control.

3.2.6. Statistical analysis

The programs Excel (Microsoft Corporation, Redmond, USA) and Prism 7 (GraphPad Software, La Jolla, USA) were used for statistical analyses. Results of experiments subjected to statistical analyses were expressed as mean ± standard deviation (SD). Student's t-tests were performed to determine significant differences between two data sets as parametric, unpaired, two-tailed tests, with a confidence level of 95%.

3.3. Results and discussion

3.3.1. Growth curves

Cultivation of *E. gracilis* outdoors under local open pond conditions (i.e. in Sydney, Australia, 33°46'26.8"S 151°07'00.4"E, in autumn) was considered as a simple and potentially economical alternative to the use of more complex bioreactors. *E. gracilis* WT and *E. gracilis* var. *saccharophila* were cultivated under PT and MT growth conditions in open ponds (tubs) without any mechanical agitation (Fig. 3.1. A and B for the set-up). The cell mass expressed as dry weight and the paramylon content along with the pH of the cultures were determined over a period of 7 d (Fig. 3.2.).

During this experimental period, the cultures did not grow significantly or at all, indicating the infeasibility of the open pond set-up. The cell mass and the paramylon content of the cultures cultivated under MT growth conditions (Fig. 3.2. C and D) reached higher values in comparison to the PT cultures (Fig. 3.2. A and B). However, only a stagnation or a mere survival of the cultures rather than (significant) growth was observed overall. The pH decreased slightly over time, most prominently during the MT cultivation of *E. gracilis* var. *saccharophila*. A decrease of pH in *E.*

gracilis MT cultures cultivated under laboratory conditions has been commonly observed by us and could be interpreted as a sign of an active metabolism of *E. gracilis*. However, the metabolic activity was not reflected in growth of the cultures (e.g., significant increase in dry weight).

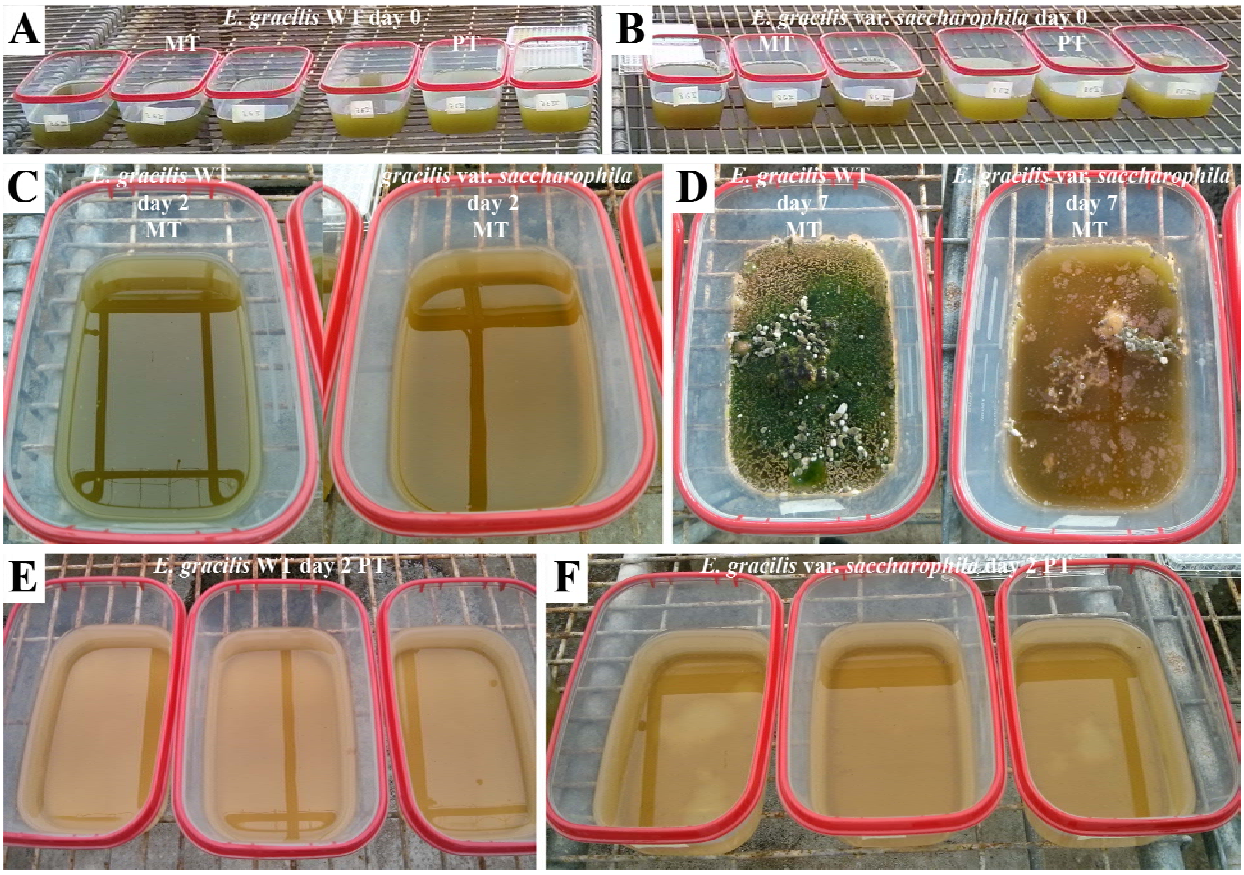

Fig. 3.1. Set-up of the open pond cultivation of *E. gracilis*. Cultures of *E. gracilis* WT (A, C and D: on the left, E) and *E. gracilis* var. *saccharophila* (B, C and D: on the right, F) in MT medium (A and B: triplicate on the left, C and D) and in PT medium (A and B: triplicate on the right, E and F) on day 0 (A and B), day 2 (C, E, F)) and day 7 (D).

Acid tolerance or acidification of the medium could be seen as a selective advantage of *E. gracilis* over other organisms and it has been suggested that the low pH of the media could prevent bacterial contamination [23]. Accordingly, bacterial cell growth was not observed over the course of the experiment. However, the low pH did not inhibit the growth of what seemed to be different kinds of moulds and filamentous fungi on the surface of the MT medium. Heavy contamination with foreign organisms was observed after 2 d of incubation and onwards (Fig. 3.1. C and D), which

were probably contributing to the growth curve measurements, and they were probably in direct competition with the *E. gracilis* cells for nutrients.

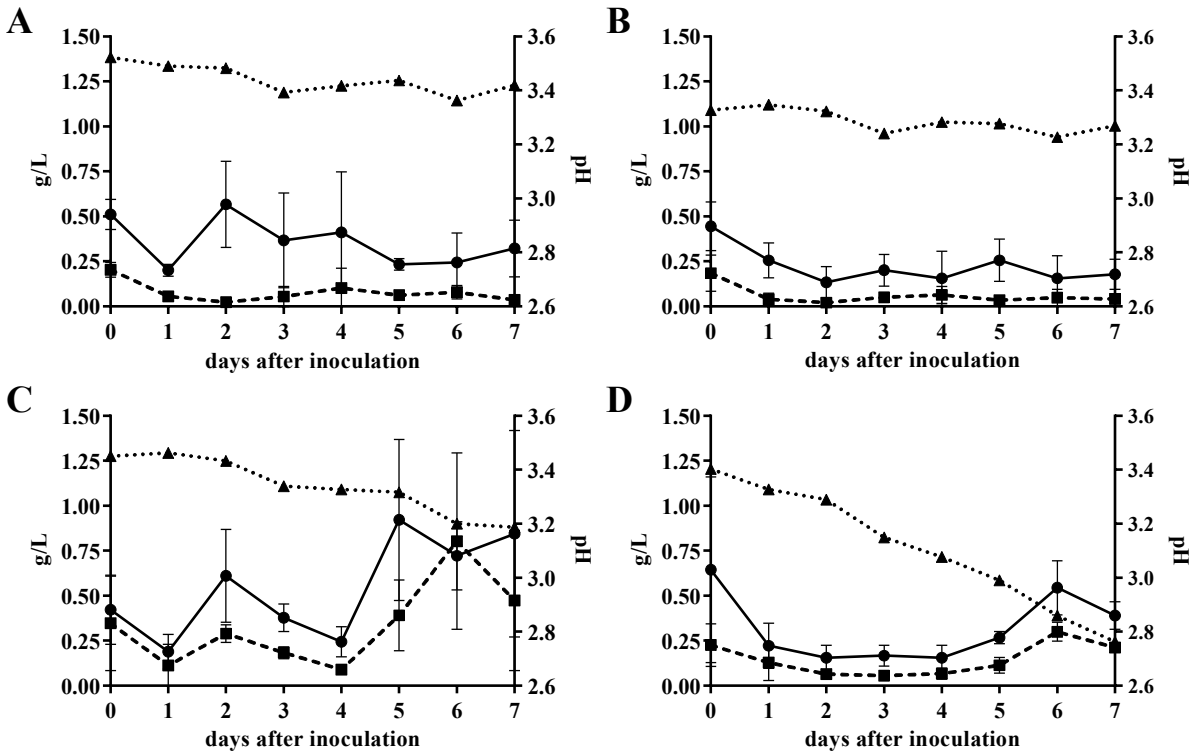

Fig. 3.2. Growth curves of the open pond cultivation of *E. gracilis*. *E. gracilis* WT (A and C) and *E. gracilis* var. *saccharophila* (B and D) under PT (A and B) and MT (C and D) growth. Error bars represent the SD. ●: dry weight, ■: paramylon, pH: ▲.

The PT cultures seemed free of contamination, most likely due to a lack of a carbon source. However, all PT cultures became bleached (Fig. 3.1. E and F). Exposure to UV light has been shown to cause *E. gracilis* cells to lose their chlorophyll and bleach [24] and could explain why these cultures did not grow, since PT cultures rely on their photosynthesis capability as a source of energy. The average light intensity at the time point of measurement (around mid-day) was 419 ± 177 µmol×m^{-2}×s^{-1} and this value was comparable to light intensities used in photobioreactors for the cultivation of *E. gracilis* [25]. Therefore, it was assumed that the light intensity in this study was suitable for growth and probably was not the cause for the observed bleaching. A more likely reason for the bleaching was probably an overheating of the cultures since an increase of the cultivation temperature to 33°C has been shown to cause a bleaching effect in *E. gracilis* [26]. The cultures in this study might have experienced temperature peaks around

mid-day through exposure to direct sunlight, even though the greenhouse they were cultivated in was temperature-controlled at 23°C. The MT cultures did not exhibit bleaching (Fig. 3.1. C and D) and maybe this was due to the availability of certain nutrients in the complex MT medium that prevented the bleaching event.

3.3.2. Biofilm formation

The degree of biofilm formation by *E. gracilis* WT and *E. gracilis* var. *saccharophila* could become a relevant factor in their commercial cultivation (e.g. in bioreactors) and has been neglected so far as a topic in basic and applied research of this organism. Thus, the two strains were grown in PT and MT media under laboratory and open pond cultivation conditions and a biofilm assay was conducted as described in Table 3.1. Controls containing only medium served as references and additional covered controls were set up for the open pond cultivation.

There was only a marginal difference between the absorbance values of the covered and uncovered controls. The classification of the biofilm would not have been affected by this difference and the influence of contamination was ruled out, also as contamination did not become apparent. As shown in Table 3.1, both strains formed a strong biofilm in medium for MT growth under laboratory and open pond cultivation conditions and a weak or moderate biofilm in medium for PT growth under both conditions. Overall, the cultivation conditions (i.e. outdoors/laboratory) did not influence biofilm formation or only slightly (*E. gracilis* WT in PT medium).

Biofilms are usually formed by secretion of extracellular polymeric substances and in the case of *E. gracilis* proposedly this substance is made up from glycoproteins, which are energy, carbon and nitrogen intensive in their synthesis [27]. The stark differences regarding the degree of biofilm formation between the cultivation media derived probably from a lack of readily available nutrients in the medium for PT growth.

Table 3.1. Biofilm assay of *E. gracilis* WT and *E. gracilis* var. *saccharophila* cultivated for 3 d. Cultivation conditions, the media used for growth and the degrees of biofilm formation are indicated for each strain.

	Laboratory		**Open pond**	
Strain	Medium	Biofilm	Medium	Biofilm
WT	PT	Weak	PT	Moderate
var. *saccharophila*	PT	Weak	PT	Weak
WT	MT	Strong	MT	Strong
var. *saccharophila*	MT	Strong	MT	Strong

3.4. Conclusions

Open pond cultivation without agitation under the low-tech and low-cost cultivation conditions (MT and PT) applied in this study as requested by the industry partner turned out to be an unfeasible method to produce *E. gracilis* cell mass and paramylon, because neither the cell mass nor the paramylon content of the cultures increased significantly during the course of cultivation. The observed heavy contamination of the MT cultures with foreign organisms and the bleaching of the PT cultures were most likely detrimental to the yields of *E. gracilis* cell mass and paramylon content. More complex set-ups (e.g. with mechanisms for cooling or stirring of the medium) or the use of different media could result in an improvement in biomass production (e.g. by providing all essential nutrients or by preventing detrimental factors like contamination and bleaching). However, guided by the results of the pilot study described in this chapter, the industry partner opted not to continue with this line of research.

To the best of my knowledge, the transfer of a biofilm assay originally designed for bacteria to *E. gracilis* it reported here for the first time. Using this method, it was possible to determine the degree of biofilm formation for *E. gracilis* WT and *E. gracilis* var. *saccharophila* under different experimental growth conditions. The formation of a biofilm by both strains was apparently only dependant on the availability of certain nutrients, as shown by the strong biofilm formation under MT cultivation conditions in comparison with the weak biofilm formed by the PT cultures. The biofilm assay could be used to assess other *E. gracilis* strains and screen for those with desired degree of biofilm formation for a specific application, for example a strain to be introduced in a biofilm membrane photobioreactor for the remediation of sewage treatment effluents [3, 28]. Using this assay, it also could be determined how different culturing conditions and the composition of different cultivation media influence the biofilm formation of *E. gracilis*.

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Chapter 4:
Microwave pretreatment of paramylon enhances the enzymatic production of soluble β-1,3-glucans with immunostimulatory activity

4.1. Introduction

Paramylon granules have been shown to be extremely resistant to enzymatic hydrolysis and would thus require pretreatment to make them amenable for degradation. Chemical pretreatments of paramylon have been used to improve the efficiency of paramylon-degrading enzymes but the recovery or neutralisation of harsh chemicals is problematic. Studies on other complex carbohydrates like cellulose have shown that hydrothermal pretreatments can be used to facilitate the enzymatic degradation of crystalline polysaccharides. Accordingly, a hydrothermal microwave pretreatment of paramylon was investigated as an alternative to the use of harsh chemicals, aiming to enhance the enzymatic degradation of paramylon to shorter-chain bioactive (immunostimulatory) soluble β-1,3-glucans. A new dye-based assay to measure the efficacy of the pretreatment was also developed and assessed. Two commercially-sourced enzymes with potential hydrolytic activity against paramylon, a *Helix pomatia* endo-β-1,3-glucanase and an *Arthrobacter luteus* exo-β-1,3-glucanase, were chosen for the enzymatic hydrolysis of the microwave-pretreated paramylon to yield soluble degradation products. The characteristics and the immunostimulatory effect of each hydrolysate produced *via* this approach was investigated and compared to hydrolysates obtained from direct acid hydrolysis of paramylon granules.

The results of this work were published in the peer-reviewed journal *Carbohydrate Polymers*.

4.2. Contribution to publication 1

The concept for this manuscript was developed in partnership with my supervisors Anwar Sunna and Helena Nevalainen, with Andrew Care and with the representative of the industry partner for this project (Agritechnology Pty Ltd) Graham Hobba. The experiments were designed by me with involvement of my supervisors. All experiments were performed and analysed by me, except for the cell culture and immunofluorescence experiments, which were performed and analysed by the collaborators Lindsay Marie Parker and Sameera Iqbal (Macquarie University). The manuscript was prepared by me and was reviewed by all the co-authors before submission to the journal. Detailed contribution of all authors is presented in Table 4.1.

Table 4.1. Author contribution summary for publication 1. Alexander Gissibl (AG), Andrew Care (AC), Lindsay Marie Parker (LMP), Sameera Iqbal (SI), Graham Hobba (GH), Helena Nevalainen (HN) and Anwar Sunna (AS).

	AG	AC	LMP	SI	GH	HN	AS
Conception	•	•			•	•	•
Experiment design	•	•				•	•
Data collection	•		•	•			
Data analysis	•		•				
Manuscript	•	•	•		•	•	•

4.3. Publication 1

Carbohydrate Polymers 196 (2018) 339–347

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Microwave pretreatment of paramylon enhances the enzymatic production of soluble β-1,3-glucans with immunostimulatory activity

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ABSTRACT

A hydrothermal microwave pretreatment was established to facilitate the enzymatic production of soluble bioactive β-1,3-glucans from the recalcitrant substrate paramylon. The efficacy of this pretreatment was monitored with a newly developed direct Congo Red dye-based assay over a range of temperatures. Microwave pretreatment at 170 °C for 2 min resulted in a significantly enhanced enzymatic hydrolysis of paramylon. The action of *endo*-β-1,3- and *exo*- β-1,3-glucanases on the microwave-pretreated paramylon produced soluble β-1,3-glucans with degrees of polymerisation (DP) ranging from 2–59 and 2–7, respectively. In comparison, acid-mediated hydrolysis of untreated paramylon resulted in β-1,3-glucans with a DP range of 2–38. The hydrolysates were assayed on their immunostimulatory effect on murine macrophages by measuring the production of the inflammation-linked marker tumour necrosis factor alpha (TNFα) using immunofluorescence. All of the tested hydrolysis products were shown to induce TNFα production, with the most significant immunostimulatory effect observed with the hydrolysate from the *exo*-β-1,3-glucanase treatment.

1. Introduction

Paramylon is the storage polysaccharide of the flagellated protist *Euglena gracilis*. It is a polymer with a high molecular weight (M_W) consisting of β-1,3-D-glucan units deposited as granules in the cytoplasm of the organism. Paramylon and other high-M_W β-1,3-glucans have been reported to display different types of bioactivity, including anticholesterol, -diabetic, -hypoglycaemic, -inflammatory, -microbial, -tumour, -viral as well as hepatoprotective and immunostimulatory activity in mammals, making them ideal candidates for nutraceuticals or adjuvants (Barsanti, Passarelli, Evangelista, Frassanito, & Gualtieri, 2011; Nakashima et al., 2017).

It is thought that high-M_W β-glucans can illicit immune responses in mammals that are comparable to those observed with β-glucans derived from the cell wall components of fungi and bacteria. Typically, high-M_W β-glucans are taken up by macrophages, degraded intracellularly and then released as short β-glucan chains to the extracellular milieu. These short β-glucans in turn stimulate macrophages and other leucocytes thereby triggering a broad immune response. One of the cytokines produced by β-glucan-stimulated macrophages is tumour necrosis factor alpha (TNFα), which has important functions in cell homeostasis and inflammation (Chan, Chan, & Sze, 2009; Kalliolias & Ivashkiv, 2016). Consequently, TNFα has been used as a target protein for assessing the immunostimulatory activity of β-1,3-glucan preparations (Miyanishi, Iwamoto, Watanabe, & Odaz, 2003).

Newly-developed drugs often fail to enter the market due to their poor bioavailability, mainly because of solubility issues (Kalepu & Nekkanti, 2015). Orally-administered, soluble β-1,3-glucans have been shown to have higher blood plasma availability and to trigger also a different immune response when compared to insoluble preparations (Rice et al., 2005). Hence, the bioavailability and bioactivity of the β-1,3-glucan paramylon may be altered or enhanced by its solubilisation.

Conventionally, strong acids are used to digest insoluble high-M_W β-glucans into soluble shorter glucan chains. However, this approach usually requires neutralisation of the acid reaction with strong bases which results in the contamination of the end-product with high amounts of salt. Other disadvantages of using acid for the hydrolysis of carbohydrate polymers include: difficulty to control the final chain

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length of the products, requirement for high temperatures and potential side-reactions that affect the purity of the hydrolysate (Heimlich & Martin, 1960; Lee et al., 2015).

Recently, enzymes have been used as an alternative to harsh chemical hydrolysis to produce bioactive compounds from high-M_W β-1,3-glucans such as curdlan (Kumagai, Okuyama, & Kimura, 2016). However, there are only few examples of successful enzyme-mediated hydrolysis of paramylon (Giese, Dekker, Barbosa, da Silva, & da Silva, 2011). Little is known about the mechanism of paramylon degradation via enzymatic hydrolysis even though the structure of paramylon is not particularly complex. In general, enzymatic hydrolysis of paramylon is hindered by its stereo-chemical structure, wherein the β-1,3-linkages result in the formation of a planar and rigid molecule. The molecules self-assemble in a triple helix, stabilising the polymer further. Ultimately, the granules are almost entirely crystalline (around 90%) and virtually insoluble in water at ambient temperature (Barsanti et al., 2011).

Only one enzyme from *E. gracilis*, an *endo*-β-1,3-glucanase, has been found to degrade paramylon granules to some degree but only after the granules were chemically pretreated (Takeda et al., 2015).

Hydrothermal pretreatments have been used to facilitate and enhance the hydrolysis of high-M_W β-glucans. For example, steam explosion and steam refining at around 200 °C were shown to enhance the enzymatic degradation of crystalline cellulose (Agudelo, García-Aparicio, & Görgens, 2016; Schütt, Westereng, Horn, Puls, & Saake, 2012). Similar results were obtained when curdlan was heat-pretreated in solution before its enzymatic hydrolysis to short-chain β-1,3-glucans, although the temperature range tested did not exceed 100 °C (Kumagai et al., 2016). Glucan incubated in suspension at increasing temperatures has been shown to undergo gradual structural changes until the melting point (T_M) is reached (Yanaki, Tabata, & Kojima, 1985). Modern microwave reactors allow accurate temperature control and the homogenous heating of a sample, minimising undesired side-reactions. In these reactors, aqueous solutions can be heated to well above 200 °C in a closed vessel under pressure. The application of microwave reactors in carbohydrate chemistry is a relatively new but already established technique that has mainly focussed on synthesis rather than hydrolysis reactions (Antonino, Ugo, Venerando, & Giovanni, 2004).

