Philosophiae Doctor (PhD)
Thesis 2019:60

Production of yeast from spruce sugars and hydrolysates of protein-rich by-products as feed ingredient

Gjær produsert fra gransukker og hydrolysater av proteinrike biprodukter som en fôringrediens

David Lapeña Gómez

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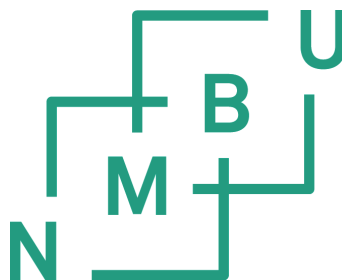
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Philosophiae Doctor (PhD) Thesis

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Ås 2019

"Caminante no hay camino,

se hace camino al andar"

Antonio Machado

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Acknowledgements

First and foremost, I would like to thank my supervisor **Vincent** for giving me the opportunity to work on this exciting project and for being an exceptional supervisor. You have inspired me to become a better scientist as well as being motivated, critical and professional at work. I would also like to thank my co-supervisor **Svein** for helpful insights and discussions, especially when it comes to fermentation. I think you made a great team of supervisors and I am very thankful to both of you.

Special thanks to **Gergely** who transferred a huge amount of his fermentation knowledge to me. You massively helped me during the fermentation experiments. Thanks a lot to **Pernille** for your motivation and help during the endless repeated fed-batch fermentations. Thanks to **Line** for always being available when things got very busy in the fermentation lab, and to **Lars** for having the pilot plant spotless and helping me with the 3-phase separator. A huge thanks to all members of the PEP and BioRef group. You have made this PhD work a very joyful ride providing a great social and working environment. I also want to thank the people working in the Dairy department, and especially to **May** who has always brought joy and happiness during all of our conversations.

A big shout out to my friends in Spain, Soria and Madrid, who have supported me in a way or another during my PhD, and to my group of friends in Copenhagen who always make me feel at home in all my visits. Thanks also to **Mathilde** for being my main support in Oslo during the writing of my thesis. Last but not the least, I would like to thank my family: my parents and my brother for always supporting me throughout my whole life.

I

Summary

The biotechnological potential and food-grade nature of yeasts are evidenced by their rich history of application in food fermentations. Thus, yeasts have been recognized as a suitable microorganism for the production of single cell protein (SCP). Production of yeast-based SCP could help meeting challenges derived from the combination of an increasing world population, limited protein availability and the need to upgrade by-products from different industries. Yeasts can utilize by-products from agriculture and forestry (lignocellulosic residues) and from food industries (by-products of meat and fish production) as carbon and nitrogen sources. Conversion of these feed stocks to yeast may not only improve protein availability in general, but could also have additional value, since yeast may have health-promoting effects. As an example, in aquaculture, yeast could replace fishmeal as a feed ingredient and/or reduce the dependency on plant-based proteins.

In the study described in this thesis, we have assessed by-products from different Norwegian industries for their potential to be used in the production of single cell protein from yeast for use in feed. The work included: 1) enzymatic hydrolysis of different meat and fish by-products using endogenous and exogenous enzymes, including a detailed characterization of the resulting hydrolysates and assessment of their potential as nitrogen source in growth media for yeasts, 2) screening of four selected yeasts as possible SCP source, using growth media composed of enzymatically hydrolysed poultry by-products and sugars derived from lignocellulosic biomass and 3) optimization and upscaling of yeast production using a semi-continuous fermentation strategy (repeated fed-batch fermentation), and use of different cell disruption methods to generate samples for future *in vitro* and *in vivo* trials.

Paper I describes a study of the enzymatic hydrolysis of three different by-products from Norwegian food industries: chicken by-products, mixed pork and beef by-products and salmon viscera. We used endogenous enzymes alone or in combination with commercial enzymes using short incubation times and a temperature gradient. Subsequently, hydrolysates were characterized by analysing the total recovery of protein, the peptide molecular-weight distribution, and the composition of total and free amino acids. The

developed protocols entailed the combined use of endogenous and small amounts of commercial enzymes and yielded protein solublization levels up to 90%. The amino acid compositions of the hydrolysates generally seemed favourable for yeast production, which was confirmed by yeast growth studies.

The study described in Paper II demonstrates that it is possible to produce protein-rich yeast biomass using hydrolysates of sulphite-pulped spruce wood in combination with hydrolysates of chicken by-products, with yields similar to, and sometimes even better than when using commercial glucose and peptones. Initially, we screened growth of four different yeasts, *Cyberlindnera jadinii* (anamorph name *Candida utilis*), *Wickerhamomyces anomalus*, *Blastobotrys adeninivorans* (synonym *Arxula adeninivorans*) and Thermosacc® Dry (*Saccharomyces cerevisiae*), on ten different media in microtiter plates. Then, the performances of the most promising medium and a commercial rich medium were compared in batch fermentations using 2.5 L laboratory fermenters. Again, the spruce-chicken medium showed good performance and the fermentations showed *B. adeninivorans* (0.9 g cells and 0.5 g protein per g of sugar) and *W. anomalus* (0.6 g cells and 0.3 g protein per g sugar) performed better than well known *Cyberlindnera jadinii* (0.5 g cells and 0.3 g protein per g sugar) on this medium. Compositional analysis the produced yeast biomass included amino acid composition, and analysis of the contents of nucleic acids, minerals, lipids, carbohydrates and ash. The produced yeasts had favourable amino acid profiles, especially for amino acids considered to be essential.