The aim of this study was to develop a new microwave pretreatment of paramylon in combination with enzymatic hydrolysis to facilitate and enhance the production of soluble immunostimulatory β-1,3-glucans.

2. Material and methods

2.1. Materials

Commercially-available paramylon granules (β-1,3-glucan from *E. gracilis*) with a M_W of around 500 kDa, *Helix pomatia* β-1,3-D-glucanase (*endo*-β-1,3-glucanase), *Arthrobacter luteus* lyticase (*exo*-β-1,3-glucanase), β-glucosidase from almonds and other chemicals were purchased from Sigma-Aldrich (St. Louis, USA) unless stated otherwise.

2.2. Differential scanning calorimetry (DSC) of paramylon granules

DSC was performed using a DSC 2010 Differential scanning calorimeter 2000 (TA Instruments, New Castle, USA) and the data generated were analysed using the software Universal Analysis provided by the manufacturer. Melting curves of paramylon and freeze-dried paramylon granules (2–4 mg each) in standard pans were recorded and compared. The heating rate was 20 °C/min and nitrogen was used as the purge gas to prevent pyrolysis. During a typical DSC experiment, the temperature was increased from the ambient temperature (around 25 °C) to 500 °C. In a comparative experiment, paramylon granules were heated on open trays to 300 °C in an oven.

2.3. Microwave pretreatment of paramylon

Paramylon granules were suspended in ultrapure water at a concentration of 1% (w/v) in a final volume of 2 mL for the microwave pretreatment. The suspension was incubated in a Discover SP Microwave Synthesizer (CEM Corporation, Matthews, USA) at different temperatures for 2 min under stirring. Pretreated samples were stored frozen until further processing.

2.4. Congo Red dye binding assay

The following Congo Red dye binding assay was developed based on previous studies (Inglesby & Zeronian, 2002; Kiss, Roberts, Brown, & Triemer, 1988; Ogawa, Dohmaru, & Yui, 1994; Pihlajaniemi et al., 2016; Teather & Wood, 1982; Wiman et al., 2012). 250 µL of 1% (w/v) Congo Red were added to 250 µL of 1% (w/v) untreated or pretreated paramylon in ultrapure water. Paramylon was substituted with ultrapure water in the control samples. All samples were incubated for 30 min with rotation followed by centrifugation at 10,000 × *g*. Supernatants were discarded and the samples washed with 1 M sodium chloride, until the supernatants became colourless. Pellets were resuspended in 500 µL of 1 M sodium hydroxide and incubated overnight under rotation. 200 µL of each sample was added in duplicate to a 96 well microtiter plate to quantify the relative amount of bound dye. 200 µL of 1 M sodium hydroxide was used as a blank. Absorbance values were measured at 498 nm and normalised against the untreated paramylon value. A calibration curve was prepared with different concentrations of Congo Red to determine the linearity of the assay.

2.5. Scanning electron microscopy (SEM)

Samples of 1% (w/v) untreated and pretreated paramylon in ultrapure water were centrifuged at 10,000 xg for 10 min. The supernatants were discarded and the pellets washed three times with ultrapure water. The final pellets were mounted on a glass cover slip and air-dried. SEM was performed on a Phenom XL instrument (Phenom-World, Eindhoven, Netherlands).

2.6. Enzymatic hydrolysis

Untreated or pretreated paramylon samples at the final concentration of 0.5% (w/v) were incubated in the presence of selected commercial enzymes for enzymatic hydrolysis in a final volume of 500 µL. Reactions with the *endo*-β-1,3-glucanase (0.2 U/mL) and the β-glucosidase (5 U/mL) were performed in 100 mM sodium acetate buffer pH 5.0, at 37 °C. The reaction mixtures were incubated at 37 °C under constant rotation at 40 rpm with mixing for different periods of time up to 24 h. Reactions using the *exo*-β-1,3-glucanase (200 U/mL) were performed in ultrapure water and the reaction mixtures were incubated as described above but at 25 °C. After incubation, all reaction tubes were centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatants obtained were mixed with two volumes of methanol to stop the enzyme reactions and vacuum-dried. The pellets were resuspended in an appropriate solvent, depending on the down-stream application. The final samples were stored at 4 °C or frozen until further processing. Controls without enzyme were prepared and treated as described above.

2.7. Clean-up of endo-β-1,3-glucanase hydrolysis products

The pellets from the *endo*-β-1,3-glucanase hydrolysis (see above) and their respective controls were resuspended in ultrapure water and subjected to a clean-up to remove buffer components and proteinaceous matter that could interfere with the down-stream analysis. Samples were passed through Dowex 50WX8 hydrogen form 200–400 mesh cation-exchange resin in Strata-X 33 u Polymeric Reversed Phase 30 mg/3 mL columns (Phenomenex, Torrance, USA), followed by

vacuum evaporation.

2.8. Direct acid hydrolysis

Untreated paramylon (1% w/v) was incubated in concentrated hydrochloric acid in a final volume of 500 µL at 50 °C with constant mixing at 1000 rpm for different periods of time. The samples were then vacuum-dried, resuspended in ultrapure water and finally centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant was stored frozen until further processing. Controls without substrate were prepared and treated as described above.

2.9. Quantification of the carbohydrate content

An established protocol was followed to determine the carbohydrate content (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). 25 µL of either a sample, a blank sample of the appropriate solvent or a control (either buffer only or enzyme/substrate in buffer) were mixed with 300 µL of 5% (w/v) phenol and 625 µL of concentrated sulphuric acid. The reaction mixtures were then incubated at 70 °C for 30 min and 200 µL were transferred in duplicate to a 96 well microtiter plate. Absorbance values were measured at 490 nm and the value of the blank was deducted together with the values of the appropriate controls. A calibration curve was prepared similarly using different concentrations of glucose and the carbohydrate content was expressed as glucose equivalents. Samples were centrifuged at 10,000 × *g* for 10 min and the supernatants were analysed for the quantification of the soluble carbohydrate content.

2.10. High-performance liquid chromatography (HPLC) of hydrolysates

The hydrolysates from enzyme and acid reactions as well as appropriate controls were subjected to HPLC using an Agilent 1290 system (Agilent Technologies, Santa Clara, USA). Saccharides were detected using a refractive index detector (RID) G1362A (Agilent Technologies). An Ultraspherogel SEC-4000 column (Beckman, Fullerton, USA) was used for the qualitative analysis of saccharides within a M_W range of 0.18 kDa (glucose) to around 642 kDa (pullulan standard), which is equivalent to the degree of polymerization (DP) range of 1 to around 3958. The HPLC was conducted at ambient temperature at a flow rate of 0.5 mL/min, using 100 mM sodium nitrate as the mobile phase. A Hi-Plex Na (300 × 7.7 mm) column and guard column (Agilent Technologies) were used for qualitative analysis of saccharides in the DP range of 1–7 and for quantification of glucose. The HPLC was conducted at 80 °C at a flow rate of 0.3 mL/min, using ultrapure water as the mobile phase. D-glucose, cello-oligosaccharides with DP 2–7 (Elicityl, Crolles, France) and the pullulan M_W standard kit P-82 (Shodex, Tokyo, Japan) were used for calibration.

2.11. Cell culture

Mouse J774 macrophage cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose that was supplemented with 10% (v/v) Fetal Bovine Serum and 1% (w/v) antibiotic-antimycotic mixture (Life Technologies, Carlsbad, USA) consisting of 10,000 IU/mL penicillin, 10,000 µg/mL streptomycin and 25 µg/mL of Fungizone Antimycotic. The murine J774 cells were kindly provided by Dr Nisha Schwarz (South Australian Health and Medical Research Institute, Adelaide, Australia). Cells were maintained in a humidified incubator with 95% ambient air and a 5% carbon dioxide atmosphere at 37 °C. All cells were sub-cultured for immunofluorescence experiments onto sterile coverslips (22 mm × 22 mm) for 24–48 h inside 6 well plates. The enzymatic hydrolysis products and the acid-produced hydrolysate in phosphate buffered saline (PBS), pH 7.2, were added to one coverslip each at a final concentration of 1 µg/mL (glucans ≥ DP 2). The coverslips were incubated for a further 8 h and then the medium was discarded. The cells were washed three times with PBS, fixed using 4% (v/v) formaldehyde for 10 min and washed again three times with PBS. Controls for each enzyme were prepared as described above with enzyme reaction mixtures which were stopped instantly without incubation. Similarly, PBS was used as the control for the acid hydrolysis and an additional sample without treatment was used as a negative control.

2.12. Immunofluorescence, microscopy and image analysis

Immunofluorescence was performed on the fixed coverslips using antibodies purchased from Abcam (Cambridge, UK). The nuclear stain 4′,6-diamidino-2-phenylindole (DAPI) was used to identify cells. DyLight 488, as a substitute for fluorescein isothiocyanate (FITC), was used to visualise indirectly the presence of TNFα. Sections were blocked for 1 h at ambient temperature with 10% (v/v) normal horse serum in PBS before adding rabbit anti-TNFα antibody at a dilution of 1:100 and incubation rocking for 16 h at 4 °C. The slides were washed subsequently three times in PBS and donkey anti-rabbit DyLight 488 secondary antibody at a dilution of 1:500 was applied before incubation for 3 h with rocking at ambient temperature. The slides were washed again three times in PBS buffer, rinsed with ultrapure water and mounted in ProLong Gold Antifade Mountant with DAPI (Life Technologies, Carlsbad, USA). The slides were imaged at 60× on an IX83 microscope (Olympus Tokyo, Japan) using FITC and DAPI fluorescence filters and cells were counted based on DAPI fluorescence. FITC fluorescence was quantified by determining integrated densities with the software ImageJ (National Institutes of Health, Bethesda, USA) using thresholding (Jensen, 2013).

2.13. Statistical analysis

The programs Excel (Microsoft Corporation, Redmond, USA) and Prism 7 (GraphPad Software, La Jolla, USA) were used for statistical analyses. Experiments subjected to statistical analyses were performed at least in duplicate, except for the immunofluorescence experiment, where at least 65 cells of each slide were analysed. Results were expressed as mean ± standard deviation (SD) or standard error of the mean (SEM). Student's *t*-tests were performed to determine significant differences between two data sets as parametric, unpaired, two-tailed tests, with a confidence level of 95%.

3. Results and discussion

3.1. Melting curve of paramylon granules

Dry heat pretreatments have been applied to high-M_W β-glucans like cellulose to make their structure more amenable to enzymatic hydrolysis (Schimper et al., 2004). Similarly, we speculated that the heating of paramylon could lead to structural changes that make it more accessible to hydrolysing enzymes. Thus, a melting curve of paramylon granules under nitrogen atmosphere was generated using DSC (Fig. 1, solid line) to determine if paramylon undergoes structural changes during heat treatment. The influence of water possibly bound in the paramylon granules was also evaluated by subjecting freeze-dried paramylon granules (i.e. water removed) to DSC (Fig. 1, dashed line).

The curve of untreated paramylon granules showed two endothermic minima. The first endothermic peak (Fig. 1, Min 1) was broad in shape, with a summit at 104.01 °C ± 4.27 °C. The peak was attributed either to the gradual melting of amorphous semi-crystalline regions within the granules, which is comparable to the melting behaviour of other amorphous compounds (e.g. polyether ether ketone), or to their dehydration as seen in DSC curves of amorphous cellulose (Bertran & Dale, 1986; Schick, 2009). The latter assumption was supported by the observation that the first endothermic peak (Fig. 1, Min 1) of the freeze-dried paramylon granules was less pronounced than the respective peak of the untreated granules and is in agreement with

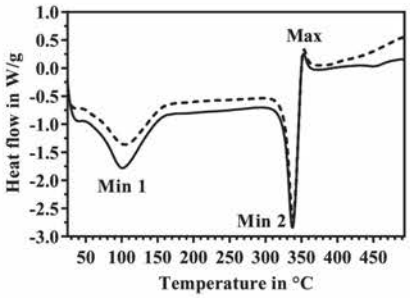

Fig. 1. Melting curves of untreated paramylon granules (solid line) and freeze-dried paramylon granules (dashed line) obtained by DSC. The two endothermic minima (Min 1 and Min 2) and the exothermic maximum (Max) are labelled.

studies suggesting that water could be part of the crystal structure of paramylon (Kiss et al., 1988). The second sharp melting peak (Fig. 1, Min 2) represented the melting of the crystalline regions of the granules and was interpreted as the complete melting of the granules. This peak onset point at 318.12 °C ± 5.37 °C was considered the T_M of the granules. This point was followed by an exothermic maximum (Fig. 1, Max) at 352.61 °C ± 0.64 °C, representing the thermal decomposition of the granules.

As a comparison, untreated paramylon granules were also dry-heated in ambient air instead of nitrogen and they showed carbonisation between 200 and 300 °C but melting was not observed (data not shown).

The major structural change observed during DSC (dry-heating under nitrogen atmosphere) was the melting of the granules. However, the T_M and temperature of thermal decomposition were very close. A dry heat pretreatment of paramylon would require a very precise temperature control and an undesired loss of substrate due to thermal decomposition probably would still be unavoidable. Thus, based on these results dry-heating did not seem to be a feasible pretreatment method.

3.2. Microscopic assessment of microwave-pretreated paramylon granules

Hydrothermal microwave pretreatment of paramylon granules was considered as an alternative to the unfeasible dry heating approach. Therefore, we investigated the effect of the microwave treatment on the crystalline structure of paramylon. The hydrothermal degradation of crystalline cellulose in suspension has been reported to occur at around 200 °C and above (Kamio, Takahashi, Noda, Fukuhara, & Okamura, 2006). Thus, the temperatures used in the microwave pretreatment of paramylon were performed below this temperature in conjunction with short incubation times to minimise pyrolysis, subsequent caramelisation or decomposition of the saccharides (Kamio et al., 2006; Sengar & Sharma, 2014). Paramylon granules incubated at 150 °C–180 °C for just 2 min appeared to be more voluminous after microwave treatment when compared to the untreated samples, indicating that structural changes had occurred as a result of the treatment and that this temperature range could be suitable to develop a pretreatment method (data not shown).

The physical effect of the microwave treatment on the overall structure of the paramylon granules was studied further by SEM (Fig. 2) and revealed that the surface structure of the granules started to deteriorate distinctively at an incubation temperature of 150 °C (Fig. 2B). At 170 °C and above (Fig. 2D–E), the original structure of the granules appeared to have completely dissolved into an amorphous mass. In this regard, microwave pretreatment had an effect similar to the dissolution of paramylon granules in a strong base followed by neutralisation (Vogel & Barber, 1968). Like other β-glucans, paramylon has a triple-helix backbone and extreme pH, high temperatures and harsh solvents

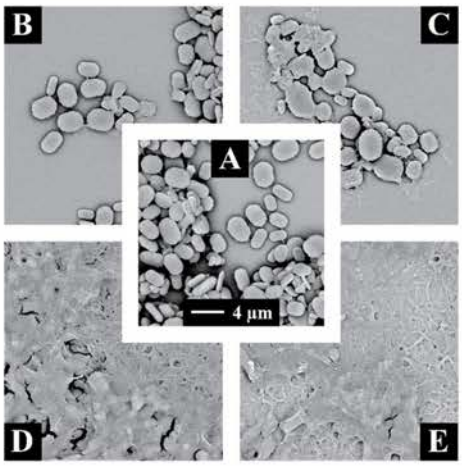

Fig. 2. SEM images of paramylon granules untreated (A) and microwave-pretreated at 150 °C (B), 160 °C (C), 170 °C (D) and 180 °C (E).

have been shown to disrupt the intra- and intermolecular hydrogen bonds within the triple-helices, causing their destabilisation. The destabilised triple-helices are capable of reforming spontaneously when these extreme conditions are reversed. However, these reformed helices are prone to imperfect assembly, leading to a loss in overall structure (Barsanti et al., 2011). Therefore, the observed changes to the physical structure of paramylon upon microwave treatment at high temperatures is likely due to the heat-induced destabilisation of its triple-helix backbone, which may also improve the accessibility of the treated paramylon to enzymes.

Microwave-assisted hydrothermal hydrolysis has been used as a stand-alone method for the production of valuable soluble β-1,3-glucans directly from substrates like curdlan. However, the hydrothermal hydrolysis of polysaccharides can often lead to the formation of side-products. Longer incubation times and higher temperatures also increases the conversion of the substrate and/or intermediates into glucose (Wang, Kim, Yoon, & Kim, 2017). We considered glucose to be an undesirable by-product of paramylon hydrolysis as it is a readily-available commodity (Clemens et al., 2016). Thus, the effect of the microwave pretreatment on the paramylon granules was further assessed by determining the soluble carbohydrate content (e.g. glucose) of the granules before and after treatment. It was established that untreated and pretreated paramylon samples did not contain significant amounts of soluble carbohydrates (data not shown), confirming that the untreated paramylon granules were completely insoluble and that liquefaction or saccharification of the granules did not occur during microwave pretreatment.

These results demonstrate that microwave treatment is effective at disrupting the structure of paramylon without degrading the glucan chains to glucose, potentially allowing the loss-free downstream production of valuable high-M_W β-1,3-glucan-based products.

3.3. Dye-based assessment of microwave-pretreated paramylon granules

The changes to the overall structure of the paramylon granules that occur upon microwave pretreatment (Fig. 2) could lead to an increase in surface area accessible to dye molecules and possibly also to paramylon-hydrolysing enzymes. Congo Red molecules bind directly to glucose subunits of glucans in a ratio of about 1:5, making the dye suitable for the determination of the surface area of high-M_W glucans in correlation with their accessibility to enzymes. However, the assays available so far require special buffers, heating and filtration, they are lengthy and dye adsorption is just measured indirectly, i.e. unbound

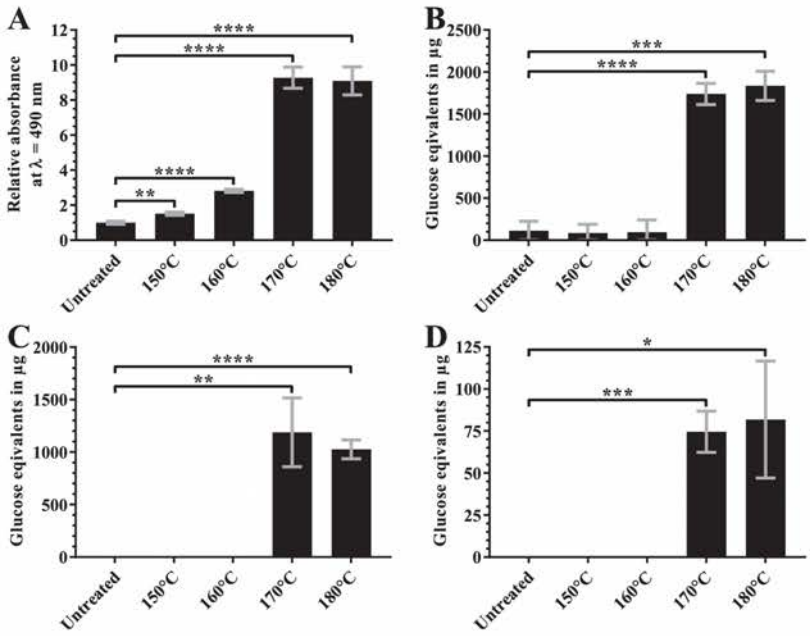

Fig. 3. Congo Red dye-binding capacity of untreated and microwave-pretreated paramylon granules as well as the respective effect of each enzyme treatments over 24 h. Enzymatic activity is expressed as glucose equivalents produced in 1 mL of reaction mixture. Congo Red dye assays (A), *endo-* (B) and *exo*-β-1,3-glucanase (C) and β-glucosidase (D). The SD is represented as error bars. Statistically significant differences ($P \leq 0.05$) are indicated: * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$ and **** for $P \leq 0.0001$.

dye (Inglesby & Zeronian, 2002; Pihlajaniemi et al., 2016; Wiman et al., 2012). We developed a new Congo Red dye binding assay based on previous studies in this field for the direct measurement of any changes to the surface area of paramylon induced by microwave pretreatment. The assay is easy and fast, it requires only few materials and dye adsorption is measured directly (i.e. paramylon-bound dye).

All pretreated paramylon samples exhibited significantly higher binding capacities for the Congo Red dye when compared to the untreated samples (Fig. 3A). Samples that were pretreated at 170 °C and 180 °C displayed the highest (about 9-fold increase) binding capacities for the dye, while a lesser but still distinct increase in dye binding was observed at both of the lower temperatures of 150 °C and 160 °C (about 1.5- and 3-fold, respectively). As expected, the dye binding capacity and therefore the available surface area of the paramylon granules was greater after microwave treatment at higher temperatures (most prominently at 170 °C and above), which directly correlates with the structural changes that were observed with SEM (Fig. 2). Thus, microwave pretreatment of paramylon at high temperatures causes structural changes that increases its available surface area.

3.4. Enzymatic assessment of microwave-pretreated paramylon granules

Next, we investigated whether the structural changes and increased surface area of microwave-pretreated paramylon enhanced its accessibility to hydrolysing enzymes. Complete enzymatic degradation of high-M_W β-glucans like cellulose (β-1,4-glucan) is usually performed by at least three types of enzymes: (a) endoglucanases, which cleave randomly within glucan chains, (b) exoglucanases, which cleave from the ends of the glucan chains and (c) glucosidases, which preferably degrade freed-up short-chain glucans to glucose (Dimarogona, Topakas, & Christakopoulos, 2012). Accordingly, the commercially-available enzymes β-1,3-D-glucanase from *H. pomatia* (annotated as an *endo*-β-1,3-glucanase), lyticase from *A. luteus* (annotated as an exoglucanase) and a β-glucosidase isolated from almonds were used as representatives of these three enzyme types. Lyticase (trade name for zymolyase) produces mainly laminaripentaose units by breaking the β-1,3-linkages in the laminarin polysaccharide (Kitamura, Kaneko, & Yamamoto, 1974). A previous study suggested that this enzyme could be used to enhance the bioactivity of paramylon but data supporting this claim were not shown (Kataoka, Muta, Yamazaki, & Takeshige, 2002).

Untreated and pretreated paramylon samples were incubated in the presence of the commercially-available *endo-*, *exo*-β-1,3-glucanase or β-glucosidase enzymes for 24 h (Fig. 3B–D). Enzyme treatment of paramylon pretreated at lower temperatures (150 °C and 160 °C) did not result in a release of soluble carbohydrates with any of the enzymes tested. However, significant amounts of soluble carbohydrates were produced by all three enzymes from paramylon pretreated at 170 °C and 180 °C when compared to untreated paramylon samples. These results correlated with the higher surface area measurements from the Congo Red dye binding assay, thus showing improved accessibility of the high-temperature pretreated samples for the enzymes independently of the enzyme type.

The virtual absence of enzymatic hydrolysis of paramylon pretreated at the lower incubation temperatures may be explained by steric hindrance of the enzymes. The *A. luteus exo*-β-1,3-glucanase and the β-glucosidase from almonds have a M_W of 21 and 135 kDa, respectively, and the *H. pomatia endo*-β-1,3-glucanase is probably in a similar M_W range (Grover, David MacMurchie, & Cushley, 1977; Kitamura et al., 1974). Congo Red dye molecules are considerably smaller in comparison, with a M_W of just 0.7 kDa (Inglesby, Zeronian, & Elder, 2002). It is possible that the structural changes allowed more dye to bind but were too limited to allow the access of the enzymes.

It has been shown that the efficacy of hydrothermal pretreatments for the enzymatic hydrolysis of glucans over the temperature range tested either reaches a maximum or a plateau. If the efficacy shows a maximum, there is only one possible optimal temperature because adverse effects are occurring after reaching the maximum. In case the efficacy is plateauing, the lowest, most efficient temperature is considered the optimal (Kumagai et al., 2016; Schimper et al., 2004). We found that a plateau was reached in our pretreatment at 170 °C with both the enzymatic assay and the Congo Red dye binding assay. This pretreatment temperature was thereby determined to be most favourable and accordingly it was chosen for all further experiments.

3.5. Enzymatic hydrolysis products from microwave-pretreated paramylon

We aimed to produce and characterise (i.e. determine their DP range) potentially immunostimulatory soluble β-1,3-glucans. To this

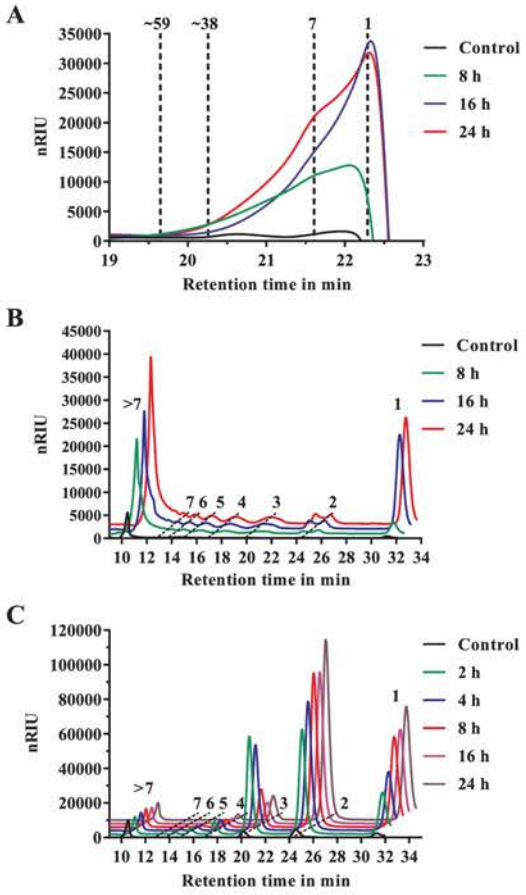

Fig. 4. Qualitative HPLC-RID analysis of the enzymatic hydrolysates. Shown are time courses of *endo*- (A and B) and *exo*-β-1,3-glucanase (C) reactions. Retention times, peaks and DP of the standards, controls and incubation times of the reactions are indicated. Ultraspherogel SEC-4000 column with retention in a broad M_W/DP range (A) and Hi-Plex Na column with resolution in the DP range of 1–7 (B and C) were used.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

end, paramylon samples were microwave-pretreated at 170 °C and then incubated for up to 24 h with the *endo*- and the *exo*-β-1,3-glucanases. The β-glucosidase was not used because only undesired low-value glucose can be expected as the hydrolysis product (Clemens et al., 2016; Dimarogona et al., 2012). The hydrolysates produced by the *endo*- and the *exo*-β-1,3-glucanase were then qualitatively analysed by HPLC-RID. Additional controls without enzyme were assayed as above and ruled out potential product formation from autohydrolysis (data not shown).

Separation using the Ultraspherogel SEC-4000 column (broad M_W/DP range) during the time course (8–24 h) of the *endo*-β-1,3-glucanase reaction mainly showed saccharides in the DP range of 1–59 (Fig. 4 A). Time progression of the reaction shifted the profiles of the chromatograms, for example the maximum at a retention time between DP 1–7 (Fig. Figure 4A, Figure 8h) shifted to the retention time of DP 1 (Fig. 4A, 16 and 24 h). Further analysis using the Hi-Plex Na column (mono- and oligosaccharides, DP 1–7 range) showed an increasing peak of DP 1 and saccharides in the DP range of 2–7, as well as an increasing peak of DP > 7 (Fig. 4B) while the controls showed negligible background signals (Fig. 4A and B).

The shift of the product profiles towards DP 1 could have been caused by a background glucosidase activity in the commercial enzyme preparation. Similar shifts have been observed when using enzyme mixtures without defined activities on β-1,3-glucan substrates (Grandpierre, Janssen, Laroche, Michaud, & Warrand, 2008). Many *endo*-β-1,3-glucanases cleave their substrates in a defined manner, leading to an accumulation of products in the oligosaccharide DP range, but not glucose (Fontaine, Hartland, Beauvais, Diaquin, & Latge, 1997). In contrast, others are randomly acting and produce increasing amounts of glucose with progressive reaction times and the enzyme used here may belong to the latter group (Wang et al., 2016).

Hydrolysates from the *exo*-β-1,3-glucanase treatment contained mainly saccharides in the DP range of 1–7, so they were analysed using the column for mono- and oligosaccharides. Peaks of DP 3–7 decreased after 2 h incubation time with a concomitant increase of DP 1 and 2. The peak of DP > 7 remained unchanged with time and was present in the control (Fig. 4C). This peak and minor signal in the controls were considered to be background signals.

The *exo*-β-1,3-glucanase produced glucose in a fashion similar to that of the *endo*-β-1,3-glucanase. Some exoglucanases have been reported to cleave off products randomly in the low-DP oligosaccharide range, including glucose (Beldman, Searle-Van Leeuwen, Rombouts, & Voragen, 1985; Creuzet, Berenger, & Frixon, 1983). Similarly, the enzyme used in our study could have exhibited a random substrate cleavage pattern although we cannot rule out the presence of a background glucosidase activity in the commercial enzyme preparation.

Based on these results, the incubation times 8 and 2 h for the *endo*- and the *exo*-β-1,3-glucanase, respectively, were considered to be optimal for further experiments under these conditions.

3.6. Acid hydrolysis products from paramylon

The conventional acid hydrolysis of paramylon was compared to our combined microwave-based pretreatment and enzymatic hydrolysis method. A time course (up to 5 h) of acid hydrolysis of untreated paramylon granules was set up and the products were analysed similarly to the enzymatically-produced hydrolysates from microwave-pretreated paramylon. Separation using the broad M_W/DP range column showed that the main range of saccharides present in the hydrolysates was DP 1–38 (Fig. 5A). Further analysis in the mono- and oligosaccharides range indicated that all hydrolysates contained saccharides in the DP range of 1–7 and > 7 (Fig. 5B). In the range of DP 8–38, the signals from the 5 h incubation were higher than the signals of the control, the 0h and the 2.5 h incubation (Fig. 5A and B), indicating the progressive acid hydrolysis of the paramylon granules with time.

The wide DP range of final hydrolysis products was rather surprising as attempts to hydrolyse paramylon granules with formic acid in a previous study have been shown to result mainly in formation of glucose and products below DP 7 (Clarke & Stone, 1960). In a previous study, the hydrolysis reactions of curdlan using trifluoroacetic acid and sulphuric acid did not result in products with a DP above 17 (Johansson et al., 2006). The choice of acid and its strength, as well as the hydrolysis conditions (e.g., time and temperature) were probably the key factors for the different outcomes in our study. Although the DP range of soluble β-1,3-glucans produced by the acid hydrolysis of paramylon granules was wide in our study (DP 2–38), it was not as wide as that of soluble β-1,3-glucans produced by the enzymatic hydrolysis of microwave-pretreated paramylon (DP 2–59, see above).

The most favourable incubation time for further experiments using acid hydrolysis under the assay conditions was after 5 h incubation, when saccharides in the DP 8–38 range could be detected unambiguously.

3.7. Glucose content of enzymatic and acid treated paramylon

Microwave pretreatment of paramylon does not release undesired glucose from paramylon under the chosen conditions described above. We also determined whether glucose is released as a by-product from

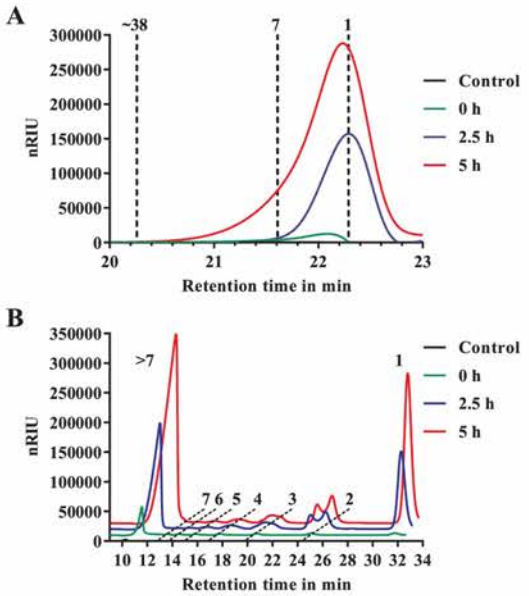

Fig. 5. Qualitative HPLC-RID analysis of the acid-produced hydrolysates. A time course is shown. Retention times, peaks and DP of the standards are indicated, as well as incubation times of the reactions. Ultraspherogel SEC-4000 column with retention in a broad M_W/DP range (A) and Hi-Plex Na column with resolution in the DP range of 1–7 (B) were used.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

the enzymatic reactions with microwave-pretreated paramylon and from the direct acid hydrolysis of paramylon granules. The respective hydrolysates were produced under optimal conditions (see above) and analysed for their glucose content.

The abundance of glucose within the hydrolysate produced by the *endo*-β-1,3-glucanase treatment was significantly higher compared to that produced by the *exo*-β-1,3-glucanase and the acid treatment (Fig. 6). The hydrolysates of the latter treatments had comparable glucose contents.

The glucose content of the hydrolysates produced by the *endo*- and *exo*-β-1,3-glucanases was 53 and 24%, respectively, and was low compared to previously published studies. The action of glucanase enzyme mixtures on untreated paramylon granules have been shown to produce glucose contents of around 70% and above, even at the shortest

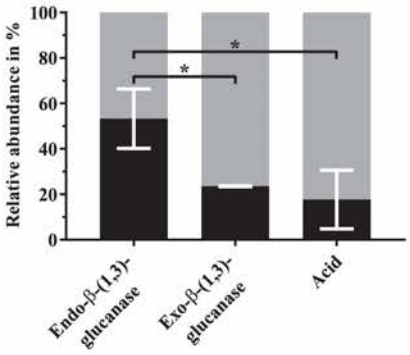

Fig. 6. Relative abundance of glucose (black bars) and ≥DP 2 saccharides (grey bars) in the hydrolysates, adding up to the total carbohydrate content (100%) determined as glucose equivalents. The SD is represented as error bars. Statistically significant differences ($P \leq 0.05$) are indicated: * for $P \leq 0.05$.

incubation times (Giese et al., 2011). These results clearly show the potential of using other purified enzyme preparations (yet to be identified) capable of degrading paramylon without generating glucose in conjugation with microwave pretreatment for the desired glucose-free production of high-value soluble β-1,3-glucans.

Our protocol for acid hydrolysis produced a hydrolysate with a glucose content of 18%, which also was low compared to hydrolysates generated by the enzyme mixtures. However, inferring from our work and previous studies it is doubtful if direct acid hydrolysis could be optimised to yield products from paramylon without glucose formation in contrast to the more promising enzymatic hydrolysis approaches (Clarke & Stone, 1960; Johansson et al., 2006).

3.8. Immunostimulatory effect of paramylon hydrolysates

Finally, we tested the paramylon-derived hydrolysates for immunostimulatory activity, a property that is desirable in many potential applications (e.g. biomedicine and nutraceuticals). The hydrolysates were prepared under the optimal conditions described above from microwave-pretreated paramylon with the two enzymes (endo- and *exo*-β-1,3-glucanase) or by direct acid treatment. Murine macrophages were then incubated with each of the hydrolysis products and the intracellular synthesis of the inflammation-linked marker protein TNFα was visualised using immunofluorescence. Microscopic images were taken and analysed (Figs. 7 and 8).

The macrophages incubated with the enzymatic hydrolysates showed higher TNFα-related green fluorescence compared to the acid hydrolysate-treated cells and cells without treatment. Cells incubated with samples of the hydrolysates produced by acid and the *exo*-β-1,3-glucanase showed significantly more intense fluorescence than their respective controls. The highest fluorescence was exhibited by cells incubated with the hydrolysate produced by the *exo*-β-1,3-glucanase. These cells had a significantly higher intensity compared to the respective controls and the untreated cells. The high fluorescence intensity of this sample indicated a strong upregulation of TNFα synthesis. The *exo*-β-1,3-glucanase produced a distinctive product profile in the DP range 1–7 whereas the hydrolysates from the *endo*-β-1,3-glucanase and the acid treatment had a wider product profile. This result could explain why the *exo*-β-1,3-glucanase hydrolysate was more immunostimulatory than the other two hydrolysates. In an earlier study, glucose was found to stimulate leucocytes in a hyperglycaemic environment, leading to increased TNFα synthesis in the cells (Gonzalez et al., 2012). However, we ruled out glucose as a TNFα-inducing factor as the hydrolysate produced by the *exo*-β-1,3-glucanase had a ower glucose concentration than the less immunostimulatory hydrolysate produced by the *endo*-β-1,3-glucanase. The DP range of the hydrolysis products seems to influence TNFα regulation but further investigation is needed to determine its relevance for other immunostimulating activities.

4. Conclusions

We have established a hydrothermal microwave pretreatment of paramylon granules that significantly enhanced the enzymatic hydrolysis of paramylon to bioactive oligosaccharides. To the best of our knowledge, we have shown for the first time that the commercially-available *H. pomatia* β-1,3-D-glucanase, *A. luteus* lyticase and a β-glucosidase from almonds are able to degrade microwave-pretreated paramylon. The Congo Red assay developed in this study was found to be a suitable, simple, sensitive and time-saving method to assess the efficacy of the microwave pretreatment on paramylon granules. Consequently, it may be used to assess optimal pretreatment conditions in an industrial setting and may be transferrable to other polysaccharides.

Future work should include further optimisation of the product profiles and of the hydrolysis yields. The scale-up of the entire process is

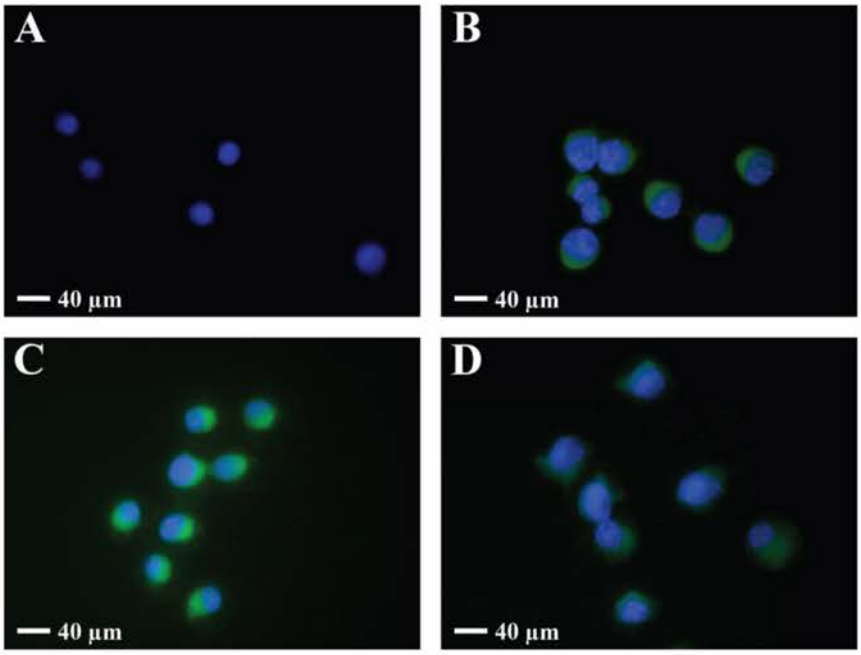

Fig. 7. Images of immunofluorescent murine macrophages after incubation with the hydrolysates of enzymatic and acid reactions. DAPI (blue) and FITC (green) fluorescence filters were used to visualise the cells and TNFα, respectively. The images were overlaid for the purpose of visualisation. Cells are shown without treatment (A) and incubated with hydrolysates from the *endo*- (B) and the *exo*-β-1,3-glucanase (C) treatments and with the acid-produced hydrolysate (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

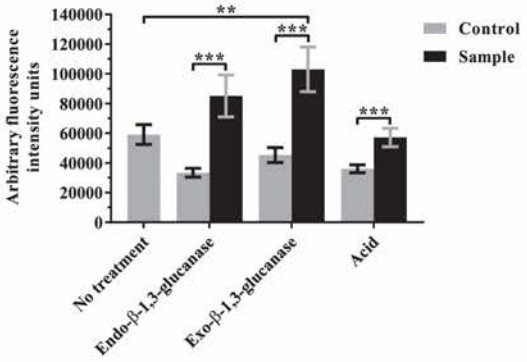

Fig. 8. Analysis of immunofluorescence of murine macrophages incubated with the hydrolysates of enzymatic and acid reactions. TNFα was visualised via the FITC filter. Fluorescence of samples and controls was quantified by image analysis. The SEM is represented as error bars and statistically-significant differences ($P \leq 0.05$) between samples and respective controls are indicated: ** for $P \leq 0.01$ and *** for $P \leq 0.001$.

desirable as a next step towards an industrial application as well as the mapping of the specific bioactivities of the hydrolysates either as a whole or fractionated in a certain DP range. The latter would make a strong case for the use of the hydrolysates as nutraceuticals and it could allow the development of new biomedical technologies.

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Chapter 5: Development of screening strategies for the identification of paramylon-degrading enzymes

5.1. Introduction

The identification of paramylon-degrading enzymes has been hampered by a lack of suitable assays and a poor understanding of the enzymatic pathway for paramylon degradation in *E. gracilis.* So far, only one enzyme from this pathway that is capable of degrading paramylon (after chemical pretreatment) has been identified. In the previous chapter (Chapter 4), the potential of a new approach for the production of soluble bioactive β-1,3-glucans through enzymatic degradation of microwave-pretreated paramylon was shown. However, the by-product formation (i.e. glucose) by the commercially-sourced enzymes used in this approach was higher than expected, even after short hydrolysis times. Therefore, additional paramylon-degrading enzymes potentially capable of producing less by-product were investigated.

Several strategies and methods for the identification of paramylon-degrading enzymes are presented in this chapter. *E. gracilis* proteins were screened for enzymes of the paramylon degradation pathway which could serve as alternatives to the enzymes used earlier (Chapter 4) and to fill gaps in current knowledge. Additionally, commercially-available enzymes other than those used earlier and in-house enzyme preparations from different organisms were screened for their activity against paramylon.

This chapter was prepared as a manuscript for submission to the *Journal of Microbiological Methods*.

5.2. Contribution to manuscript 2

The concept for this manuscript was developed in partnership with my supervisors Anwar Sunna and Helena Nevalainen. They were involved also in designing experiments and troubleshooting. Angela sun was also involved in the conception in a supervisory role. I performed all of the experimental work, data collection and analysis, and prepared the initial draft of the manuscript. Detailed contribution of all authors is presented in Table 5.1.

Table 5.1. Author contribution summary for manuscript 2. Alexander Gissibl (AG), Andrew Care (AC), Angela Sun (AS^1), Graham Hobba (GH), Helena Nevalainen (HN) and Anwar Sunna (AS^2).