Paper III describes a follow-up study to Paper II, where fermentation of three of the yeasts discussed in Paper II, *Cyberlindnera jadinii*, *Wickerhamomyces anomalus*, and *Blastobotrys adeninivorans* was partially optimized and upscaled from 1.5 to 25 L scale, using an aerobic repeated fed-batch strategy. The tested fermentation media composed of enzymatically saccharified sulfite-pulped spruce wood, enzymatic hydrolysates of poultry by-products and urea was optimized for the production of single cell protein. In this set-up, *W. anomalus* was the most effective candidate in terms of substrate consumption, yields of cells and protein, and productivity, whereas the repeated fed-batch procedure was least effective for *B. adeninivorans*. Upscaling of repeated fed-batch fermentation of *W. anomalus* on a medium where 80 % of the nitrogen source came from the poultry hydrolysate and 20 % from urea yielded 0.6 g of cell dry weight and 0.3 g of

protein per gram of glucose, with cell and protein productivities of 3.92 g/L/h and 1.87 g/L/h, respectively. The protein content of the yeast biomass was 48 %, and the amino acid profiles were similar to those of fish and soybean meal, apart from low levels of sulphur-containing amino acids. Preliminary experiments to assess possible down-stream processing steps showed that *W. anomalus* cells were susceptible to commonly used disruptive methods, such as homogenization. Importantly, while there is still room for further optimization, the results of Paper III indicate that industrial production of *W. anomalus* in amounts required for its utilization in fish feed may indeed be technically feasible.

Sammendrag

Det bioteknologiske potensialet og matkvaliteten av gjær fremgår av deres rike anvendelseshistorie i matfermenteringer. Gjær har således blitt anerkjent som en egnet mikroorganisme for produksjon av encelleprotein (SCP). Produksjon av gjærbasert SCP kan bidra til å møte utfordringen fra kombinasjonen av en økende verdensbefolkning, begrenset tilgang til protein og behovet for å oppgradere biprodukter fra ulike bransjer. Gjær kan bruke biprodukter fra jordbruk og skogbruk (lignocellulose-rester) og fra næringsmiddelindustrien (biprodukter fra kjøtt og fisk) som karbon og nitrogenkilder. Omdannelse av disse substratene til gjær vil ikke bare forbedre tilgjengeligheten av protein, men kan også ha en tilleggsverdi siden gjær også kan ha helsefremmende effekter. Som eksempel kan gjær i akvakultur erstatte fiskemel som fôringrediens og/eller redusere avhengigheten av plantebaserte proteiner.

I studien som er beskrevet i denne oppgaven har vi vurdert muligheten for å bruke biprodukter fra forskjellige norske næringer til produksjon av encelleprotein i form av gjær og bruke dette som fôr. Arbeidet omfattet: 1) enzymatisk hydrolyse av forskjellige kjøtt- og fiskebiprodukter ved bruk av endogene og eksogene enzymer, inkludert en detaljert karakterisering av de resulterende hydrolysatene og vurdering av deres potensial som nitrogenkilde i vekstmedium for gjær, 2) screening av fire utvalgte gjær som mulig SCP-kilde ved bruk av vekstmedium bestående av enzymatisk hydrolyserte biprodukter fra kylling og sukker avledet fra lignocelluloseholdig biomasse og 3) optimalisering og oppskalering av gjærproduksjon ved hjelp av en halvkontinuerlig fermenteringsstrategi (gjentatt matet batchfermentering) og bruk av forskjellige metoder for ødeleggelse av cellevegg for å generere prøver for fremtidige *in vitro* og *in vivo* studier.

Artikkel I beskriver en undersøkelse av enzymatisk hydrolyse av tre forskjellige biprodukter fra norsk næringsmiddelindustri: kyllingbiprodukter, blandete biprodukter av svin og storfe, og lakseinnvoller. Vi brukte enten endogene enzymer alene eller i kombinasjon med kommersielle enzymer og benyttet korte inkubasjonstider og en temperaturgradient. Hydrolysatene ble så karakterisert ved å analysere total utbytte av protein, peptidmolekylvektfordeling og sammensetningen av totale og frie aminosyrer. De utviklede protokollene innebar kombinert bruk av endogene enzymer og små mengder kommersielle enzymer, og resulterte i opptil 90% løselig protein.

Aminosyresammensetningen av hydrolysatene virket generelt gunstig for gjærproduksjon, noe som ble bekreftet i dyrkingsforsøk av gjær.

Studien beskrevet i Artikkel II viser at det er mulig å produsere proteinrik gjærbiomasse ved bruk av hydrolysater fra sulfittbehandlet gran i kombinasjon med hydrolysater av kyllingbiprodukter, med lignende eller høyere utbytter enn det som oppnås ved bruk av kommersiell glukose og peptoner. Først testet vi veksten av fire forskjellige gjær, *Cyberlindnera jadinii* (Navn på anamorf er *Candida utilis*), *Wickerhamomyces anomalus*, *Blastobotrys adeninivorans* (Synonymt navn er *Arxula adeninivorans*) og Thermosacc® Dry (*Saccharomyces cerevisiae*), på ti forskjellige vekstmedier i mikrotiterplater. Deretter ble mikrobiell vekst på det mest lovende vekstmediet og et kommersielt rikt vekstmedium sammenlignet i batch-fermenteringer ved bruk av 2.5 liter laboratoriefermentorer. Igjen viste gran-kyllingmediet god ytelse, og *B. adeninivorans* (0.9 g celler og 0.5 g protein pr. gram sukker) og *W. anomalus* (0.6 g celler og 0.3 g protein per gram sukker) vokste bedre enn mer kjente *cyberlindnera jadinii* (0.5 g celler og 0.3 g protein per gram sukker) på dette vekstmediet. Analyser av sammensetning i den produserte gjærbiomassen inkluderte aminosyrer og analyse av innholdet av nukleinsyrer, mineraler, lipider, karbohydrater og aske. De produserte gjærene hadde gunstige aminosyreprofiler, spesielt for aminosyrer som anses å være essensielle.