	AG	**AC**	**AS^1**	**GH**	**HN**	**AS^2**
Conception	•	•	•	•	•	•
Experiment design	•	•			•	•
Data collection	•					
Data analysis	•					
Manuscript	•				•	•

5.3. Manuscript 2

Development of screening strategies for the identification of paramylon-degrading enzymes

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Keywords

Euglena gracilis, paramylon, enzymatic degradation, fluorescence-activated cell sorting, proteomics analysis, Congo Red plate assays

Abstract

Enzymatic degradation of the β-1,3-glucan paramylon could produce bioactive compounds and feedstocks for biofuel production. However, the enzymatic degradation of paramylon is poorly understood and there is a lack of enzymes able to degrade it efficiently. Thus, the aim of this work was to find paramylon-degrading enzymes and ways to facilitate their identification. Towards this end, a *Euglena gracilis*-derived cDNA expression library introduced into *Escherichia coli* was generated and a flow cytometry-based screening assay for these enzymes was developed using the fluorogenic substrate fluorescein di-β-D-glucopyranoside in combination with time-saving auto-induction medium. From proteomic data, four amino acid sequences of potential *E. gracilis* β-1,3-glucanases were identified. The open reading frame light_m.20624 encoding one of these candidate sequences was heterologously expressed in *E. coli*. Finally, a Congo Red dye plate assay was developed for the screening of enzyme preparations potentially able to degrade paramylon. The assay was validated with enzymes assumed to have paramylon-degrading activity. Four commercial preparations were found to show previously unknown activity against paramylon.

1. Introduction

Paramylon is a high molecular weight (M_W) β-1,3-glucan produced by the microalga *Euglena gracilis*, deposited as insoluble granules in the cytoplasm. The granules are surrounded by a biomembrane containing the paramylon synthase complex and other proteins (Bäumer *et al.*, 2001, Kiss *et al.*, 1988). Paramylon, like other high-M_W β-1,3-glucans, has been reported to exhibit various bioactive properties. Amongst others, it has immunostimulating, hepatoprotective, antiinflammatory and antitumor effects on mammalian health (Barsanti *et al.*, 2011,

Nakashima *et al.*, 2017). However, the mammalian immune system has to first break down high-M_W β-1,3-glucans before a broad immune response is induced, whereas shorter-chain β-glucans stimulate macrophages and other leucocytes directly (Chan *et al.*, 2009). Therefore, it would be highly desirable to be able to hydrolyse high-M_W β-1,3-glucans to shorter-chain glucans to use them as adjuvants, drugs and nutraceuticals.

Microalgal biomass is considered as a bioresource for third-generation biofuel and fermentable sugars (obtained from microalgal biomass) have been used for the production of bioethanol (Chen *et al.*, 2013, Lee and Lavoie, 2013). Grown under optimal conditions, *E. gracilis* can accumulate paramylon to approximately 90% of its dry weight (Barsanti *et al.*, 2001). This ability to accumulate large amounts of paramylon makes *E. gracilis* an attractive source for the production of fermentable sugars and soluble bioactive glucans. Hydrolysis of paramylon for the production of bioactive compounds and fermentable sugars should preferably be carried out solely with enzymes or in combination with them to eliminate or reduce the use of harsh chemicals as these chemicals need recovering in the process (Mussatto *et al.*, 2010).

Paramylon granules are exceptionally recalcitrant to enzymatic hydrolysis. The *in vivo* enzymatic degradation pathway remains mostly unknown and so far only one enzyme from *E. gracilis* able to degrade paramylon, an endo-β-1,3-glucanase, has been identified functionally (Takeda *et al.*, 2015). However, the enzyme is only capable of hydrolysing chemically pretreated paramylon (i.e. dispersed paramylon, Vogel and Barber, 1968). It is likely that *E. gracilis* degrades paramylon synergistically by employing endoglucanases, exoglucanases and β-glucosidases, similarly to organisms degrading the β-1,4-glucan cellulose (Dimarogona *et al.*, 2012).

Identification of more enzymes taking part in paramylon degradation in *E. gracilis* would provide fundamental insight into the carbon metabolism of *E. gracilis* and could provide a suite of new

enzymes for the *in vitro* digestion of paramylon. Comparative transcriptomic data from *E. gracilis* has suggested a multitude of candidate proteins that are potentially involved in the paramylon catabolism (O'Neill *et al.*, 2015). However, there is a lack of experimental data linking these *E. gracilis* sequences to an enzyme function and little advance has been made in the last 50 years to overcome this knowledge gap. Enzymes from other organisms than *E. gracilis* have been reported to act on paramylon to some extend *in vitro*, mostly from the genus *Trichoderma* (Giese *et al.*, 2011, Suzuki *et al.*, 2017). Thus, an alternative approach would be the screening of potential paramylon-degrading enzyme preparations from different organisms other than *E. gracilis*

Here we report three approaches to facilitate the functional identification of new paramylon-degrading enzymes: a high-throughput fluorescence-activated cell sorting (FACS) assay for the screening of enzymes from *E. gracilis*, analysis of the *E. gracilis* proteome and an activity plate assay to test commercially-available enzymes for their potential to degrade paramylon.

2. Materials and methods

2.1. Materials

Paramylon granules (β-1,3-glucan from *E. gracilis* with a M_W of around 500 kDa) and other chemicals were purchased from Sigma-Aldrich (St. Louis, USA) unless stated otherwise. Culture supernatant of *Trichoderma reesei* QM6a, grown for 3 d in medium containing minimal salts, 1% (w/v) cellobiose, 1% (w/v) lactose and 3% (w/v) soybean flour extract (Lim *et al.*, 2001), was kindly provided by W. Babar (Macquarie University, Lim*, et al.*, 2001).

2.2. Cultivation, harvest and cell lysis of E. gracilis

E. gracilis wild type strain Z (strain 25 in the original publication by Pringsheim and Pringsheim, 1952) was obtained from Southern Biological Pty Ltd (Australia). A cultivation medium containing glucose as carbon source was prepared as described previously (Hasan *et al.*, 2017).

Cultures were incubated at 23°C with orbital shaking at 150 rpm, after inoculation to a final concentration of ca. 5% (v/v) with a culture grown in the same medium until the cell density plateaued. The total working volume of the cultures was 50-500 mL. The pH of the growing culture was measured at different time intervals using a Eutech Instruments pH 510 pH meter (Thermo Fisher Scientific, Waltham, USA). Cultures were centrifuged at 4200×g for 10 min at ambient temperature and the pellets containing the cells were lysed with either a Branson Ultrasonics Sonifier S-450 (Emerson Electric Co., St. Louis, USA) or a French pressure cell press (Thermo Fisher Scientific). For sonication, the cell pellet was washed two times with ultrapure water and then resuspended in appropriate buffer depending on the downstream-application. The suspension was sonicated in an ice bath five times for 10 s at 25% of the maximum amplitude and with 30 s rest in between runs. The lysate was centrifuged at 15,000×g for 30 min at 4°C. The supernatant obtained is from here on referred to as crude extract and was used in the Congo Red dye plate assay (see sections 2.12 and 3.3.). If necessary, crude extracts were concentrated further using an Amicon Ultra-15 centrifugal ultrafiltration unit (≤10 kDa cut-off, Merck, Darmstadt, Germany). For French pressure cell lyses, the cell pellet was washed as described above and disrupted by three passages through the pressure cell at 1000 psi. The lysate was centrifuged at 100,000×g for 60 min at 4°C. The supernatant was concentrated further as required using an Amicon Ultra-15 centrifugal ultrafiltration unit (≤10 kDa cut-off, Merck) and is referred to from here on as cell extract.

2.3. Dry weight and paramylon content determination

The dry weight of an *E. gracilis* culture was determined by harvesting 3 mL cell culture in triplicate daily. The cells were centrifuged at 5000×g for 10 min at ambient temperature and the supernatants were discarded. The pellets were washed in ultrapure water, transferred to pre-weighed aluminium dishes and dried at 70°C for 2 d before weighing.

Paramylon was isolated daily by acid and sodium dodecyl sulphate (SDS)-treatment from samples of an *E. gracilis* culture following a published protocol (Rodríguez-Zavala *et al.*, 2010). The total carbohydrate content was determined by an UV-absorbance assay after hydrolysis of the paramylon pellets in sulphuric acid (Albalasmeh *et al.*, 2013). In short, samples were transferred in duplicate to a microtiter plate and absorbance was measured at 315 nm. Blank samples were processed similarly. A calibration curve was prepared using different concentrations of glucose and the total carbohydrate/paramylon content was expressed as glucose equivalents.

2.4. Preparation of a cDNA expression library

E. gracilis was cultivated as described above and the dry weight and paramylon content of the culture was determined daily for the preparation of a cDNA expression library. Cell mass was collected daily, snap-frozen in liquid N_2 and stored at -80°C. The cell mass harvested 8 d after inoculation (i.e. the cells were degrading paramylon) was sent frozen in dry ice to Express Genomics (Frederick, USA) for the construction of a cDNA library. In short, the mRNA was extracted, followed by cDNA synthesis, library construction, quality control and determination of the number of primary transformants. The cDNA fragments were directionally inserted into the plasmid pExpress 1 for lactone-inducible pro- and eukaryotic expression (Forman and Samuels, 1991). For quality control, the cDNA insert was excised from the plasmid of 24 random transformants using the restriction enzymes *Xho*I and *EcoR*I and the size of each cDNA fragment excised was determined by gel electrophoresis. The number of primary transformants was determined by plating on selection plates containing antibiotics. *E. coli* BL21 (DE3) chemically competent cells (New England Biolabs, Ipswich, USA) were transformed with the cDNA library (delivered as a plasmid preparation from the primary transformants) in our laboratory following the protocol of the manufacturer and the number of transformants was determined by plating on selection plates.

2.5. Flow cytometry screening

E. coli cells harbouring the cDNA library were initially grown at 37 °C with shaking at 200 rpm in lysogeny broth (LB) medium supplemented with 50 µg/mL carbenicillin. Cultures were grown until the A_{600} reached 0.6 and then heterologous expression under *lac* promoter was induced by the addition of 1 mM isopropyl β-D-thiogalactoside (IPTG). Cultures were incubated overnight at 20°C with shaking at 200 rpm. Approximately after 15 h, a sample of 1 mL of a culture was adjusted to $OD_{600} = 0.2$, centrifuged for 1 min at 11,000×g at ambient temperature and the supernatant was discarded. The pellets obtained were resuspended in a solution containing the fluorogenic substrate fluorescein di-β-D-glucopyranoside (FDGlu) at a concentration of 100 µM in 50 µL phosphate buffered saline (PBS, Plovins *et al.*, 1994). The cells in the substrate solution were incubated with shaking at 150 rpm at 37°C for 1 h and then 950 µl PBS were added to bring the final volume to 1 mL at a cell density of OD_{600} = 0.2 before FACS. *E. coli* Tuner (DE 3) expressing the β-glucosidase gene *bglA* from *Caldicellulosiruptor saccharolyticus* and *E. coli* BL21 (DE 3) cells without expression plasmids served as a positive and negative control, respectively (Care *et al.*, 2017, Hardiman *et al.*, 2010). FACS was performed on a BD Influx flow cytometer (Becton Dickinson, Franklin Lakes, USA) with a 200 mW 488 nm laser excitation and 540 ± 30 nm emission filter. The *E. coli* BL21 (DE 3) cells containing the cDNA library were treated similarly to the controls and 20×10^6 cells were sorted using a gate encompassing the positive population of the positive control (Fig. 3 B) in an enrichment step. The sorted cells were then grown in LB medium, expression was induced and the cells were sorted again as described above.

In order to validate the assay, six cells of each control were sorted into single wells of a 96 well plate containing 100 µL LB medium using gates encompassing the positive and negative populations (Fig. 3 A and B), respectively. The 96 well plate was incubated overnight at 37°C with shaking at 200 rpm. Approximately after 15 h, a black replica 96 well plate containing auto-

induction medium ZYM-5052 was inoculated and incubated at 37°C with shaking at 250 rpm. Expression was induced automatically in this medium after *E. coli* had consumed all the original glycerol and started to utilise α-lactose instead (Studier, 2014). After overnight incubation, the plate was centrifuged at 1,800×g for 10 min at 4°C and the supernatant was discarded. The pellets were incubated in 50 µL of 100 µM FDGlu in PBS with shaking at 150 rpm at 37°C for 1 h. Fluorescence (enzymatic activity against FDGlu) was then measured directly on a plate reader at 485 nm excitation /520 nm emission.

2.6. Protein analysis

Proteins were separated by SDS-polyacrylamide gel electrophoreses (PAGE) on NuPage 4-12% (w/v) Bis-Tris Gels (Life Technologies, Carlsbad, USA) and visualised with Coomassie Brilliant Blue staining. Protein samples were analysed by liquid chromatography electrospray ionisation tandem mass spectrometry (LC ESI MS/MS) at the Australian Proteome Analysis Facility (APAF, Macquarie University) as described elsewhere (Atack *et al.*, 2015).

2.7. Preparation of dispersed paramylon

Dispersed paramylon was prepared following a protocol outlined elsewhere (Vogel and Barber, 1968). In short, 50 mg/mL paramylon granules were dissolved in 500 mM potassium hydroxide, dialysed against ultrapure water at 4°C for 2 d and either used immediately or stored at -20°C.

2.8. Reducing sugar assay

Release of reducing sugars was determined using the dinitrosalicylic acid (DNS) assay (Miller, 1959). Reaction mixtures were prepared for the fractions obtained from the cation exchange chromatography (CIEX) of *E. gracilis* proteins (see below), following a modified protocol (Miller, 1959). In short, samples were prepared containing either 2.5% (w/v) dispersed paramylon or 2.5% (w/v) paramylon granules and 10% (v/v) protein solution (fraction 1-10), in 50 mM sodium

acetate buffer, pH 5.2. Samples and controls were incubated at 37°C for 10 h with shaking at 200 rpm. Reactions were stopped by incubation on ice and the addition of one volume of the DNS solution. Samples and controls were then incubated at 99°C for 10 min with shaking at 600 rpm. Finally, samples were centrifuged at 15.000×g for 15 min at ambient temperature and the resulting supernatants were transferred in duplicate to a microtiter plate before absorbance values were measured at 540 nm. A calibration curve was prepared similarly using different concentrations of glucose and the reducing sugar content was expressed as glucose equivalents.

2.9. Preparation of proteins for proteomic analysis

CIEX was conducted on an ÄKTA Pure system using a HiTrap SP HP 5 mL column (both from GE Healthcare, Chicago, USA). The flow rate was 5 mL/min with initially 145 mM sodium acetate buffer, pH 5.2, as mobile phase. *E. gracilis* cell extract in 50 mM sodium acetate buffer, pH 5.2, was passed through a 0.2 µm syringe filter (Merck) and injected into the column. Proteins were eluted with a gradual increase of the salt concentration of the mobile phase to 287.5 mM. Protein fractions were collected during elution in intervals of 2 mL and numbered 1-10 (i.e. the number of fractions was chosen to ensure there was enough protein in each fraction for any downstream applications). Samples were concentrated and then buffer-exchanged with 50 mM sodium acetate buffer, pH 5.2, using an Amicon Ultra-15 centrifugal ultrafiltration unit (10 kDa cut-off, Merck). All fractions (1-10) were subjected to SDS-PAGE analysis and the fractions 6-10 additionally to LC ESI MS/MS analysis.

A standard binding assay (SBA) was conducted as previously described by (Sunna *et al.*, 2013) with *E. gracilis* cytosolic proteins and paramylon granules as follows. A 300 µL portion of *E. gracilis* cell extract in 100 mM sodium acetate buffer, pH 5.2, was mixed with 10 mg paramylon granules and incubated with shaking at 300 rpm with occasional rotations at ambient temperature for 1 h. The unbound fraction was removed after centrifugation at 5000×g for 15 min at ambient

temperature. The pellet was washed three times with 200 µL of 100 mM sodium acetate buffer, pH 5.2, containing 100 mM sodium chloride. The bound protein fraction was eluted after addition of 2X SDS-PAGE loading buffer to a total volume of 200 µL and incubation at 99°C with shaking at 600 rpm for 10 min with occasional rotations. A control assay was performed without substrate. All fractions of the SBA were subjected to SDS-PAGE and the bound protein fractions additionally to LC ESI MS/MS analysis (see section 2.6.).

Paramylon granules with an intact surrounding membrane were isolated as described by Bäumer*, et al.*, 2001, for the isolation of the paramylon membrane. First, *E. gracilis* cells were resuspended in 100 mM sodium acetate buffer, pH 5.2, and lysed using a French pressure cell press as described above. Then, the paramylon granules obtained were washed with buffer containing 100 mM sodium chloride, boiled at 99°C for 10 min in PBS buffer containing 2% (w/v) SDS and finally centrifuged at 14.000×g for 20 min. The supernatant was subjected to LC ESI MS/MS analysis (see above).

2.10. Database searches

The program Global Proteome Machine (http://www.thegpm.org/) was used to compare LC ESI MS/MS spectra with a non-redundant proteome of *E. gracilis* (http://jicbio.nbi.ac.uk/euglena/sequences) containing translated open reading frames (ORFs), generated from a non-redundant transcriptome (Craig *et al.*, 2004, O'Neill*, et al.*, 2015). Full-length sequences of proteins present in the samples were thereby identified and compared with entries in the carbohydrate active enzymes (CAZy) database (Lombard *et al.*, 2014) using the basic local alignment search tool (BLAST, Altschul *et al.*, 1990). Hits with positive e-values were discarded and the remaining hits were compared with the BLAST database of non-redundant protein sequences. Hits with high sequence similarities to sequences of proteins not involved in carbohydrate metabolism were discarded. Sequences were categorised during BLAST based on

conserved protein domains or by the category of the sequence from the CAZy database with the highest similarity (Marchler-Bauer *et al.*, 2017). The theoretical M_W of proteins coded by the identified ORFs were calculated using the expert protein analysis system (ExPASy) Compute pI/M_W tool (Gasteiger *et al.*, 2003).

2.11. DNA sequence design, cloning and heterologous expression

The nucleotide sequence of ORF light_m.20624 was designed with flanking restriction sites for the enzymes *Nde*I and *Xho*I (New England Biolabs) and synthesised codon-optimised for expression in *E. coli* by Integrated DNA Technologies (Skokie, USA). The synthesised sequence was delivered in the plasmid pUCIDT and ligated in-frame after excision with respective restriction enzymes into the expression plasmid pET-22b(+) (Merck, Green, 2012). *E. coli* α-Select (Bioline, London, UK) was used as a host for propagation of plasmids while BL21 (DE3) (New England Biolabs) and Tuner (DE3) cells (Merck) were used for recombinant protein expression. *E. coli* cultures were grown with shaking at 200 rpm at 37 °C in LB medium supplemented with 50 μg/mL carbenicillin. Cultures of *E. coli* harbouring the expression plasmids were grown until the A_{600} reached 0.6 and then recombinant protein expression was induced by the addition of 1 mM IPTG. The kit QIAexpress Ni-NTA Fast Start (Qiagen, Hilden, Germany) was used for the attempted His-tag purification of the recombinant protein under native and denaturing conditions.

The recombinant expression of the ORF light_m.20624 was also tested in *Saccharomyces cerevisiae*. Plasmids were constructed and introduced into the *S. cerevisiae* strain CEN.PK2-1C (MATa ura3-52 trp1-289 leu2-3,112 his3Δ 1 MAL2-8C SUC2) by yeast assembly employing the homologous recombination system of the organism (Joska *et al.*, 2014). The plasmid pRS426 containing a phosphoglycerate kinase promotor sequence, the secretion signal XYNSEC and a cytochrome c terminator sequence was used as a PCR template. The strain and the template were kindly provided by Dr. H. Kroukamp (Macquarie University). Overlapping fragments were

generated using the primers in Table 1. Primers 1 and 2 were used for fragment 1 of an expression plasmid without XYNSEC, the primers 1 and 3 for fragment 1 of an expression plasmid with XYNSEC as well as the primers 4 and 5 for fragment 2 of both expression plasmids (Lilly *et al.*, 2009, Sikorski and Hieter, 1989, Yamanishi *et al.*, 2011). The His-tagged ORF light_m.20624 was amplified from the pUCIDT plasmid as fragment 3 with homologous regions to the expression plasmid fragments 1 and 2, respectively, using the primers 6 and 7 for the expression plasmid without XYNSEC and the primers 8 and 7 for the expression plasmid with XYNSEC.

Table 1: List of primers used in this study. The IDs of the primers are indicated. The primer sequences are displayed in 5'-3' direction.

IDs	Sequences
1	TATACTAAGAAACCATTATTATCATGACATTAACC
2	TATAGACGATGTCTTAATTAAATTTGTTGTAAAAAGTAGATAATTACTTCC
3	TAATAGATCTACCTTGATCAGGATCCTATCTTTTTTCTACAGCAACGGAC
4	TAATGCTAGCCATCACCATCACCATCACTAGTAGGGCGCGCCTTTTCCTTTGT
5	TATATGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGG
6	TAATGGAAGTAATTATCTACTTTTTACAACAAATATGTACGGTTCTGTTCCGGCG
7	TATACGCGCCCTACTAGTGATGGTGATGGTGATGCGGAGAGGTCGCACGTTCTTTTT
8	TTATAGAAGTCGAGTCCGTTGCTGTAGAAAAAAGATACGGTTCTGTTCCGGCGGA

The fragments were combined as needed with the yeast to generate respective episomal expression plasmids. Transformed yeast was cultivated for expression in double-strength buffered synthetic complete medium without uracil at 30°C with shaking at 200 rpm (Kroukamp *et al.*, 2013).

Expression was monitored by subjecting cell mass and medium, respectively, at different time points after inoculation (up to 5 d) to SDS-PAGE, compared to samples of cells without an expression plasmid.

2.12. Congo Red dye plate assay

Commercially-available enzymes were acquired for the Congo Red dye plate assay. Viscozyme L from *Aspergillus aculeatus*, Celluclast 1.5 L from *T. reesei* and Pulpzyme HC from *Bacillus agaradhaerens* were acquired from Novo Nordisk (Bagsværd, Denmark). Pyrolase 160 1X and 3X from a non-disclosed thermophilic microorganism and hemicellulase from *Aspergillus niger* were acquired from BASF (Ludwigshafen, Germany).

The Viscozyme L enzyme preparation was buffer-exchanged with 50 mM sodium acetate buffer using a Sephadex G-25 column (GE Healthcare) and the proteins were eluted in 50 mM sodium acetate buffer, pH 5. The preparation was then subjected to size exclusion chromatography (SEC) on an ÄKTA Pure system (GE Healthcare) to enrich for potential paramylon-degrading enzymes. The SEC was conducted with a Superdex 200 10/300 GL column (M_W range of 10-600 kDa, GE Healthcare) with 50 mM sodium acetate buffer, pH 5, at a flow rate of 0.5 mL/min and fractions were collected during elution in intervals of 1 mL and numbered 1-11 (i.e. the number of fractions was chosen to ensure there was enough protein in each fraction for any downstream applications). The fractions were concentrated around 50 times using an Amicon Ultra-15 centrifugal ultrafiltration unit (10 kDa cut-off, Merck).

Substrate plates containing 0.5-2% (w/v) agarose, supplemented with 0.5% (w/v) of either carboxymethyl-cellulose (CM-cellulose), xylan, dispersed paramylon or paramylon granules, were prepared in ultrapure water. Hot suspensions of the insoluble substrates paramylon granules and dispersed paramylon were homogenised using a T 10 basic ULTRA-TURRAX disperser (IKA,

Staufen, Germany). The homogenised suspensions were quickly poured in plates placed on a precooled metal tray, thereby speeding up agar solidification to prevent the settling of the substrate.

5 µL of each commercial enzyme, in addition to *T. reesei* culture supernatant (see section 2.1.) and *E. gracilis* crude extract in 100 mM sodium acetate buffer, pH 5, were spotted onto the surface of a substrate plate. The plates were incubated overnight at 37°C or 70°C, depending on the nature of assayed enzymes (mesophilic or thermophilic origin). Substrate hydrolysis was visualised by flooding the surface of the agar plates with a solution of 1% (w/v) Congo Red dye for 10 min. Unbound dye was removed after incubation with 1M sodium chloride for 30 min on a rocking shaker. When necessary, the plates were additionally rinsed with 0.5% (v/v) glacial acetic acid to turn the colour of Congo Red to violet/dark purple and increase the intensity of the clearing zones.

2.13. Statistical analysis

The programs Excel (Microsoft Corporation, Redmond, USA) and Prism 7 (GraphPad Software, La Jolla, USA) were used for statistical analyses. Experiments subjected to statistical analyses were performed in technical or biological replicate (in duplicate or at least in triplicate, respectively) and qualitative experiments were repeated several times. Results of experiments subjected to statistical analyses were expressed as mean ± standard deviation (SD). Student's t-tests were performed to determine significant differences between two data sets as parametric, unpaired, two-tailed tests, with a confidence level of 95%.

3. Results and discussion

3.1. High-throughput screening

The first approach for the identification of paramylon-degrading enzymes produced by *E. gracilis* based on function was a high-throughput screening of a cDNA library. The cDNA library was prepared from cell mass of an *E. gracilis* culture (Fig. 1) in a growth phase where the overall dry

mass stayed nearly constant while the paramylon content was declining (i.e. 8 d after inoculation). Consequently, transcription of genes coding for paramylon-degrading enzymes were likely to be upregulated and the cDNA library potentially enriched with respective mRNAs. Plasmids containing the cDNA fragments were transformed into *E. coli* BL21 (DE3) for heterologous expression, yielding 2.3×10^7 transformants from initially 3×10^6 primary transformants with 63% recombinants. The average insert size was 0.7 kb with a maximum of 1.5 kb. The percentage of recombinants was low and the average insert small compared to cDNA libraries prepared from other organisms (personal communication Express Genomics) and could have been due to problems during RNA extraction caused by the high concentrations of lipids and/or polysaccharides commonly occurring in microalgae (Poong *et al.*, 2017).

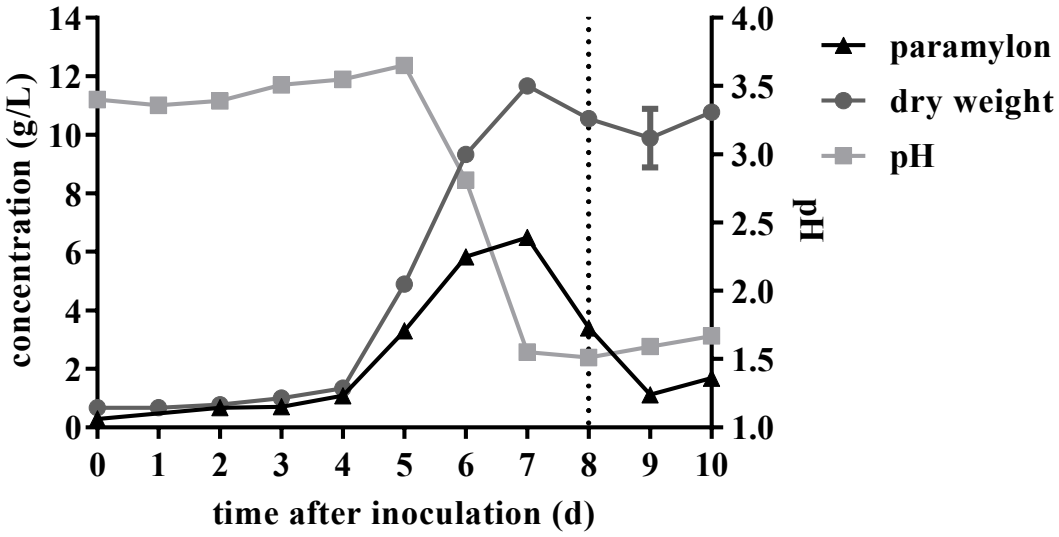

Fig. 1. Growth curve and paramylon content of the *E. gracilis* culture used for the cDNA library preparation. The paramylon content was declining while the dry weight was plateauing 8 d after inoculation (indicated by the dotted line). Cell mass harvested on this day was used for the cDNA preparation. Error bars represent the SD.

High-throughput FACS assays have been shown to be powerful tools for the identification of certain enzyme activities (e.g. cellulase activity) in large populations (Ostafe *et al.*, 2014). *E. gracilis*-derived cDNA libraries have been used widely to screen for single genes or enzymes. However, the target sequences of the screenings have been identified based on sequence homologies, either at the DNA or amino acid sequence level, without establishing a link between

genotype (DNA sequence) and phenotype (function of the enzyme, Houlné and Schantz, 1988, Nowitzki *et al.*, 2004, Sharif *et al.*, 1989).

FDGlu is a fluorogenic substrate mimicking paramylon as both have β-glycosidic bonds and it is suitable for the detection of β-glucosidase and β-1,3-glucanase activity (Cid *et al.*, 1994, Hardiman*, et al.*, 2010). We developed an FDGlu-based FACS assay in combination with auto-induction medium in order to screen the *E. gracilis* cDNA expression library for paramylon-degrading enzymes directly linking genotype and phenotype (Fig. 2). The advantage of using an auto-induction medium is that there is no need for lengthy, labour-intensive monitoring of growth before the induction of expression (Studier, 2014).

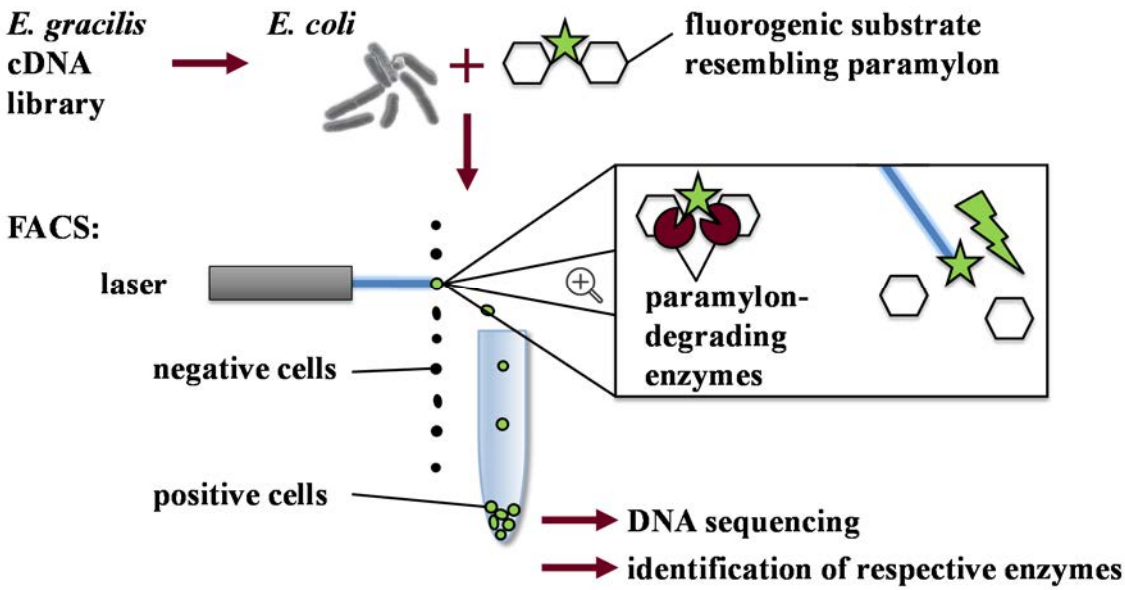

Fig. 2. Overview of the FACS strategy for the screening of paramylon-degrading enzymes possibly coded in the *E. gracilis* cDNA library. *E. coli* transformants containing the cDNA library are incubated with the fluorogenic substrate FDGlu and cells producing enzymes capable of catalysing the hydrolysis of β-glycosidic linkages (positive cells) are sorted out *via* FACS. The plasmids of sorted cells can then be sequenced to identify the gene (cDNA) sequences of respective enzymes and their translated amino acid sequences.

Initial FACS experiments leading to the development of the concept have been performed with *E. coli* cells expressing the β-glucosidase gene *bglA* from *C. saccharolyticus* (Hardiman*, et al.*, 2010).

Flow cytometry results confirmed here that negative (*E. coli* cells without expression plasmid) and positive cells (*E. coli* harbouring the *bglA* expression plasmid) could be distinguished based on an increase in fluorescence intensity upon FDGlu hydrolysis by the β-glucosidase enzyme (Fig. 3). A distinctive population representing negative cells was easily identified (Fig. 3 A) while the supposedly positive cells displayed a main population of cells expressing the β-glucosidase (Fig. 3 B, upper population) in addition to a subpopulation of negative cells (Fig. 3 B, lower population). This subpopulation of negative cells probably represented cells lacking the capability to express *bglA* due to mutations on the respective plasmid. Recovery of potentially positive cells was tested by sorting negative and positive cells into auto-induction medium. This extra validation step was capable of unambiguously confirm the positive and negative status of the previously sorted cells (Fig. 3 C).

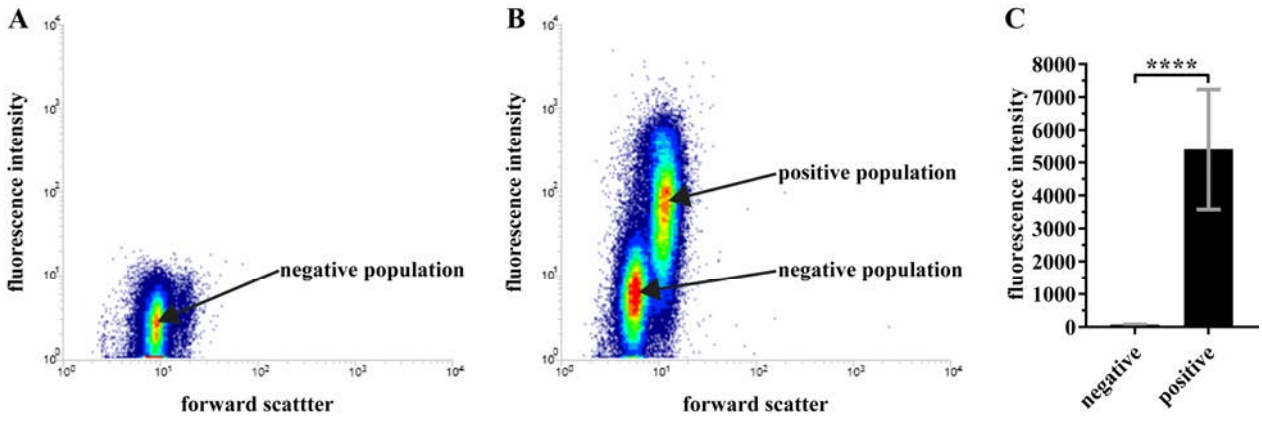

Fig. 3. Flow cytometry of control cells and assessment of the validation step of the FACS/auto-induction medium system. Negative control cells without expression (A) and of the positive control cells expressing bglA (B) were incubated with FDGlu before the analysis. Positive and negative populations of cells are indicated. Previously sorted cells of the negative (A) and the positive control (B, positive population) were incubated with FDGlu in the validation step (C) before the analysis. Error bars represent the SD. A statistically significant difference ($P \leq 0.05$) is indicated: **** for $P \leq 0.0001$.

The *E. gracilis* cDNA expression library was sorted after incubation in auto-induction medium as described above. A thousand cells of a potentially positive population were sorted in an enrichment step but virtually none of this population were identified as positives in the next round of FACS intended for the isolation of single positives cells. This result probably meant that only false-

positive cell were sorted before within the variation of the assay and that the cDNA library did not actually contain positive cells in the first place. The lack of positive cells in the last FACS round might be directly related to the quality of the cDNA library. The catalytic domain of most glucanases have an average size around 1.5 kb (Gilkes *et al.*, 1991). Similarly, the only functional *E. gracilis* β-1,3-glucanase identified so far has a similar average size (Takeda*, et al.*, 2015). The lower than expected average insert size (0.7 kb) of the cDNA library meant that there were only few inserts long enough to code for a functional glucanase. The screening potential of the cDNA expression library was thereby reduced and in concurrence its capacity for identification of functional glucanase genes, probably to such a degree that the FACS approach became infeasible.

3.2. Proteomics

3.2.1. Subsets of the proteome of E. gracilis

A proteomic-based analysis was investigated as an alternative to the high-throughput strategy presented above to identify paramylon-degrading enzymes from *E. gracilis*. Analysis of a subset of the *E. gracilis* proteome that is specifically enriched with proteins of interest could establish a link between a particular amino acid sequence and its function. A similar approach was previously used for the identification of the first functional paramylon-degrading enzyme from *E. gracilis* and of a β-1,3-glucan phosphorylase involved in the β-1,3-glucan metabolism of *E. gracilis* (Kuhaudomlarp *et al.*, 2018, Takeda*, et al.*, 2015).

Three different *E. gracilis* protein fractions were prepared for LC ESI MS/MS analysis and subsequent database searches were conducted to identify potential paramylon-degrading glucanases by enriching candidate proteins as described in Figure 4.

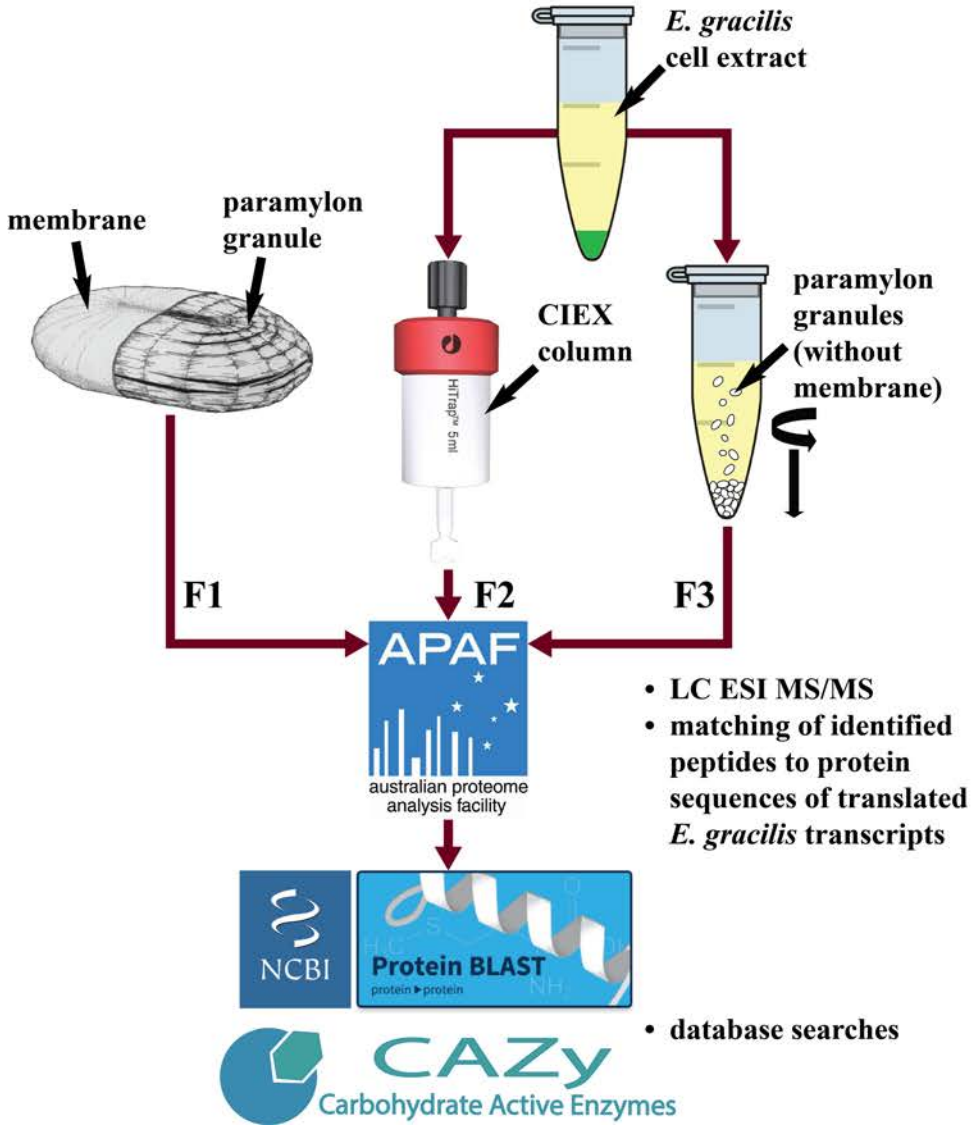

Fig. 4. Overview of the proteomics strategy. Protein fractions 1-3 (F1-F3) are indicated. F1: paramylon membrane. F2: fractions of *E. gracilis* cell extract. F3: proteins bound to paramylon in an SBA. The image of the paramylon granule with part of the paramylon granule membrane omitted is from Kiss *et al.*,1987, Structure of the euglenoid storage carbohydrate, paramylon, *American Journal of Botany* © 1987 by John Wiley Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.

Fraction 1 comprised proteins from the paramylon membrane sample (Fig. 5 A) as it has been suggested that proteins of the paramylon membrane could be involved not only in the synthesis but also in the degradation paramylon (Barras and Stone, 1969, Bäumer*, et al.*, 2001).

Fraction 2 was from *E. gracilis* cell extract subjected to CIEX to enrich soluble cytosolic glucanases (Barras and Stone, 1969). After CIEX, 10 fractions were obtained with distinctive protein profiles (Fig. 5 B) and their enzymatic activity against paramylon granules (Fig. 5 D) and

dispersed paramylon (Fig. 5 E) was determined using the DNS reducing sugar assay. A previous study showed that enzymatic activity of paramylon-degrading enzymes is usually higher against dispersed paramylon than against paramylon granules (Vogel and Barber, 1968). Similarly, the CIEX fractions obtained here showed higher activity (expressed as glucose equivalents) against dispersed paramylon. CIEX fractions 6-10 were most active against dispersed paramylon and paramylon granules. Accordingly, these fractions were combined before submission to LC ESI MS/MS, assuming that they contained the highest concentrations of paramylon-degrading enzymes of all the fractions.

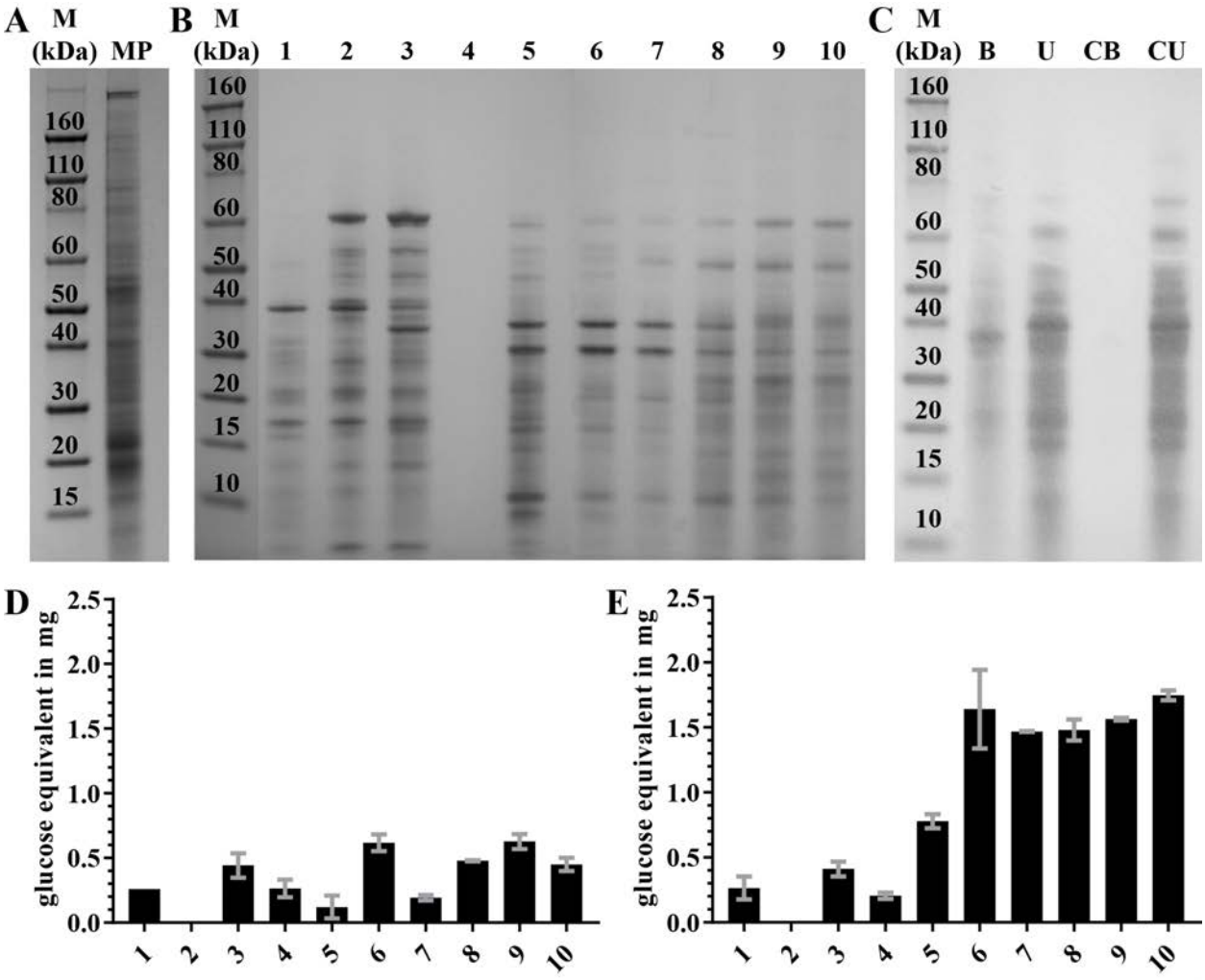

Fig. 5. SDS-PAGE images of the protein fractions 1-3 (A-C, respectively) and reducing sugar (in glucose equivalents) produced enzymatically by the CIEX fractions within 10 h from paramylon granules (D) and dispersed paramylon (E). A-C: the lanes of the markers (M) and the following samples are indicated. Membrane proteins (MP), numbered CIEX fractions (1-10), bound fraction (B), unbound fraction (U), bound fraction of the control (CB) and unbound fraction of the control (CU). D and E. The SD is represented as error bars.