Artikkel III var en oppfølgingsstudie til Artikkel II, hvor fermentering av tre av gjærene brukt i Artikkel II, *Cyberlindnera jadinii*, *Wickerhamomyces anomalus* og *Blastobotrys adeninivorans*, ble delvis optimalisert og oppskalert fra 1.5 til 25 liter skala ved bruk av en aerob gjentatt fed-batch-strategi. De testede vekstmediene bestod av enzymatisk sakkarifisert sulfittbehandlet gran, enzymatiske hydrolysater av biprodukter fra fjærfe og urea, og sammensetningen ble optimalisert for produksjon av encelleprotein. *W. anomalus* viste seg å være den mest effektive kandidaten når det gjelder substratforbruk, utbytter av celler og protein og produktivitet, mens den gjentatte fed-batchprosedyren var minst effektiv for *B. adeninivorans*. Oppskalering av gjentatt fed-batch-fermentering med *W. anomalus* på et medium hvor 80% av nitrogenkilden kom fra fjærehydrolysatet og 20% fra urea ga 0.6 g celler tørrvekt og 0.3 g protein per gram glukose, med celle og proteinproduktiviteter på henholdsvis 3.92 g/L/h og 1.87 g/L/h. Proteininnholdet i gjærbiomassen var 48%, og aminosyreprofilen lignet de av fisk og soyabønnemel bortsett

fra lave nivåer av svovelholdige aminosyrer. Foreløpige eksperimenter for å vurdere mulige nedstrøms prosesseringstrinn viste at *W. anomalus* celler var mottakelig for vanlige metode før å ødelegge cellevegg, som for eksempel homogenisering. Selv om det fortsatt er rom for ytterligere optimalisering er det viktig å legge merke til at resultatene fra Artikkel III viser at industriell produksjon av *W. anomalus* i mengder som kreves for bruk i fiskefôr faktisk kan være teknisk mulig.

Abbreviations

AA: Amino acid

ABPs : Animal by-products

B: BALI^{TM} spruce hydrolysate

CDW: Cell dry weight

CH: Chicken by-products hydrolysate

DM: Dry matter

DO: Dissolved oxygen

DSP: Downstream processing

EAA: Essential amino acid

NEAA: Non-essential amino acid

Q_p : Productivity, g yeast protein per liter and hour (g/L/h)

Q_x : Productivity, g dry yeast per liter and hour (g/L/h)

SCP: Single Cell Protein

TCA: Trichloroacetic acid

TFA: Trifluoroacetic acid

YCW: Yeast cell wall components

YNBAS: Yeast nitrogen base without amino acids with ammonium sulfate

YNBU: Yeast nitrogen base without amino acids with urea

$Y_{P/glucose}$: Yield, g dry yeast protein per g consumed glucose (g/g)

$Y_{P/sugars}$: Yield, g dry yeast protein per g sugar fed (g/g)

YP+G: Commercial yeast extract, peptone from meat and glucose

$Y_{X/glucose}$: Yield, g dry yeast per g consumed glucose(g/g)

$Y_{X/sugars}$: Yield, g dry yeast per g sugar fed (g/g)

List of papers

Paper I

Lapeña, D., Vuoristo, K.S., Kosa, G., Horn, S.J. & Eijsink, V.G.H. 2018, 'A comparative assessment of enzymatic hydrolysis for valorization of different protein-rich industrial by-products', *Journal of Agricultural and Food Chemistry*, vol. 37, pp. 9738–49.

Paper II

Lapeña, D., Kosa, G., Hansen, L.D., Mydland, L.T., Passoth, V., Horn, S.J. & Eijsink, V.G.H. 2019, 'Production and characterization of yeasts grown on media composed of spruce-derived sugars and protein hydrolysates from chicken by-products', Manuscript submitted to *Microbial Cell Factories*.

Paper III

Lapeña, D., Olsen, P.M., Arntzen, M.Ø., Kosa, G., Passoth, V., Eijsink, V.G.H., Horn, S.J. 2019, 'Spruce sugars and poultry hydrolysate as growth medium in repeated fed-batch fermentation processes for production of yeast biomass', Manuscript submitted to *Journal of Industrial Microbiology and Biotechnology.*

1 Introduction

1.1 General introduction

The global demand for protein is expected to double by the year 2050, when the world would need to generate 1,250 million tons of meat and dairy products per year to meet global demand for animal-derived protein if current consumption levels prevail (Ritala et al. 2017). The increase in protein demand is not only due to the increase in population and increased consumption of meat and dairy products, but also relates to increased appreciation of the importance of proteins in a healthy diet, especially for children and the elderly population (Boland et al. 2013). Fish and meat products are important sources of proteins for human consumption, but increasing the production of fish, meat and dairy products will not cover the projected increase in protein consumption (Boland et al. 2013). Increased production of protein-rich agricultural products is a potentially more efficient way of providing humans with proteins directly, and will also be needed to generate more feed for the fish and meat industry. Direct use of plant proteins for human consumption is more efficient, since it takes approximately 6 kg of plant protein to produce 1 kg of meat protein (Pimentel & Pimentel 2003), but may not be compatible with consumer demands. Furthermore, while moving towards increased use of plant proteins may be beneficial, increased agricultural production may lead to environmental problems, such as deforestation. It is thus of utmost importance to use available protein sources in the best possible way, and to develop novel routes for production of good protein sources.

Increased use of plant proteins is well illustrated by recent developments in the aquaculture industry in Norway, which is the largest supplier of Atlantic salmon in the global market. Based on intense scientific research (Collins et al. 2013), the use of plant proteins in salmon feed has increased drastically in the last two decades (Figure 1).
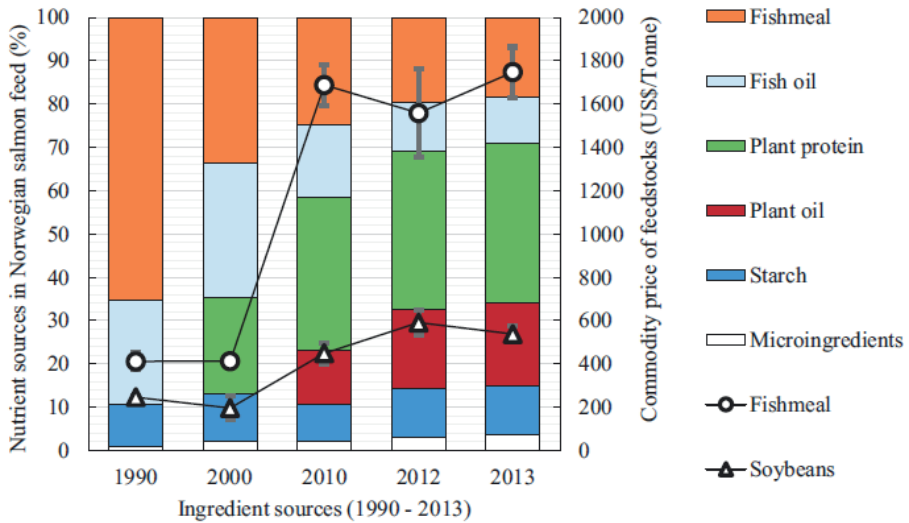
Figure 1. Ingredient sources in Norwegian salmon feed and commodity price of fishmeal and soybean feedstocks. The Figure illustrates the increase in use of plant proteins, which was accompanied by an approximately four-fold increase in the price of fish meal. This Figure was taken from Ytrestøyl, Aas & Åsgård 2015.

Interestingly, while the increased use of soya protein in salmon feed is considered an important improvement, more could be gained by using other protein sources, for example proteins derived from bacteria or yeast, as illustrated in Figure 2.
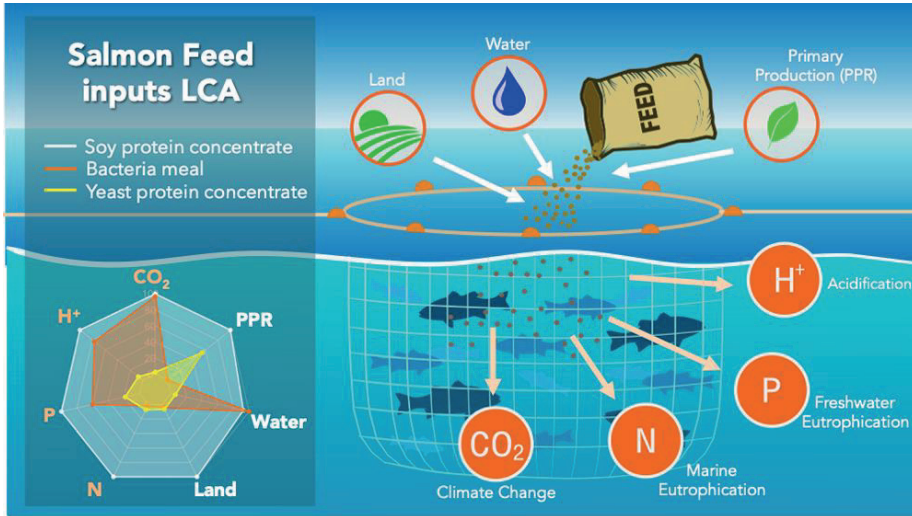
Figure 2. Schematic life cycle analysis for salmon production. The panel to the right shows various inputs and outputs, whereas the panel to the left shows the life cycle analysis of inputs based on the use of soy protein concentrate, bacteria meal or yeast protein concentrate as feed ingredient. This Figure was taken from Couture et al. 2019.

The meat and aquaculture industries generate huge amounts of protein-rich residual raw materials, which need to be properly treated due to environmental concerns and may be used as a source of value-added products for animal and human consumption (Figure 3). In USA, by-products derived from slaughtering and processing of cattle, pigs and chicken represent around 49 %, 44 % and 37 % of the total live weight of these animals, respectively (Meeker 2009). In Norway, production of Atlantic salmon, and white fish and pelagic fisheries resulted in 32, 43 and 12 % residual raw materials in 2016 (Richardsen 2017), while in tuna processing rest fractions can amount to above 50 % (Gamarro et al. 2013). These residual raw materials generate environmental challenges, but also possibilities. In fact, despite decades of research (Aspevik et al. 2017; Jayathilakan et al. 2012; Lynch et al. 2018), most of these by-products and co-products remain under-utilized and mostly end up as low value-added products, if not as waste.

According to European regulations, the residuals described above can be divided into co-products, which can be used for human consumption, and by-products which cannot. The regulatory framework for ABPs (Animal By-Products) divides the by-products into three

categories depending on their origin and potential risks for humans, animals and environment (Cat. 1-3; Figure 3). No ABP can be used for human consumption, and only category 3 may be used for animal feeding purposes (*The European Parliament and The Council of the European Union - Official Journal of the European Union L 300/1* 2009). Category 1 ABPs (Cat. 1) are categorized as high risk by products (derived from circus animals, pets or animals used in experiments), which need to be incinerated or used as fuels in combustion plants. Category 2 ABPs (Cat. 2) are categorized as high risk by products (carcasses of dead livestock or digestive tract content), which can be used for landfill only after sterilization and which cannot be used for animal consumption. Category 3 ABPs (Cat. 3) are categorized as low risk by products (by-products from slaughterhouses for human consumption or domestic catering waste), which can be used as animal feed or organic fertilizers but not for human consumption.
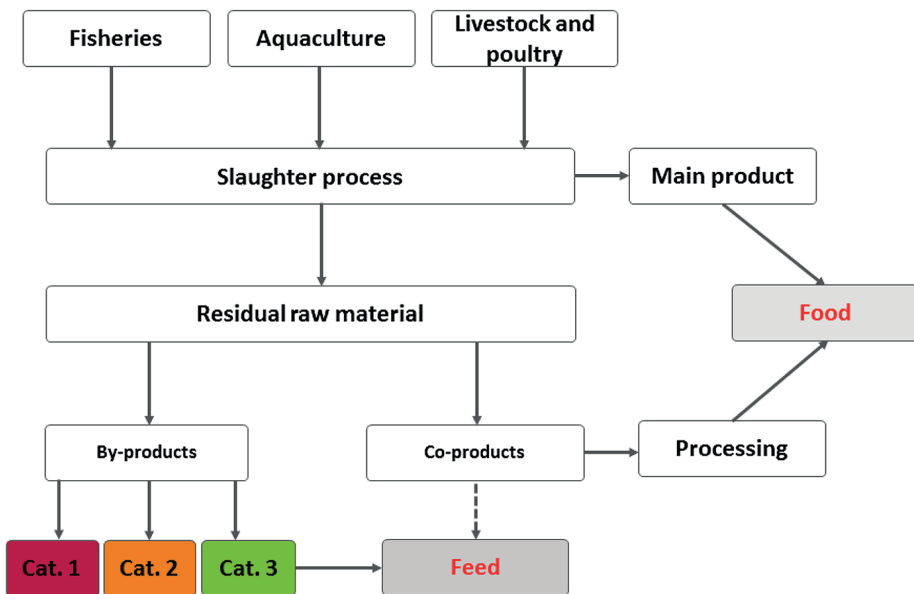
Figure 3. Process from fisheries and farming of fish and livestock to co-products and by-products. This Figure was taken from Aspevik et al. 2017