Many cellulases and β-1,3-glucanases are multi-domain proteins with catalytic domains and associated carbohydrate-binding modules (CBMs). CBMs have been shown to increase the concentration of enzymes on the carbohydrate substrate, thereby aiding an effective substrate utilisation (Hashimoto, 2006, Várnai *et al.*, 2014, Watanabe *et al.*, 1992). We assumed that paramylon-degrading enzymes could interact with their substrate in a similar or comparable fashion and conducted a binding assay to isolate potential paramylon-degrading enzymes from *E. gracilis* cell extract using paramylon granules (membrane-less) as substrate. Fraction 3 were the proteins obtained by this SBA and SDS-PAGE analysis (Fig. 5 C) indicated that several proteins remained bound to the paramylon granules (Fig. 5 C, lanes B and U). No detectable amount of proteins was found in the final bound fraction of the sample of the control SBA without substrate (Fig. 5 C, lane CB), confirming that proteins from the *E. gracilis* cell extract were indeed bound to the substrate rather than the result of precipitation during the SBA. The bound fraction from the *E. gracilis* cell extract and its corresponding control were subjected to LC ESI MS/MS.

Five ORFs of putative enzymes with potential activity against paramylon were identified through database searches (see Supplementary data for the DNA sequences of the ORFs and their translated protein sequences). The M_W of the translated proteins were calculated and the proteins were categorised by glycoside hydrolase (GH) families (Table 2) according to the CAZy database. The IDs of the identified ORFs referred to the *E. gracilis* transcriptome used for the database search, which contained entries from light and dark-grown *E. gracilis* cultures. However, respective ORFs could have been expressed under both conditions even though they were labelled either 'light' or 'dark' as the transcriptome was compiled non-redundantly. ORF dark_m.440 was identified to code for the enzyme Egcel17A, the only functional endo-β-1,3-glucanase so far identified from *E. gracilis*, while the other ORFs were also annotated to code for endo-β-1,3-glucanases (Takeda*, et al.*, 2015). Similar to cellulose hydrolysis, paramylon degradation might require the concerted action of several enzymes. Paramylon is most likely first digested extensively by endoglucanases

before exoglucanases and β-glucosidase hydrolyse the freed-up glucan chains, ensuring an efficient degradation of the paramylon polymer (Dimarogona*, et al.*, 2012). The theoretical M_W of the proteins coded by the ORFs were in the range of the M_W of fungal endoglucanases (e.g. from *Trichoderma* spp., Kim *et al.*, 1994, Messner *et al.*, 1988, Nobe *et al.*, 2004). The GH families of the identified ORFs include mostly members either from bacteria (GH 55 and 64) or from eukaryotes (GH 17 and 81) but also from archaea (GH 81). This diversity of origins and/or relationships probably reflects the complexity of the genomic history of *E. gracilis* as a result of many horizontal gene transfer events (O'Neill *et al.*, 2015). Remarkably, most of the candidate sequences were from the membrane fraction, substantiating further the hypothesis that not only paramylon synthesis but also its degradation is associated with the membrane.

Table 2. Experimental origin, IDs, calculated Mw, category and (predicted) enzymatic activity of the ORFs identified by LC ESI MS/MS and database searches.

Origin	ORF ID[a]	Mw (kDa)	GH family[b]	enzymatic activity
CIEX	light_m.26257	110.5	81	endo-β-1,3-glucanase
membrane	light_m.20624	46.1	64	endo-β-1,3-glucanase
membrane	light_m.63754	63.8	64	endo-β-1,3-glucanase
membrane/SBA	dark_m.22013	67.1	55	exo-/endo-β-1,3-glucanase
SBA	dark_m.440 (Egcel17A[c])	40.8	17	endo-β-1,3-glucanase[c]

[a]O'Neill*, et al.*, 2015, [b]Lombard*, et al.*, 2014, [c]Takeda*, et al.*, 2015

ORF light_m.20624 was considered for heterologous expression as the theoretical M_W of the protein it is coding for was closest to the M_W of the enzyme coded by ORF dark_m.440 (i.e. Egcel17A), which has been produced functionally in *E. coli* (Takeda*, et al.*, 2015).

3.2.2 Heterologous expression of a potential endo-β-1,3-glucanase from E. gracilis

ORF light_m.20624 was heterologously expressed in *E. coli* with six 3'-end-coded histidine residues (referred to as His-tag) to produce and purify the recombinant protein to verify its assumed endo-β-1,3-glucanase activity. Expression was monitored at different time points by SDS-PAGE. A 40 kDa protein band appeared 1 h after induction of protein expression (Fig. 6), which matched the theoretical M_W of the putative protein coded by ORF light_m.20624 (Table 2). The protein band (after 3 h expression) was excised from the SDS-PAGE gel and subjected to LC ESI MS/MS, which confirmed that this protein was encoded by ORF light_m.20624.

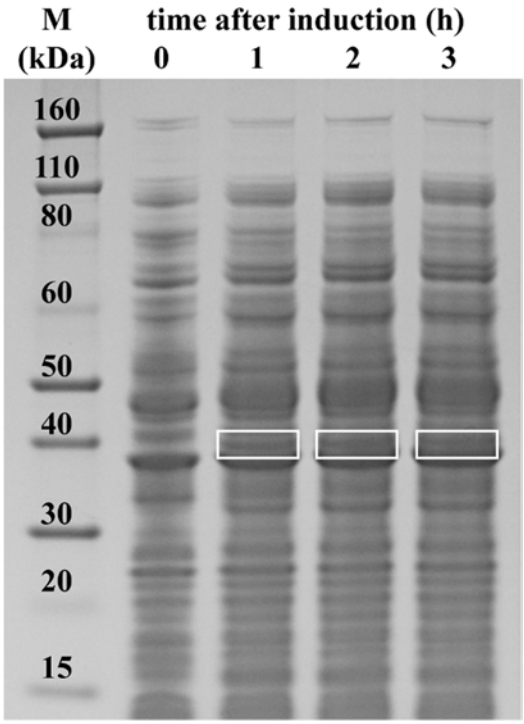

Fig. 6. SDS-PAGE of the expression of ORF light_m.20624 in *E. coli* BL21 (DE3). The lanes of the marker (M) and of the samples taken at different time points (0-3 h) after the induction of expression are indicated. The bands of the protein encoded by ORF light_m.20624 appearing 1 h after induction are indicated by boxes.

Most of the 40 kDa ORF light_m.20624 protein was present in the insoluble protein fraction (data not shown). Several attempts to increase the production levels of soluble protein were performed including different induction temperatures (37 and 20°C) and times (1-3 h and overnight, respectively) as well as expression in *E. coli* Tuner (DE3) at the same conditions. However, none of these increased the final yield of soluble ORF light_m.20624 protein. His-tag purification did not result in concentration of the recombinant protein and the levels of soluble protein were deemed too low for enzyme activity assays. Furthermore, expression of ORF light_m.20624 was attempted in *S. cerevisiae* under the control of a constitutive promotor (for up to 5 d, with and without a secretion signal) but protein production was not detectable (data not shown). Previous reports indicated the difficulty of producing *E. gracilis* proteins heterologously in different prokaryotic and eukaryotic host organisms like *Aspergillus oryzae*, *Brevibacillus choshinensis*, *E. coli* and *Nicotiana benthamiana* (Kuhaudomlarp*, et al.*, 2018, Ntefidou *et al.*, 2006, Takeda*, et al.*, 2015). The possible reasons for these difficulties have not been attracting much attention so far and could be manifold. There are various complex or unique mechanisms in *E. gracilis* for the regulation of gene expression which are little understood (e.g. the possible utilisation of the hypermodified Base J as a transcription terminator) and the presence of these mechanisms might be crucial for the expression of some genes (Borst and Sabatini, 2008, O'Neill*, et al.*, 2015). Considering the unsuccessful attempts described above, it was decided not to pursue further heterologous expression of the identified ORFs but rather to assess the paramylon-degrading capabilities of readily-available enzymes from commercially-resourced preparations.

3.3. Congo Red dye plate assays

Measuring the activity of potential paramylon-degrading enzymes can be quite cumbersome and laborious quantitative (colourimetric) assays are usually employed (Giese*, et al.*, 2011, Takeda*, et al.*, 2015, Vogel and Barber, 1968). We considered a simple dye-based agarose plate assay as an alternative to the quantitative assays. Congo Red dye has been shown to have strong interaction

with β-1,4 and β-1,3-linkages in several polysaccharides and has been used in plate screening assays to identify enzymes that are able to hydrolyse cellulose and other high-M_W β-glucans (Sutivisedsak *et al.*, 2013, Teather and Wood, 1982, Wood and Fulcher, 1978). However, to our knowledge, a Congo Red-based plate assay has not been established with paramylon as substrate. We developed a paramylon hydrolysis plate assay using dispersed paramylon or paramylon granules in combination with Congo Red visualisation to assess the ability of several enzyme preparations (see section 2.12) to hydrolyse paramylon. The enzyme preparations included commercially-available enzymes, *T. reesei* QM6a culture supernatant and *E. gracilis* cell extract (Fig. 7 and 8).

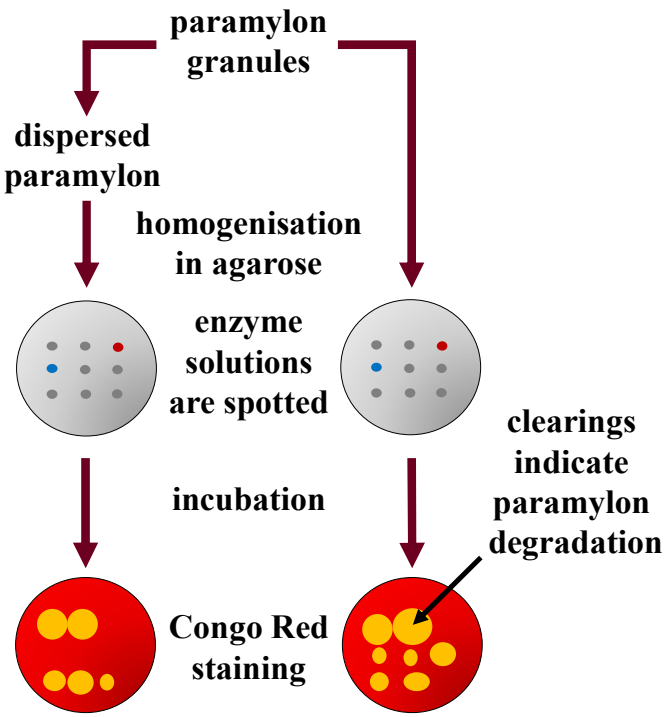

Fig. 7. Overview of the Congo Red dye plate assay strategy. Paramylon granules and dispersed paramylon are homogenised in hot agarose to allow the pouring of plates. Selected enzyme solutions are spotted on the plates to test if they contain paramylon-degrading enzymes. The plates are then incubated at temperatures estimated to be suitable for the respective enzymes. The staining of the plates with Congo Red dye followed by a wash step reveals activity against paramylon indicated by yellowish clearings.

Cellulose, hemicellulose and xylan are polysaccharides with β-glycosidic linkages and enzymes able to degrade these substrates might also show promiscuity towards paramylon (Naidu *et al.*, 2018, Tian *et al.*, 2016). The commercially-available enzymes and *T. reesei* QM6a culture

supernatant comprised (hemi)cellulase and/or xylanase activities targeting these and other linkages (FAO/WHO, 2004, Gibbs *et al.*, 2010, Novozymes, 2009, Novozymes, 2015, Sheir-Neiss and Montenecourt, 1984, Verenium, 2012). Plates containing CM-cellulose or xylan (Fig. 8 A and B) served as controls for the activity of these enzymes. In addition to β-1,4-specific glucanases, *T. reesei* also produces β-1,3-specific glucanases (Bamforth, 1980). For example, Celluclast 1.5 L, a preparation of *T. reesei* enzymes, was shown to have activity against paramylon. (Suzuki*, et al.*, 2017, Vogel and Barber, 1968). Also, the enzymes in *E. gracilis* cell extract are capable of degrading paramylon *in vitro* (Suzuki*, et al.*, 2017, Vogel and Barber, 1968). Therefore, Celluclast 1.5 L, the *T. reesei* QM6a culture supernatant and *E. gracilis* cell extract were regarded as positive controls for the hydrolysis of paramylon in the Congo Red-based plate assay. All enzyme preparations except Pyrolase 200 and Pulpzyme were active against dispersed paramylon (Fig. 8 C and F). However, only Celluclast 1.5 L, *T. reesei* culture supernatant and *E. gracilis* cell extract showed activity against paramylon granules. Pyrolase 160 (like Pyrolase 200) is derived from thermophilic microorganisms and has a relatively high temperature activity optimum at around 70°C (Levine, 2013, Verenium, 2007). Accordingly, the Pyrolase 160 1X and 3X showed higher activity against dispersed paramylon when the incubation temperature of the plate assay was increased to 70°C (Fig. 8 F). However, neither of the Pyrolase enzymes was able to hydrolyse paramylon granules and Pyrolase 200 did not degrade dispersed paramylon even at this assay temperature (data not shown).

In summary, Viscozyme L, Pyrolase 160 (1X and 3X) and hemicellulase from *A. niger* were identified to be able to degrade paramylon. Viscozyme L displayed the highest activity against paramylon of all the enzyme samples tested with the Congo Red plate assay (Fig. 8). This preparation contained a range of polysaccharide degrading enzymes including cellulases, xylanases, β-glucanases and hemicellulases (Guan and Yao, 2008, Novozymes, 2015).

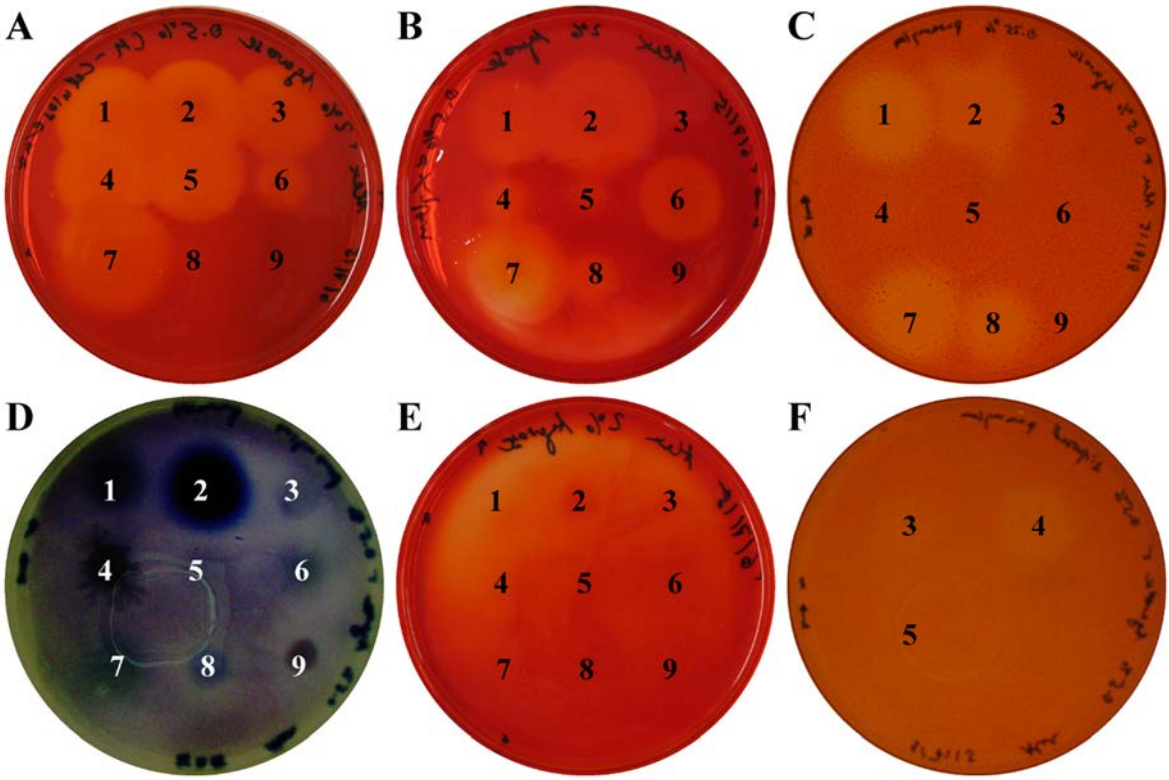

Fig. 8. Congo Red dye plate assay. The plates contained different substrates, indicated as follows. CM-cellulose (A) and xylan (B) as controls for enzymatic activity, dispersed paramylon (C and F) and paramylon granules (D). The negative control did not contain any substrate (E). Plates A-E were incubated at 37°C overnight, plate F at 70°C. Clearings on plate D were visualised using acid. 5 µL each of various commercial enzymes and other enzyme preparations were applied. Viscozyme L (1), Celluclast 1.5 L (2), Pyrolase 160 1X (3), Pyrolase 160 3X (4), Pyrolase 200 (5), Pulpzyme HC (6), hemicellulase (7), *T. reesei* culture supernatant (8), *E. gracilis* cell extract 1X (A-C 9) and approximately 4X concentrated (D 9).

SEC fractionation coupled with the Congo Red plate assay was implemented in an attempt to enrich the enzyme(s) responsible for paramylon degradation from the Viscozyme L complex enzyme preparation. Several distinctive protein fractions were obtained from the SEC fractionation as shown by SDS-PAGE (Fig. 9 A). The original enzyme preparation (before fractionation) and SEC fractions 2-7 showed activity against dispersed paramylon and paramylon granules (Fig. 9, C and D). The enzymatic activities of the fractions were deducted from the different diameters of the clearing zones and the different degrees of substrate clearing. Fractions 4-6 had the highest enzymatic activities against both paramylon substrates and they displayed protein profiles with prominent protein bands between 40-60 kDa. These bands could belong to the proteins of interest as *A. aculeatus* has been shown to produce glycolytic enzymes with similar M_W, for example a 45 kDa endoglucanase (Naika *et al.*, 2007). Their analysis by LC ESI MS/MS might reveal the

enzyme(s) responsible for the high activity of respective fractions against paramylon in a fashion similar to the proteomics approach presented above. Viscozyme L showed undoubtedly activity against paramylon granules in this assay, even though it did not do so in the earlier activity plate screening (Fig. 8 D).