These residual raw materials from fish and meat industry represent a rich source of protein and other nutrients, but their use is restricted, as described above, which are in part due to concerns related to allergy and transmissible prion diseases such as bovine spongiform encephalopathy (BSE) in cattle. One strategy to overcome these limitations is the

production of protein hydrolysates by enzymatic treatment, thus altering the protein components by cleaving them into smaller peptides and amino acids. Ruminant or non-ruminant ABP material can be used as a substrate for protein hydrolysates as long as the resulting peptides of the hydrolysate have a molecular weight below 10.000 Da (Jędrejek et al. 2016). In order to increase the "distance" between the original by- or co-product, a next step could be to use these protein hydrolysates as nitrogen source for production of protein rich microbial biomass, here referred to as single-cell protein or SCP. Although the regulatory status of SCP produced in this manner remains unclear, production of SCP seems a promising strategy for reutilization of ABPs. It has even be proposed that microorganisms could upgrade ABP components to SCP in the form of yeasts that would be suitable for human and animal consumption (Jalasutram et al. 2013). Likewise, lipids and oils in the by- and co-products could be upgraded to microbial lipids with favourable fatty acid compositions using oleaginous fungi (Kosa et al. 2018). The use of microbes could not only solve an environmental problem regarding disposal of by-products, but could also provide a technology for converting non-edible meat and fish by-products into new sources of proteins and lipids (Figure 4).

Production of SCP will require sugar. In the past decade, within the same environmental framework and as part of a foreseen transition from an oil-based economy to a bioeconomy, there has been much focus on development of technologies for converting non-edible lignocellulosic biomass into platform sugars. Such sugars may be converted to ethanol, i.e. a second generation biofuels, but could also be used in the production of SCP. Production of SCP using lignocellulosic biomass sugars and by-products from the meat and fish industry as sources of carbon and nitrogen, respectively, could provide a sustainable route towards production of proteins for animal feed (Spalvins, Ivanovs & Blumberga 2018) (Figure 4).
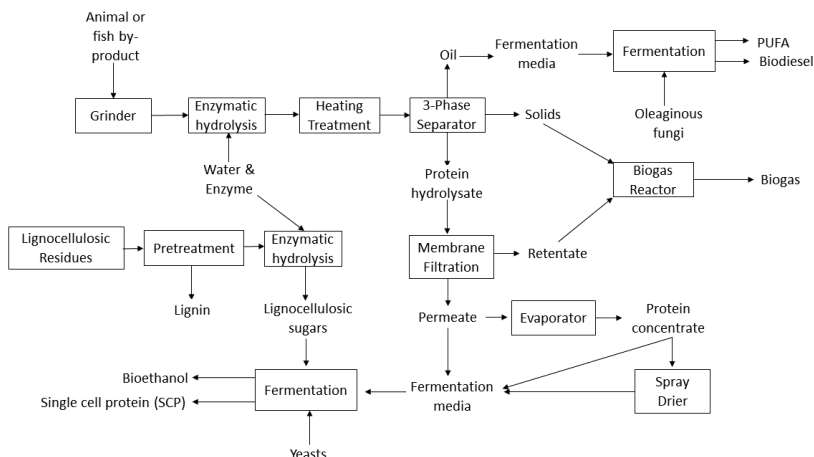
Figure 4. Process diagram showing operations applied in a future biorefinery. The scheme shows scenarios based on combining hydrolysis of proteins in non-edible meat and fish by-products with hydrolysis of polysaccharides in non-edible lignocellulosic biomass to produce single cell protein and other value-added products.

This thesis describes research on the production of yeast-based SCP using protein hydrolysates and lignocellulose-derived sugar.

1.2 Single cell protein

1.2.1 Background

The term "single cell protein" was first used by researchers at MIT in 1968 (Mateles & Tannenbaum 1968), and it refers to dead, dry cells of microorganisms such as algae, yeast, fungi or bacteria which are produced by fermentation systems, and then used as a protein source in human foods or animal feeds. However, the use of yeasts as a source of protein dates back to 1910, when researchers at the Institut für Gärungsgewerbe in Berlin used surplus brewing yeasts as feeding supplement for animals (Delbrück 1910). The latter use of yeast proved to be useful during World War I, when Germany managed to replace half of its imported protein sources by yeasts (Ugalde & Castrillo 2002). More than a century

later, the shortage of protein availability worldwide and environmental and economic concerns, such as the need for valorisation of by-product streams from different industries, are driving the development of novel SCP products.

Table 1. Protein production potential for different protein sources. The Table shows the efficiency of protein production for several protein sources, as protein production per 24h. This table was taken from Israelidis 2015.

Organism (1000kg)	Amount of protein
Beef cattle	1.0 kg
Soybean	10.0 kg
Yeast	100.0 tons
Bacteria	1000,000,000 tons

In terms of production potential, particularly space-time yields, microorganisms are an excellent protein source, compared to conventional sources of plant an animal origin. This is illustrated by Table 1, which shows that the productivity of protein production that may be achieved by growing microorganisms is orders of magnitude higher than the productivity that can be achieved by producing agricultural crops or in animal farming. Microorganisms can be produced in bioreactors, not requiring agricultural land, and SCP can be produced continuously throughout the year, independent of the season. In terms of nutritional value, the protein content of certain types of dried SCP, such as bacteria, may reach levels up to 80 % (Øverland et al. 2010), with favourable amino acid profiles, for example similar to animal feed (Skrede et al. 2003). Importantly, microbial biomass also contains free amino acids, carbohydrates, fats, vitamins, minerals and nucleic acids, which affect the nutritional value of the SCP, possibly in a positive manner (Panda et al. 2017). For example, the cell wall of yeasts contains different polysaccharides such as β-glucans, mannans and chitin, which are known to have immunological and health benefits for fish (Øverland & Skrede 2016) and mammals (Chen, Seviour & Ramsdale 2007).

1.2.2 The choice of microorganism

Microalgae, fungi (filamentous fungi and yeast), and bacteria can all be used as SCP (Anupama & Ravindra 2000) (Table 2). The choice of microorganism is critical and depends on multiple factors. It is important that the selected microorganism is able to utilize cheap available substrates, especially by-products and side-streams, and does not require additional grow factors, which normally increase the price of the final product. The microorganism should be robust during production in the bioreactor, easy to harvest, amenable to straightforward downstream processing with a good outcome, and, finally, maintain its essential characteristics when blended into feed and food formulas for animal and human consumption. Safety and the ability to be converted into a nutritionally rich and non-toxic end-product are also key aspects to consider during the selection of the microorganism.

Table 2. Compositional analysis of different microorganisms used in the production of SCP. The numbers in the Table show % of dry weight and are derived from (Miller & Litsky 1976). Note that the table shows typical numbers as they were known in 1976; today in most cases, examples of higher protein content exist, as discussed in the text.

	Fungi	Microalgae*	Yeasts	Bacteria
Protein	30-45	40-60	45-55	50-65
Fat	2-8	7-20	2-6	1.5-3.0
Ash	9-14	8-10	5-9.5	3-7
Nucleic acids	7-10	3-8	6-12	8-12

*Cyanobacteria are usually included under microalgae

1.2.3 SCP from Microalgae

Microalgae are interesting as SCP for animal and human consumption, since they may have high protein contents of up to 70% (Ritala et al. 2017). In addition, the amino acid profiles of microalgae are well balanced, with the exception of sulphur-containing amino acids, as is common for all microorganisms used for SCP production. Microalgae are a good source of vitamins A, B, C, D and E, free amino acids, minerals and crude fiber (Bajpai 2017). Microalgae are special in that they normally contain high amounts of lipids compared to fungi and bacteria used for SCP production, being very rich in

essential fatty acids (Garcia-Garibay, Gomez-Ruiza & Cruz-Guerrero 2014). Nutritional disadvantages include relatively high amounts of heavy metals and the cellulosic nature of the cell walls, which hampers degradation (Nasseri et al. 2011).

Many microalgae are autotrophic microorganisms feeding on carbon dioxide and light, which is potentially highly attractive but comes with technological challenges (Smetana et al. 2017). However, there are also heterotrophic species that can use organic carbon sources while being produced by traditional fermentation methods. Traditional fermentation allows better control of several parameters and contamination can be easily avoided. The advantages of using algae as SCP are linked to the relatively low-cost substrate requirements, fast growth and rich protein content (Arora, Mukerji & Marth 1991). The disadvantages are linked to the technological challenges of large-scale production, for example due to the low solubility of carbon dioxide in water. Additionally, outdoor production of algae is limited by several factors such as the control of temperature and sunlight, and contamination (Harun et al. 2010).

1.2.4 SCP from Bacteria

Bacteria typically have very high protein contents compared to algae and fungi, and it has been claimed that protein content may reach 80% in some cases (Nasseri et al. 2011). Bacterial proteins typically show favourable amino acid profiles that are comparable to animal feed (Skrede et al. 2003). The nucleic acid content of bacterial biomass tends to be high, especially RNA, and nucleic acids may need to be removed before use in food or feed (Strong, Xie & Clarke 2015). Bacteria possess the highest growth rate among microorganisms used for SCP production (Bajpai 2017), and can utilize a wide variety of C1-C6 compounds as carbon source, including methane (Anupama & Ravindra 2000). Methane is an interesting substrate since it is abundantly available, cheap, and it may be sustainably produced in biogas plants (Øverland et al. 2010). One of the biggest disadvantages of the use of bacteria as a SCP is related to bacterial morphology. Bacterial cells are difficult to recover due to their small size and low density, making the downstream processes problematic. They must be flocculated to increase the concentration of the slurry before centrifugation (Trehan 1993), increasing the production costs. Finally, the public opinion regarding bacteria is linked to diseases and infections,

which may create a psychological barrier for consumers to accept the use of bacteria as a SCP in feed or food.

1.2.5 SCP from Fungi

Fungi, and especially yeasts, are the most widely used microorganisms for SCP production (Mondal et al. 2012). Filamentous fungi have lower protein contents (30% - 45%) (Bajpai 2017) than yeasts (45% to 65%) (Nasseri et al. 2011). Both filamentous fungi and yeast contain beneficial amino acid profiles, compared to animal feed, with relatively higher levels of threonine and lysine but lower levels of methionine compared to bacteria (Ritala et al. 2017). Filamentous fungi and yeast are easy to harvest in comparison to bacteria, due to their larger cell size and flocculation properties. Fungi are a good source of vitamins, especially the B complex, essential minerals and dietary fiber (Pacheco 1997). In addition, yeast cell walls, which represent 26 – 32% of the cell dry weight, contain different proportions of mannan-oligosaccharides, ß-glucans and chitin, depending on the strain (Nguyen, Fleet & Rogers 1998), which are thought to have beneficial health effects and are sometimes marketed as functional foods or nutraceuticals (Rakowska et al. 2017). Beneficial health effects are in part ascribed to the ability of these polysaccharides, or fragments thereof, to stimulate the immune and antioxidant systems, for example in fish (Navarrete & Tovar-Ramírez 2014). Next to potentially favourable cell wall components, yeast usually contain higher amounts of protein than filamentous fungi, and also have higher growth rates. However, the perhaps most important advantage of yeasts is their popularity. Yeasts have been used and consumed by humans for centuries and therefore the degree of acceptance among consumers is high.

Some key properties of the various potential SCP sources discussed above are summarized in Table 3. The research described in this thesis concerned the production of yeast.

Table 3. Comparison of various parameters for SCP production from algae, fungi and bacteria. This Table was adapted from (Anupama & Ravindra 2000).