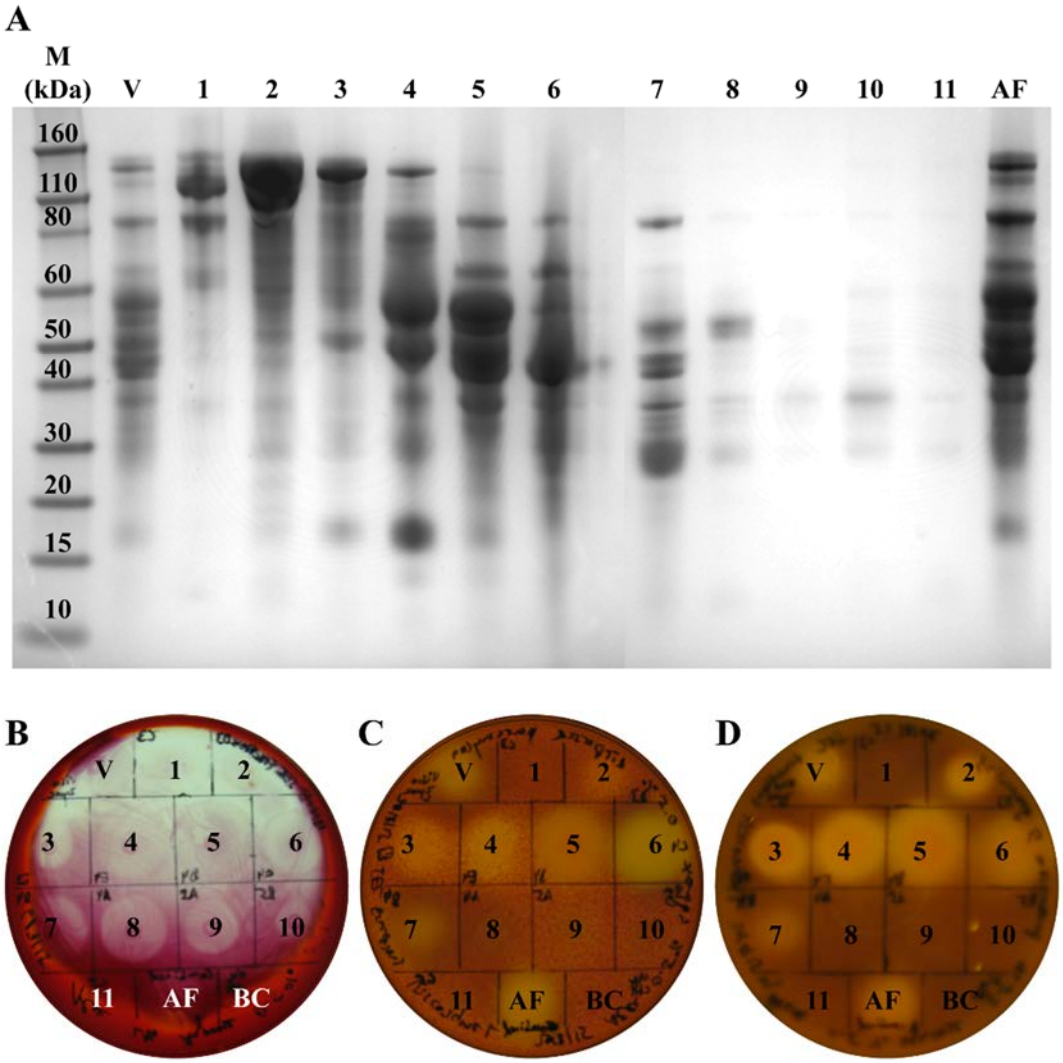

Fig. 9. SDS-PAGE images (A) and Congo Red dye plate assays (B-D) of buffer-exchanged Viscozyme L (V), its SEC fractions (1-11) and of all fractions combined (AF). The SDS-PAGE gel lanes of the marker (M) and of the samples are indicated. The plates contained the following substrates. Xylan (B) as control for enzymatic activity, dispersed paramylon (C) and paramylon granules (D). 50 mM sodium acetate buffer, pH 5, was the buffer control (BC). 5 µL each of the fractions, of AF and of the BC were applied.

The only difference between these assays was the inclusion of an additional buffer exchange step before the second assay. The optimal pH condition for Viscozyme L with its several activities

reportedly was in the pH range of 3.3-5.5 (Novozymes, 2015). Accounting for the different results obtained in both plate screening tests, it is possible that the formulation of the original buffer of the Viscozyme L enzyme complex with pH 3-4 was optimised for storage rather than activity or that especially the enzymes responsible for the degradation of paramylon are more active at pH 5.

4. Conclusions

To the best of our knowledge, we report here the first cDNA library prepared from a culture of *E. gracilis* during the growth phase of paramylon degradation, aimed at expression of the library in *E. coli* with a quest for finding genes encoding paramylon degrading enzymes. A high-throughput cDNA library screening assay based on a fluorescent substrate analogue for paramylon and FACS in combination with time-saving auto-induction medium was developed to identify potential paramylon-degrading enzymes and ultimately their respective genes. This assay could be extended to screening of gene libraries from other organisms or to screen for other enzyme activities by using a different enzyme-specific fluorogenic substrate.

Five ORFs were identified *via* the proteomic analysis approach. Four of these ORFs (dark_m.22013, light_m.20624, light_m.26257 and light_m.63754) could potentially code for paramylon-degrading β-1,3-glucanases and warrant further investigation. The fifth ORF (dark_m.440) was already identified to encode the enzyme Egcel17A, the only functional endo-β-1,3-glucanase so far identified from *E. gracilis*, and confirmed the validity of the entire proteomic approach (Takeda*, et al.*, 2015).

The Congo Red plate assay developed here with dispersed paramylon and paramylon granules as a substrate, respectively, was shown to work with commercial enzymes and laboratory preparations and thereby provides a new tool for the direct screening of paramylon-degrading enzymes or microorganisms capable of degrading paramylon. Plates containing dispersed

paramylon were more sensitive to enzymatic degradation than those containing intact paramylon granules. Therefore, the dispersed paramylon plates could be used to identify single enzymes with β-1,3-glucanase activity whereas the plates containing granules could be used to identify and study mixtures of synergistic paramylon-degrading enzymes. The simplicity of the plate screening assay has several advantages over lengthy and more complex approaches such as proteomics. The plate-based method allows the screening of multiple preparations simultaneously and provides flexibility since the screening parameters (temperature, pH and time) can be adjusted easily as required. The plate assay possibly could be improved to a quantitative method by measuring the intensity of the clearing with imaging software.

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Appendix A. Supplementary data

S.1. E. gracilis ORFs of putative enzymes with potential activity against paramylon

S.1.1. ORF dark_m.440

Table S.1. Nucleotide sequence of ORF dark_m.440.

ATGATTCGCCTTTTGGGTCTGGCATTGTTGGCATGCACAAGCCACGCCACGGAGCTGGGATGGAATATCA
ACTTCGGCTTCCAGCAAAATCCGCTGAACCCGGATGTTGCGGTTGCAAAATTGAAGTCCCTCATTCCACA
GCTGAACTATTCCAAGACGTTCGACTACAATGCAACAGTGTTGGCAGCATTGCACACACATGGAATTCGT
AACATGGTCGTTGGCATTCCCAACTCCGACCTGCAAAGTATTGCAACAAGTGGAAACCCCCTGGTGGGAA
CGATCCTTGATGGGCTGAAGCCACTGTACGATGACGGTGTCCAACTCACCATCGCCGTGGGGAACGAACC
AACCCTTGCCACTTATGGAACTGCATACTCCCCTTGGGTGTATCCTGCCCTGCTCAATGTCCGCTCCACA
TTGTCCAACAAGTACATGAACAAAGTCAAATTGACTGTCCCCTTCGATTCCGGCATCCTTGGGACCAGCT
ATCCGCCCAGCCAGGGAATTTTTTCAATCAGCACAGCCAGTGTTGTCACCACCGTTGCAGAATTTCTGAA
GAATGAAGGGTCACCTTTCACTGTGAACCTGTATCCTTTCTTCTCACTGGTTGACAACCCCACTGATGTG
AGTGTTGCTTATGCTACATTGCAGACTGGCCTCACTGCCTCGGATGGCATCACGTACCCCAACATGCTGG
CGGCGATGGTGGCTGCTGTGCGCGCTGCGCTGTTGCACCAGGACCCAGTCCTGACAGAGGCAAACTTGCC
CATCATTGTCGGTGAGACTGGCTGGCCAACTTCAGGCAACACCTACGCCACGGTGGAAAACGCCCAGACG
TACGTCAACAACGCCGTCAACTGCGGGATTCCGCTGTATGGCTTCGAGGCCTTCGACGAGAAGCTGAAGA
CGAGCGGCAGCGGCAGCGGATCCACCAGTTCTGTTGAGGGCAGCTGGGGATGGATGTCAGAGGGAGGCGA
CCCCAAGTTTCCCATCAACTGGCCCACGGGACCAGTGGCGCCAGCGGAGACGTGCGATTCCAAATTCCCA
CCTGCCACTGGAGAGTTCGTGAAACTTGTTTGCCCACCCAACACCCTCGCAGGGTGGTTGCAATCTGGCT
CTTGTCAGCAGGATTCGGACTGCGATGTCATCTCCTGCCCCGAGGTGCCCAAGGATACGGTGGTGGCCAC
CTGTTCCAGCGTCTAA

Table S.2. Translated amino acid sequence of ORF dark_m.440.

MIRLLGLALLACTSHATELGWNINFGFQQNPLNPDVAVAKLKSLIPQLNYSKTFDYNATVLAALHTHGIR
NMVVGIPNSDLQSIATSGNPLVGTILDGLKPLYDDGVQLTIAVGNEPTLATYGTAYSPWVYPALLNVRST
LSNKYMNKVKLTVPFDSGILGTSYPPSQGIFSISTASVVTTVAEFLKNEGSPFTVNLYPFFSLVDNPTDV
SVAYATLQTGLTASDGITYPNMLAAMVAAVRAALLHQDPVLTEANLPIIVGETGWPTSGNTYATVENAQT
YVNNAVNCGIPLYGFEAFDEKLKTSGSGSGSTSSVEGSWGWMSEGGDPKFPINWPTGPVAPAETCDSKFP
PATGEFVKLVCPPNTLAGWLQSGSCQQDSDCDVISCPEVPKDTVVATCSSV

S.1.2. ORF dark_m.22013

Table S.3. Nucleotide sequence of ORF dark_m.22013.

ATGTCCCTCGCCATGCCCCGGCCCCTGCCGGCCTTCCTCTGGCTGCTGCTGGTGCTGCCCCACGGGCGCC
CCACGGGGGCCGCCGTCGCGCCCGTCCAGCCGCAGCCCAACCCGCCGCAGTGGCCCCCCGGCGTGACGGT
GTTTGGCCCGGGGGACCCCACCATTGAGCAGACCGTCAATGCCGCGTACCGGCGCAACGGCGGCCACGAC
CCCCCATGCAATGGCCAGTTCAGCGACGAGCGGTATGCGTTCCTCTTCAAGCCGGGGGTGTACCCGGTGG

ACGTGCCGGTGGGCTACTACACCCAGGTGCTGGGGCTGGGTGAATCCCCGGACGACGTCGTGTTCACGGG
CCCAAAGGGGGTCCACGCCGAGGAGGGGGATTATGCTTTCACCGTCGGGGCATTGAACAACTTCTGGCGG
TCGGCGGAGAACTTCCAGACAACCTCGGCGTACCCGTGGTTTCCGGGCTTCAACGGCATGCTGTGGGCGG
TTTCCCAGGCATCCCCGCTGCGCCGGGTCCACGTCCAGAGCAACCTCATCCTCTTTCAGTACACATATGG
CGATGCCGCCGGGTTTGCCTCCGGCGGCTACCTTGGCAACTCTCAGGTGGATGGGAAGGTGGTCTCCGGG
TCGCAGCAGCAATGGGTGACACGGAACTGCCGCCTCGGGGCTTGGGACGGAGCGGTGTGGAACATGGTGT
TTGTCGGATCCACTGGCACGCCCACCGCCCACTGTGGAAACGTGGACGGCCGGCCATATGTCGTGGAGCC
CGCCACCCCCGTGGTGGCGGAGAAGCCGTTCCTCACCATCGACAGCGCGGGGCGGTATTACCTGAATGTC
CCGGGGCCGAAGTATGAGTCCATCGGGGTGGACTGGTCCTCGGCCTCCCGGGGCCGTCGCATCCCCTTCG
AAGAGGTGTACGTGGCGGACCCCCGGCGTGACACCGCCGCATCCCTCAACGCCCGCCTCGCTGCGGGTCT
CCACCTTGTGTTTGCCCCCGGCGTGTACGGCCTCGACGCTCCGCTGGACGTGGTGCAGGACGACCAGGTG
CTGCTCGGCCTGGGGCTGGCGACGCTGGTGTCCCTCAACGGCAACGCGGTGGTGAGGGTGGGGAAGGCCA
GCGGCGTGCGCATCGCGGGACTTCTGCTGGAGGCAGGGCCCAAGCCAACCGCTGTGTTGCTGCAGTTCGG
TCCAGCCGGTGAGGCAGAGGACGGCTCGTCGGCGGCCGCCACAGGAGACCCAGCCAACCCAGGTGTTATT
TCTGACGTGTACGCCCGTGTCGGCGGCGCGCGACCGGGGCAAGTGGACACAATGGTGCGGGTGACGAGCG
GCAACGTGGTGCTGGACAACGTGTGGCTGTGGCGGGCGGACCACGACGCGCAGGGCCTCGTGTTCAACCG
CAGCAATCCGTGTACCACTGGGCTGGTGGTGGACGGGCACAACGTCACCGCGTACGGGCTGGCGGTGGAG
CACACCCTGGGGGACCTGGTGCGCTGGAACGGCGACCACGGCCGCACATATTTCTTCCAGGCGGAGATGC
CCTACGACGTGCGGCAGGAGGACTATGGTGACCACGACTTCGTGGGGTACCGGGTGGGCGACGCAGTGAC
GGCACACGAGGCACACGGGGCCGGCGTGTACCACTTCTTCCGGGACCACCCAGTGGCGGTACGCCGCGGC
ATCGCCGCCCCCGCCAGCCCGGGGGTGCGCTTCGTGTCGCCGCTGGCGGTGTACCTCAGCGGCTTTGGGA
TCCTGCATCATGTCGTCAACGAGGAGGGATCTGAAACATCCGCGAAGTCTCCAACGACGGACCCCGGAGC
TCACATTGCTTGGCTATGCTGA

Table S.4. Translated amino acid sequence of ORF dark_m.22013.

MSLAMPRPLPAFLWLLLVLPHGRPTGAAVAPVQPQPNPPQWPPGVTVFGPGDPTIEQTVNAAYRRNGGHD
PPCNGQFSDERYAFLFKPGVYPVDVPVGYYTQVLGLGESPDDVVFTGPKGVHAEEGDYAFTVGALNNFWR
SAENFQTTSAYPWFPGFNGMLWAVSQASPLRRVHVQSNLILFQYTYGDAAGFASGGYLGNSQVDGKVVSG
SQQQWVTRNCRLGAWDGAVWNMVFVGSTGTPTAHCGNVDGRPYVVEPAAPVVAEKPFLTIDSAGRYYLNV
PGPKYESIGVDWSSASRGRRIPFEEVYVADPRRDTAASLNARLAAGLHLVFAPGVYGLDAPLDVVQDDQV
LLGLGLATLVSLNGNAVVRVGKASGVRIAGLLLEAGPKPTAVLLQFGPAGEAEDGSSVAATGDPANPGVI
SDVYARVGGARPGQVDTMVRVTSGNVVLDNVWLWRADHDAQGLVFNRSNPCTTGLVVDGHNVTAYGLAVE
HTLGDLVRWNGDHGRTYFFQAEMPYDVRQEDYGDHDFVGYRVGDAVTAHEAHGAGVYHFFRDHPVAVRRG
IAAPASPGVRFVSPLAVYLSGFGILHHVVNEEGSETSAKSPTTDPGAHIAWLC

S.1.3. ORF light_m.20624

Table S.5. Nucleotide sequence of ORF light_m.20624.

ATGTACGGCTCTGTGCCCGCGGACCAGGTGGCGCCGGACCCCCGGCCGAGGGACGGCGCCGAGGGGCAGG
GGCTGCTGCGCGGCCAGCCCCAGCCCCGGCCCACCGGCACCCAGTTCTCCTTCTGCGCGCTTCTGCTGGG
GCTTGCCCTCCTGGTGACCCTCGTACTTTACCACTTCCTGCAGCCGCCCCCCAAGATCCCAAAGCACTGG

GAGCCCCGCTGCCCGAAGGGCAACCTGGACATCATGGGGGAGTGCCGGGACCGCGGGGAGTTCGGGGTGG
ACGGTGCCTCCCGGGCGTTTGTGTTCGAGAACCACGCGAACTTCCCTGTGTGGGTGGCCGGCATCGGGCC
GCCCACCTACCACGCCGACCCCGCCATTACCGGCTTCCAGCTGCAGCCGGGGCAGTACAAGGTGATCTCC
CTGCCCTCCACGCTGGAGTCCGTCCGCTTCTGGCCCCGCACCGGCTGCCGGTACGTGCCCGACACGGTGT
CGCTCAGCGGCATCGACGTCAACATCACGAAGTTCCGGTGCGACACAGGGGACTGCGGCAGCATCCTGAA
CATGTACGGCATGGAGTGCCAGATGGTCTCCGGGCTGCCGCCGGCCACCCTGGCGGAGTTCTCCCTCTCC
ACCAAGGGGAACGACTTCTACGACTTGAGTAATGTGGACGGGTATAATGTCGGCATGAAGATCGAGCCGT
ACAACTACGAGAAGGCCAACATCGAGGACCCCGACTTCGACTGCGGGGCCCCGGGCTGCGTCATGGACAC
CTCCAAGTGCCCGGACGAGCTGAAGTTCAAGGACAGCCTGGGGGTGACCCACTGCCTCAGCATCTGCCAG
GCCATCTACAACGAGAAGCAGCGCGCCCGCCACCCGCGACTGCAGGAGCTGTGGGACATGCAGACCGACC
GCGGCTTCATGCGGGACCAGCTGTGCTGCACGTGCGGCACCGGCGGCGGGTTGTGCGAGTCCCTGGGGTC
CCCCTGCACCTACGGCTGCTCGCCCTTCGTGGACAGCTACTCCGATGCGGGTTACAGCACCCGCAAGTGT
GAGAACATCCCTGGCACGTACCTCCCCAAGTGGCCGAAGGCGTCCAACGGCGAGAACTACGCCTCCGTGT
TCAACAAGCAGTGCCCGGAGGCGTACAGCTGGCAGTTCAACGACGACCAGTCCACGTACCAGTGCCACAA
GGCGGACTACAAGATCAGCTTCCTGAGCACCCACAAGAAGGAGCGGGCCACCAGCCCCTAA

Table S.6. Translated amino acid sequence of ORF light_m.20624.

MYGSVPADQVAPDPRPRDGAEGQGLLRGQPQPRPTGTQFSFCALLLGLALLVTLVLYHFLQPPPKIPKHW
EPRCPKGNLDIMGECRDRGEFGVDGASRAFVFENHANFPVWVAGIGPPTYHADPAITGFQLQPGQYKVIS
LPSTLESVRFWPRTGCRYVPDTVSLSGIDVNITKFRCDTGDCGSILNMYGMECQMVSGLPPATLAEFSLS
TKGNDFYDLSNVDGYNVGMKIEPYNYEKANIEDPDFDCGAPGCVMDTSKCPDELKFKDSLGVTHCLSICQ
AIYNEKQRARHPRLQELWDMQTDRGFMRDQLCCTCGTGGGLCESLGSPCTYGCSPFVDSYSDAGYSTRKC
ENIPGTYLPKWPKASNGENYASVFNKQCPEAYSWQFNDDQSTYQCHKADYKISFLSTHKKERATSP

S.1.4. ORF light_m.26257

Table S.7. Nucleotide sequence of ORF light_m.26257.

ATGAAGGCACTTGTCCTTTTACTGCTCATTGCTTGTGGCCTGCTGCATGCTGATCCACTGCAGCCTCAGG
GACCGAAATGCGTCTGTGTGGAAGGCGTGTCCTATGAAGGCCACGACCTAAAGTCGTTGCCAGTAAATGA
CGAAGGGGCCTGCTGCGGCCTCTGCACCACCACCCCAGGCTGCAAGATGGGAGTGATGGCAGAAGGAAAG
CATGGCCAAATGATTTGCCGGCTGACAGGGAGCAAGGCTCATCTGCAGAAGGTCTCGGGGGCAAAGTCAT
GCTATTGGGATCGCACTGGTGGGCACTCTCCTAAGCCAAAGTTGGCACCAAGGCCCAAACCCAAACCTCT
GCCAAAGCCCCGGCCCAGCGATCCGTTGCCAAAGGAATTCCCCCCAGTGTCCACCCAAAACCCGGCGGCC
TCGGGATACACTCCCACTGCTCGCAGTGGCGAAGCGGTGCCCCGGTACAACCTGATGTGCTTCGATGACA
AGCGCAAGCCACTGTCGACCAACCACTGGTGGGTGCCGGCCGTGCTCGGCCCCAACGCCGCAGGCAAGAA
CTACATCACCACCCTTCCCTACGTTGTGAATTGTGAGAAGTACGGGCTGGAGTTGGCCTACCCGTGGATC
ATGTCTCAGAAGAAGATTGTCCAGAACATCGTCAACCGGCACTGGAGCATCAGCCCCGGCCACGGGGAAC
GGTACTGCGTCCGCCTGGCCGATGAGGTGACGTTCACCGTGACGTGGGGGGGCACCATGGAGAGCACCAT
CGTGCGGGGGTCGCCGTACATCACGGCGACGTTCCACAAAGCCCCCGGGGCCACGGTCGGCACCGCGCAA
GGGCTTCGCACACTGTTCATCGACGAGGATGAGATCCCGGCTCCCACCACTGTTGGTGCGGAATACACTC
CTAAACGCAAGATTACTGCGGTGCTGCGGGACAGCGATGAGTCGTGGGTCATCTACCTACCACCCTCCAC