Parameter	Microalgae	Bacteria	Fungi (Yeast)	Fungi (Filamentous)
Growth rate	Low	Highest	Quite high	Lower than bacteria and yeast
Substrate	Light, CO_2 and a wide range of inorganic and organic substrates	Wide range	Wide Range	Wide Range
pH range	6.5 - 11	5-7	5-7	3-8
Contamination risks	High and serious	Precautions needed	Low	Low if pH is below 5
*S-containing amino acids	Low	Deficient	Deficient	Low
Nucleic acid removal	-	Sometimes Required	Sometimes Required	Sometimes Required
Toxins	Some species	Endotoxins from Gram-negative bacteria	-	Mycotoxins in some species

* Compared to fish and soybean feed ingredients

1.2.6 Nutritional requirements of microorganisms

Microbial growth, metabolism and quality of the final product in the production of SCP are strongly influenced by the composition of the fermentation medium. Microorganisms require water, sources of energy, carbon, nitrogen, minerals, vitamins and oxygen, if aerobic. However, the specific nutritional requirements used in industrial fermentation are as heterogeneous and diverse as the microorganisms under discussion. Microorganisms require a variety of elements, including trace elements, which in some cases may need to be identified (Vasey & Powell 1984) before composing fermentation media. Compositional analysis of cells grown on a rich medium maybe a good starting

point for development of balanced growth media. Table 4 shows the elemental composition of some microorganisms. The vitamins most frequently required are thiamine and biotin. Required in the greatest amounts are usually niacin, pantothenate, riboflavin and some (folic derivatives, biotin, vitamin B12 and lipolic acid) are required in smaller amounts (Zabrisky et al. 1980).

Table 4. Elemental composition of some microorganisms. The numbers represent the average mass in percent of dry cell mass. The third row shows the carbon/nitrogen ratio. This table is derived from Rhodes & P.F. Stanbury 1997.

Element	Bacteria	Yeast	Fungi
Carbon	48	48	48
Nitrogen	12.5	7.5	6
Carbon/Nitrogen ratio	3.84	6.4	8
Phosphorous	2.5	1.7	2.5
Sulfur	0.6	0.1	0.3
Potassium	2.8	2.5	1.4
Magnesium	0.3	0.3	0.2
Sodium	0.8	0.06	0.26
Calcium	0.56	0.2	0.75
Iron	0.11	0.26	0.15
Copper	0.02	$6.0 \cdot 10^{-3}$	-
Manganese	$6.0 \cdot 10^{-3}$	$4.0 \cdot 10^{-3}$	-
Molybdenum	-	$0.2 \cdot 10^{-3}$	-

It is relatively easy to design a medium composed of pure compounds on a laboratory scale, but the resulting medium might be unsuitable for use in an industrial scale process. Depending on the characteristics of the components used, fermentation media can be chemically defined and composed of pure chemicals in known proportions, or be more undefined, when formulated by ingredients of natural origin, for which exact compositions are not known. Undefined complex natural media containing inexpensive carbon and nitrogen sources have been used in industrial fermentation processes (Miller & Churchill 1986; Stanbury, Whitaker & Hall 2017a). However, variation in the concentration of components and impurities between different batches can cause undesirable variability in productivity. As a consequence, it is difficult to control and optimize those processes. One approach to decrease the variability in productivity is to use chemically defined media. However, these media are expensive and they are not commonly used in industrial processes (Zhang 1999).

The yeast *Saccharomyces cerevisiae*, frequently utilized in biotechnology worldwide, can ferment a variety of sugars (i.e., glucose, fructose or sucrose), as long as demands for niacin, nitrogen (amino acids, small peptides, ammonium salts or urea), oxygen, sulphur, phosphorous, potassium and magnesium are met (Kampen 2014). Ammonia and ammonium salts are assimilable by all commonly used yeasts. Several yeasts can also assimilate urea in two different ways, either by extracellular degradation by an urease, leading to ammonia production, or by transport and assimilation through the urea and amydolyase pathway. In this latter case, addition of biotin is necessary in order to assimilate urea since it works as a cofactor of the urea amidolyase (Roon & Levenberg 1972). Certain yeasts can utilize organic nitrogen sources, such as amino acids, not only as nitrogen sources but also as sources of carbon and energy (Freese et al. 2011). The trace element requirements of yeasts are generally in low and the need for these elements can sometimes even be met by the low quantities of minerals occurring in water (Atkinson & Mavituna 1991). The correct vitamin balance can be achieved by the correct blending of complex materials (Rhodes & Fletcher 1966).

Typical examples of cheap bulk components used in industrial fermentation media include cane molasses, beet molasses, cereal grains, starch, glucose, sucrose and lactose as carbon source, and inorganic or organic sources of nitrogen such as ammonium salts, urea, nitrates, corn steep liquor, soya bean meal, slaughter-house waste, and fermentation residues (Kampen 2014). In fermentations for production of commodities such as ethanol or single cell protein, raw materials make up more than 50 % of the production cost, which means that the selling price of the product will for a large part be determined by the cost of these raw materials (Stanbuty, Whitaker & Hall 1995). Therefore, cost and sustainability of the raw materials are major factors in determining the success of SCP production, and, thus, there is great interest in developing better and more sustainable medium ingredients. In this respect, biodegradable agro-industrial by-products are a promising source of nutrients (Spalvins, Ivanovs & Blumberga 2018). Yeasts can utilize by-products from agriculture and the food industry as nitrogen and carbon sources. These are cheap and potentially even food-grade sources of nutrients and their controlled use in the fermentation industry would help controlling environmental pollution, eliminate waste-disposal problems and reduce production costs. The most important considerations when selecting by-products for production of SCP are:

- Geographic and seasonal availability, and their derivatives such as freshness, microbial contamination, possible conservation measures, and transportation and storage costs.

- The technological feasibility and costs of pre-treatment processes that are needed before the industrial side streams or by-products can be utilized in the fermentation process.

- Production yields in space and time and quality of the final product generated upon utilization of the alternative substrate.

Maria *et al.*, 2017, categorized different agricultural by-products into four groups: monosaccharide- and disaccharide-rich sources, starch-rich sources, structural polysaccharide-rich sources and protein- or lipid-rich sources (Figure 5).
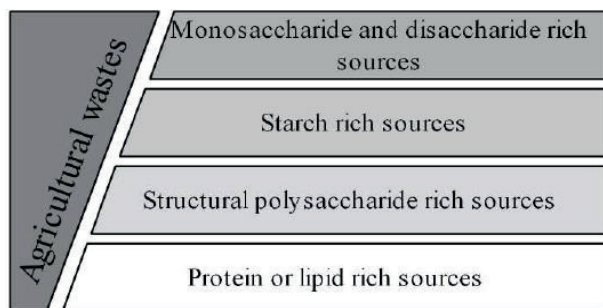
Figure 5. Categorization of agricultural wastes for possible use in SCP production according to Maria et al. 2017.