CCCAGTTACCATCAAAGCCAGCCCATCGAGCTTCGGAATTGGCTTCCATGAGGGCTTCAGCGGCGTGGTG
CGGTTGGCGATGATCAACAACTGCACCACCGGGCTGGACGGGGTGTCCCCCCACTGCCCGGCGAAGGACA
CACCCAACGAGGAAACCGACCCATACATCTCTGACTACGCCACCGCCATCGACGAGGGCAGCAACGTTTG
CACAGAGAAGGCGGTCGTGACCATGGAAAAGGCCCCGGGAGGCACCCTCGTGCAGTACCACTACAAGGAC
TACCGCTGCTGGCCCAGCCTGCCTGACGGGCTGCTGACGATGGTCGCGCTGCCCCACCACCTCGCCGTCA
CCCCCTGCGAGGGGCAGACGCGGGTCATCCTCAGCGGCGGCCACCGCAACCTGCGCGGCCTCAGCCTCGC
CCTGCAGCTGGCGGGCCACACCTGGCCGCTGCTGTACCCGGATTACTCCATTGACTGGATTGGGATCCCC
GACAAGTCGAAGATCCCCACCATCTTGTGGGCACTCAAGGGTCGGAACGAGTCGTCAGACGAGCATTATG
ACATCCGGTATGACATGAAAGACGGCATGATCGATCCTTACAATGCTGGCAAGCTGTTTGCGAAGTTGGG
GCGGTTGGTGCTTATCTCTGACCAGCTGGGAGAGGTGGAAATCCGGGACAAGTTGCTTGGCCGGTTGAGA
ACGTACATTTCCAGGTGGTACGACCACACCACCAAGAACCTCCTCATTTATGACAAGTCCTGGGGTGGTA
TCATTTCCTGTGGCTGCCGGTACATTTGGATCAGCCAGCCGGAGAAGCCGGAGCCCCACGGCTACCCCAT
CTGTGCCAACAACGGCAGCCACCTGGAGTGCCCCACGTTCCAGGACCCTAATTTTGACTTCGGGAATGCG
TATTACAACGACCACCACTTCCACTATGGGTATTTCATCTATTCCTCTGCCGTCATCGCCAAGTTCGATC
CCGCATGGGCCGAGCAGTATAATGAAAAAGTGCTGGCACTGATCCGGGACATTGCCAACCCGAGCCCCAA
GGACCCCTACTTCACCACCTTCCGTTATTTTGATTTCTTTGTGGGGCACTCCTGGGCTCTGGGCATCTAC
TCTGACCCGAACGGCAAGGGGCAGGAGTCTACCAGTGAGGCCGTCAACGCGTGGTACGGCATCCACCTCT
ACGGCAAGGCCACCAACCAACCCCTGCTGGCCCTGGTGGGTGAGACACTTGTCCAGATGGAGGTGCACAG
CACCAATTACTACTGGCACGTCCCCTCCAACATGGACATCTACCCCAGGGAGTTCAAGCATACCATCGTG
GGCATTGTACACGACCTCATCATCGAGTTCCAGACGTATTTCGGATCGGCGGGCTTCTTCGTCCACGGCA
TCCAGCTGCTGCCCATCACGCCAGTGGTCCACCTCATGTTCCACCCGTTCTGGGTGCAGTGGGGCTACCC
CCGCTTCCACGAGTACTGCAGCAACGACCCGTTCTGTACGGAAAGTGGCTTCGTGACGTTCATGATTGCG
GAGCAGGCGATGATTGACAAGGAGGGTGCCTGGGAGAGTGCCCTCAAGCTGCCCGACCACGTCTTCTCCC
TGGAGTGTGCGGGGGGCAACGGCAACAGCCTGACCAACACGCTGTATTTCATCTCCGCCTGGGGCAACGA
GAGGCGGGAGAAGTGGCTGTCCGACGAGCCGCTGTGCTCCAACTGGGAGTTCGGCTCCGACACCACCGAC
ACGGCGGCGCTCCGGGACGCCACCAACCCGGCGGTGGCCGGGGACCACCCGGTCGCCCTAAACTCTGACC
CCCGGGCCCGCCGCGGCGGCGGGTGGCTGTGGCTGGTACTGCTCCCGGCGCTGGCGCTGGCCGCCGCCGC
GGCTGTCTACACCCACCGCGGCGGGGACCTCCCGGCGCTGGCCGCCTCCGCGCTTGCCTGGCGGCCCGGG
CGGCCGGACGAGGCCGCCAGCCTGGCGTCACAGGGGCCGCTGTATGGGACCGGTGAGTAA

Table S.8. Translated amino acid sequence of ORF light_m.26257.

MKALVLLLLIACGLLHADPLQPQGPKCVCVEGVSYEGHDLKSLPVNDEGACCGLCTTTPGCKMGVMAEGK
HGQMICRLTGSKAHLQKVSGAKSCYWDRTGGHSPKPKLAPRPKPKPLPKPRPSDPLPKEFPPVSTQNPAA
SGYTPTARSGEAVPRYNLMCFDDKRKPLSTNHWWVPAVLGPNAAGKNYITTLPYVVNCEKYGLELAYPWI
MSQKKIVQNIVNRHWSISPGHGERYCVRLADEVTFTVTWGGTMESTIVRGSPYITATFHKAPGATVGTAQ
GLRTLFIDEDEIPAPTTVGAEYTPKRKITAVLRDSDESWVIYLPPSTPVTIKASPSSFGIGFHEGFSGVV
RLAMINNCTTGLDGVSPHCPAKDTPNEETDPYISDYATAIDEGSNVCTEKAVVTMEKAPGGTLVQYHYKD
YRCWPSLPDGLLTMVALPHHLAVTPCEGQTRVILSGGHRNLRGLSLALQLAGHTWPLLYPDYSIDWIGIP
DKSKIPTILWALKGRNESSDEHYDIRYDMKDGMIDPYNAGKLFAKLGRLVLISDQLGEVEIRDKLLGRLR
TYISRWYDHTTKNLLIYDKSWGGIISCGCRYIWISQPEKPEPHGYPICANNGSHLECPTFQDPNFDFGNA
YYNDHHFHYGYFIYSSAVIAKFDPAWAEQYNEKVLALIRDIANPSPKDPYFTTFRYFDFFVGHSWALGIY
SDPNGKGQESTSEAVNAWYGIHLYGKATNQPLLALVGETLVQMEVHSTNYYWHVPSNMDIYPREFKHTIV

GIVHDLIIEFQTYFGSAGFFVHGIQLLPITPVVHLMFHPFWVQWGYPRFHEYCSNDPFCTESGFVTFMIA
EQAMIDKEGAWESALKLPDHVFSLECAGGNGNSLTNTLYFISAWGNERREKWLSDEPLCSNWEFGSDTTD
TAALRDATNPAVAGDHPVALNSDPRARRGGGWLWLVLLPALALAAAAAVYTHRGGDLPALAASALAWRPG
RPDEAASLASQGPLYGTGE

S.1.5. ORF light_m.63754

Table S.9. Nucleotide sequence of ORF light_m.63754.

ATGGATCTGGTTCGCTCACCCTGGAACTGGAGCATCTGGAATGCGCTGGCAGGCGTGTCAGGCACTGCCA
CCCCTTCCGGACCGTTCACGGCGGGCGCCACCGGGCCCTCCCAGTACTTCACCCTCAAGGTGCGGAACGG
CACCAAGCTGGACGCGCCGCTCTTCGTGCTGGGCAAGGGTCAAGACCCTGGCCACCACAAGGAAGGGCAG
TACGCCATGGTGGAGGCGTACCTCCACTTCAACGATGGCGGCGTGGCGTCCTTCGTGCCCGCCACCACCG
CCGGGCCCTCGGGGGCGTACGCCCGGAAGCTGTCCGACTTCCCTAGCGACCCGAAGGACCCCTCTCTGCG
GGTGCTCACCATCCCGAAGACCATCGGCGGCCGCCTGTACTTTTCCTTCGCCGAGCCCGTGGTGCTGTCG
GTGGTGGAGGGAAAGGACCCCCAGGGGCGTGCCGTTTCCAAGACCCTCGCCGAGCCCGACCCGTTCAAGT
TGGACGACCCCAGCTACTACGTCCTGTATGACAAGGTGGAGTTCACCTTCAACGATCTGGGGGTGTGGAT
CGACACCACTGCGGTGGATTTCTTCTGCATGCCGGTCGCCCTCCACTTCGAGGGCCGGCCCGGCACCGCC
GTCGGCCTCGCCCAGCCCCGCGCCGCCGTGCTGCGCACCGTGGCGGACGCCTTCGCCGGCCCCTGGGGCC
GCCTGCGCCTCACGCACCCCAACCCGGACGGCAAAGAAGCGGTCCTCCGCATCCTGGCGCCCAACAAGGC
CATTGGCCGTCGGGACCCAGCCGAGAACTTCCCCGCAGACTACCTGCAGACCGCCATCCAGGCTGTCTGG
ACCCACTACCGAGCCTCCGGGGCCAGCCCGAGCCCCGCGGACCACGTGCTGCGGGTGGACTGCGGCGAGC
TGATCGGCTTCGGCTTCCCGCTGAGCGACGTGGACGGCGGCAAGCTGACCGCCGCCAACGCGCTGTTCAC
CGGCTGGGTCACCGCAGACGGCGCCACCTTCCACTTCGAGAACTCCACAAAGGACGTGGTGAGGGTCGCC
CTCCCGCAGTCCAACCAGGTCTTCGGCTGCGACGGCGGCTCGATGACGTGCGTCCCCAACACCGCGGCCT
CCGTTGTGGTGCGGGAGTTGGGGGCGGCGATGACGGTCGGGCTGCTGCCGTTGCCGGACCGGATGAAGAA
CCCGGCCACCGGGAAGCTGGAGGACGTCGCCCTCTGTCGCTCCTTCTTCACCCGGCCGGACGTCAAGAAG
CTCTTCTACACCAACAGCCCGGCCCTCCCACCCACCCCGGCCGGCCCGGCCTACAACCTGTACTCCAAGG
TGCTCCACAGCTTCGGCGACAAGGTGTACGCGTTTGCGTTCGACGATGCGGTGGGAGAGGATAGCACGCT
GCACTCCAGTGACACTGACCCAAATGTCCCGGACGTCAGCACACCGTACGTCGTGGTCCTGGGGGACATG
ACCGGCTCCGTCCTGCCTGACATCCACGACGACACGCACTACCACGTCACCCTCACTCCGGGCTGGGGTG
GCAAGGGGACCTACGTAGCGCCGGATGGCAAAACGTTGGACTTTGCATGGCCGTCCAACTGTGTCATCCC
GGACGTGACGTGCCCCTTCCGCTTCCAGTACGACGTCGGGGCGGGGCGGGACACGTTCGAGGTGGCGGTC
AACGTGCGGCGGGTGCGGTGCCTCAGCAGCGCGGCGAAGGTCTCCCTCGGCGTGGACTGGCACGTGGAGG
GGACGGCGGTGACCCTTTCCTTGGAGGGCGCCCCACGCTGA

Table S.10. Translated amino acid sequence of ORF light_m.63754.

MDLVRSPWNWSIWNALAGVSGTATPSGPFTAGATGPSQYFTLKVRNGTKLDAPLFVLGKGQDPGHHKEGQ
YAMVEAYLHFNDGGVASFVPATTAGPSGAYARKLSDFPSDPKDPSLRVLTIPKTIGGRLYFSFAEPVVLS
VVEGKDPQGRAVSKTLAEPDPFKLDDPSYYVLYDKVEFTFNDLGVWIDTTAVDFFCMPVALHFEGRPGTA
VGLAQPRAAVLRTVADAFAGPWGRLRLTHPNPDGKEAVLRILAPNKAIGRRDPAENFPADYLQTAIQAVW
THYRASGASPSPADHVLRVDCGELIGFGFPLSDVDGGKLTAANALFTGWVTADGATFHFENSTKDVVRVA

LPQSNQVFGCDGGSMTCVPNTAASVVVRELGAAMTVGLLPLPDRMKNPATGKLEDVALCRSFFTRPDVKK
LFYTNSPALPPTPAGPAYNLYSKVLHSFGDKVYAFAFDDAVGEDSTLHSSDTDPNVPDVSTPYVVVLGDM
TGSVLPDIHDDTHYHVTLTPGWGGKGTYVAPDGKTLDFAWPSNCVIPDVTCPFRFQYDVGAGRDTFEVAV
NVRRVRCLSSAAKVSLGVDWHVEGTAVTLSLEGAPR

Chapter 6:
Conclusive summary and future research directions

This thesis aimed to explore the approach of using enzymes for the degradation of paramylon to high-value soluble bioactive β-1,3-glucans as an alternative to conventional degradation methods such as acid hydrolysis. Key objectives to this approach were the development of a new non-chemical pretreatment method for paramylon to enhance the efficiency of its subsequent enzymatic hydrolysis. Further objectives included analysing the composition of the hydrolysates obtained after the enzymatic hydrolysis of the pretreated paramylon and to determine their immunostimulatory effects. In addition, investigation of potential suitable enzymes that could be used in such an approach was carried out including the conception of strategies for their identification.

In Chapter 3, an open pond (outdoor) cultivation of the *E. gracilis* wild type (WT) and *E. gracilis* var. *saccharophila* (sugar loving variant strain) was attempted as an inexpensive alternative to a more complex bioreactor cultivation, as requested by the industry partner. The rationale of this approach was to exploit the metabolic diversity of *E. gracilis* by utilising sunlight as a direct source of energy as opposed to closed heterotrophic (HT) cultivation, which is requiring a more complex set-up and the supplementation of an organic carbon source. It was considered that the biomass obtained from the open pond experiments could be used as a source of paramylon granules for the subsequent experiments on the enzymatic hydrolysis of the granules. However, the performance of the strains under both photoautotrophic (PT) and mixotrophic (MT) growth (i.e. utilisation of an organic carbon and energy source with additional light energy) was rather poor. The MT cultures were heavily contaminated with foreign organisms soon after the start of the experiments and the cultures under PT growth became bleached.

The technical set-up of this preliminary study probably played a major role in the low performance of the open pond cultures. The company euglena Co, Ltd. (http://www.euglena.jp) claims to be able to produce industrial-scale quantities of *E. gracilis* biomass in an open pond system equipped

with a stirring mechanism, whereas the open ponds in this work were designed without agitation [1]. Stirring aerates the cultures and would probably contribute to even heat distribution and prevent extreme temperature fluctuations during the incubation period. The bleaching observed in PT cultures in these experiments was probably due to overheating caused by daily temperature peaks. Thus, stirring should be an integral part of any further experimental design to study outdoor cultivation of *E. gracilis*.

Other outdoor cultivation systems have been designed for microalgae, including raceway tanks with a constant flow for aeration. These systems offer higher exposure to sunlight compared to open ponds and are worth considering for future outdoor cultivation experiments with *E. gracilis* [2].

The requirement of an organic carbon source for MT or HT cultivation could be addressed by introducing industrial effluents, especially those from local food-producing companies, because they are usually a rich source of nutrients and may represent a low-cost alternative to the currently used complex media. In the case of HT cultivation, this alternative source of nutrients could make cultivation of *E. gracilis* in a closed bioreactor economically feasible.

Also in Chapter 3, the degree of biofilm formation of *E. gracilis* was investigated by introducing an assay originally developed to categorise bacterial biofilms. Both strains, *E. gracilis* Z and *E. gracilis* var. *saccharophila,* produced strong biofilms only under MT growth, indicating that the availability of certain nutrients such as organic carbon could be a requirement for a higher degree of biofilm formation by *E. gracilis*. In an industrial setting, biofilm formation in photobioreactors might be detrimental to the biomass yield (e.g. shading effects) whereas an outdoor cultivation could profit from cultures forming strong biofilms because these cultures are likely more resilient to environmental stresses. Further investigation into the main factors that determine the formation

of biofilms by *E. gracilis* cultures could enable control over biofilm production in different applications. This investigation may include the comparison of biofilm formation by different *E. gracilis* strains in different media.

Our preliminary outdoor cultivation of *E. gracilis* did not produce enough paramylon material for the subsequent enzymatic hydrolysis studies. Thus, paramylon used in all following studies was obtained from commercial sources.

In Chapter 4, a new approach for the hydrothermal pretreatment of paramylon granules was investigated. The microwave pretreatment method developed in this study was shown to significantly enhance the hydrolysis of paramylon by enzymes. Microscopy studies revealed that major structural changes in the paramylon granules occurred during the pretreatment, making the substrate more accessible to enzymes. A new simple and time-saving direct Congo Red dye assay was also developed to assess the efficacy of the paramylon pretreatment. This dyed-based assay proved to be more sensitive than the enzymatic activity assays conducted in this project [3-5].

The soluble β-1,3-glucans produced enzymatically from the originally recalcitrant substrate were shown to induce tumour necrosis factor alpha (TNFα) production in murine macrophages, thereby confirming their immunostimulating activity. The degree of polymerisation (DP) range of the hydrolysis products seemed to have a major effect on TNFα production in that products with a lower DP range were the most effective. The initial results presented in this work could form the basis for a more in-depth study that would elucidate the precise nature of the bioactivity (e.g. immunostimulatory or anticancer effects) of each hydrolysate. Individual fractions of the hydrolysates could be assessed in multiplexed cellular assays with different target molecules (e.g. cytokines) using cell lines such as human leukocytes or cancer cells, which could possibly reveal a correlation between the DP of the produced β-1,3-glucans and a certain bioactivity. The

hydrolysates could also be tested for their systematic effect using animal models to make sure that they are safe for consumption as nutraceuticals or safe for injection as adjuvants for cancer therapy or immunoregulators to treat certain diseases.

Any commercial application of the combined paramylon microwave pretreatment and enzymatic hydrolysis method would require the scale-up of the process. Industrial-scale microwave reactors would need to be tested to assure that results obtained in the laboratory experiments are transferable and reproducible in a large-scale set-up. The Congo Red assay developed here could be introduced to monitor the efficacy of the large-scale pretreatment of paramylon. In addition, this simple dyed-based assay might find applications with other recalcitrant crystalline polysaccharides like cellulose by helping to determine the efficacy of other large-scale hydrothermal pretreatments (e.g. steam explosion vs. microwave reactor) on these substrates.

The yield of hydrolysis products was not a focus of this work but it would be highly relevant for any scale-up of this approach. In this study, it was shown for the first time that enzymes from *H. pomatia* (endo-β-1,3-glucanase) and *A. luteus* (exo-β-1,3-glucanase) display activity against microwave-pretreated paramylon. The endo-β-1,3-glucanase was the most efficient enzyme, probably due to a higher stability of the enzyme over the initial incubation time of 24 h. However, only an incomplete substrate conversion of 15-20% was obtained after 24 h hydrolysis with the endo-β-1,3-glucanase. The identification and use of new paramylon-degrading enzymes that have longer half-lives and/or higher specific activities would increase the substrate conversion. However, the overall yield of soluble carbohydrates is not the only factor to be considered since the composition of the hydrolysate is equally important.

Ultimately, the hydrolysis of paramylon in *E. gracilis* most probably requires a consortium of different enzymes with defined activities similar to those required for the hydrolysis of crystalline

cellulose [6]. Only few enzymes able to degrade paramylon have been identified to date and currently there is a lack of suitable assays for the screening and identification of new ones.

In chapter 5, three strategies for the identification of paramylon-degrading enzymes are presented. One strategy relied on the screening of a cDNA library derived from *E. gracilis* cells during the growth phase of paramylon degradation for genes or gene fragments encoding potential β-1,3-glucanases. For this purpose, an assay was developed in which fluorescence-activated cell sorting (FACS) was combined with an enzyme activity assay on a microtiter plate. Detection was based on the activity of the recombinant protein produced in *Escherichia coli* cells against the fluorogenic β-glucan analogue substrate fluorescein di-β-D-glucopyranoside (FDGlu). However, despite the high-throughput nature of this screening, no potential paramylon degrading enzymes were identified. This was probably due to the small average insert size (0.7 kb) of the cDNA, especially since the minimum size of typical glucanase genes is around 1.5 kb [7]. Additionally, the cDNA library may have lacked diversity due to mRNA degradation during RNA extraction. The possible success of further studies using this screening methods would require cDNA libraries with stricter quality control requirements and a bigger average insert size. It is also possible that the FACS assay was not sensitive enough leading to an increased number of false-positive cells. Alternative approaches like encapsulation of the *E. coli* cells could help to avoid loss of signal possibly caused by the diffusion of the fluorophore [8]. Another possibility to consider is the suitability of the prokaryotic organism *E. coli* as the host to produce functional proteins of eukaryotic origin. However, the assay can be easily adapted to eukaryotic host organism such as the single-cell *S. cerevisiae*.

The second strategy for the identification of paramylon-degrading enzymes was to utilise proteomics to identify respective *E. gracilis* enzymes. From this approach, four potential new β-1,3-glucanases were identified and an additional one matched an endo-β-1,3-glucanase recently

reported elsewhere, confirming the overall potential of this screening strategy [9]. The heterologous expression of one of the putative β-1,3-glucanase genes (ORF light_m.20624) was attempted in *E. coli* and *S. cerevisiae* [10]. However, the levels of soluble recombinant protein were barely detectable by gel electrophoresis. Problems with heterologous expression of *E. gracilis* genes have been encountered frequently across this field of research [9, 11, 12]. Currently, there is no customised molecular toolbox available for the simple and direct genetic manipulation of *E. gracilis*. Such a toolbox would provide efficient gene transformation and expression systems to facilitate homologous expression of *E. gracilis* proteins. Development of several elements for an *E. gracilis* molecular toolbox is currently underway in our laboratory and amongst several other groups [13, 14].

In our third strategy, a Congo Red dye assay using agarose plates supplemented with paramylon granules or dispersed paramylon as substrate was developed for the screening of enzyme preparations potentially able to degrade paramylon. The assay was validated with commercially-available enzymes previously suggested to have paramylon-degrading activity. Four commercial preparations of (hemi)cellulases were found to display activity against paramylon, two from *Aspergillus* spp. and two from undisclosed thermophilic organisms. The sensitivity of the assay varied depending on the choice of substrate (i.e. dispersed paramylon or recalcitrant paramylon granules). It is also possible to combine the plate assay with the proteomics approach, for example by analysing protein fractions for their activity followed by mass-spectrometry of the fraction(s) with the highest activity. This assay could also be used for the direct screening of axenic cultures of microorganisms potentially able to degrade paramylon by spotting them directly onto the agarose screening plates.

In conclusion, the microwave pretreatment approach developed in this study was shown to be effective in turning paramylon into a viable substrate for the enzymatic production of soluble

bioactive β-1,3-glucans. The new strategies explored in this research will enable the identification of enzymes with higher activities against paramylon than those currently available. This study set the foundations for further development and optimisation of the methods presented here, which was not possible with the time constrains of this PhD project.

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Appendix: Biosafety approvals

Organism	Biosafety approval notification number
Escherichia coli	5201400124
Euglena gracilis	5201500511
Saccharomyces cerevisiae	520180283404