Monosaccharide- and disaccharide-rich sources include molasses (i.e., sugar solutions) derived from sugar cane (Samadi, Mohammadi & Najafpour 2016), sugar beet (Nigam & Vogel 1992), soy protein concentrate production (Sirilun et al. 2017), fruit processing waste (Uchakalwar & Chandak 2014) and dairy by-products such as whey (lactose-rich) (Sandhu & Waraich 1983), which all have been used in production of SCP. These by-products can usually be utilized directly by the microorganisms and do not require pretreatment steps. Molasses would normally be used as the cheapest carbohydrate to

grow yeast biomass in a large scale process, while it is not accepted for recombinant protein production because in this case molasses complicate in the subsequent purification processes, thus increasing costs (Kampen 2014).

The most widely available carbohydrates are starch-rich sources from agricultural by-products such as brans from grain processing, and potato residues. In contrast to molasses, these carbohydrate sources normally need to be processed to convert their polymeric components to monosaccharides before use in a fermentation medium, which increases the costs of SCP production. The use of starch particles as an inexpensive medium for ethanol production has been reported by Bawa et al. 2010, while wheat and rice bran (Sandhu & Waraich 1983) and potato starch (Liu et al. 2014) have been used for SCP production.

The use of structural polysaccharide-rich sources and protein-rich sources is discussed in detail in separate sections, below.

1.3   Lignocellulose-derived carbon sources

Structural polysaccharide-rich sources include lignocellulosic biomass, like wood and agricultural by-products, and represent the most abundant renewable carbon source on the planet (Spalvins, Ivanovs & Blumberga 2018). Lignocellulosic biomass is primarily composed of cellulose, hemicellulose and lignin. The proportions of these three components differ between different plant species, varying from 33 - 51% for cellulose, 19 - 37% for hemicellulose and 14 - 28% for lignin (Pauly & Keegstra 2008). Cellulose, is a linear unbranched homo-polysaccharide composed of β-(1-4)-linked glucose residues. Unlike cellulose, the term hemicellulose refers to a heterogeneous class of branched polysaccharides, of which xyloglucans, xylans and mannans are the most common. Lignin is a complex aromatic polymer composed of three different phenylpropane units: p-coumaryl, coniferyl and sinapyl alcohol, that are held together by different kinds of linkages (Hendriks & Zeeman 2009). Lignin provides structural support and it represents a chemical-physical barrier for enzymatic degradation and microbial attack. Figure 6 shows how the different elements in lignocellulosic substrates are organized.
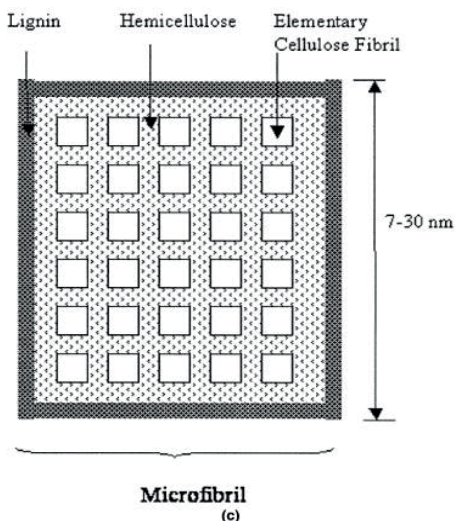
Figure 6. Highly schematic illustration of the organization of lignocellulose. The picture shows cellulose (white), hemicellulose (dotted) and lignin (grey) which form elementary fibrils and microfibrils. This picture was adapted from Zhang & Lynd 2004.

1.3.1 Pretreatment of lignocellulosic biomass

The lignocellulosic feedstock has to undergo a pretreatment in order to achieve efficient enzymatic hydrolysis of the different polysaccharides to yield fermentable sugars, which can then be utilized by the selected microorganism during SCP production. Common pretreatment methods include physical (e.g. chipping), physico-chemical (e.g. steam explosion), chemical (e.g. dilute acid or base) or biological (e.g. enzymes or fungi) processes. An in-depth discussion of pretreatment technologies is not within the scope of this thesis and several reviews are available on this topic (Chandra et al. 2007; Dyk & Pletschke 2012; Prasad, Ankit & Negi 2015). Each pretreatment method has its advantages and disadvantages, and selection of an optimal method is usually feedstock dependent.

During pretreatment, the lignocellulosic structure is disrupted, lignin and hemicellulose are released or modified, and the porosity of the whole structure increases (Figure 7). The subsequent enzymatic degradation of cellulose is improved because the cellulose becomes more accessible, as reflected in e.g. an increased surface area (Hendriks &

Zeeman 2009). Pretreatment methods differ in terms of how much of the hemicellulose remains in the cellulose- and lignin rich solid material. The amount of hydrolysable carbohydrates recovered, energy consumption, cost-effectiveness and the generation of by-products that may inhibit enzymes and/or microorganisms (see below) determine the suitability of a pretreatment method (Kumar et al. 2009), and such suitability may vary depending on the further use of the liberated sugar.

Several pretreatment processes with a primary focus on lignin removal are known under the common term "pulping". One option is soda pulping (Huang, Shi & Langrish 2007), which entails that lignin is removed by cooking the lignocellulosic biomass in an alkaline solution. Sulphite pulping is another technology of which various variants exist. Recently, the Norwegian biorefinery company Borregaard, developed a novel pretreatment technology that includes a sulphite cooking step utilizing calcium and sodium as counter ions, which solubilizes lignin into water by sulfonation while most of the hemicellulose is washed out. Consequently, the remaining solid fraction primarily consists of cellulose. This process is called BALI (Borregaard Advanced Lignin)[TM] (Rødsrud, Lersch & Sjöde 2012) and can be applied to a wide range of lignocellulosic substrates. After this process, relatively modest amounts of cellulolytic enzyme cocktails are needed to convert the glucan polymers into fermentable sugars (Chylenski et al. 2017).
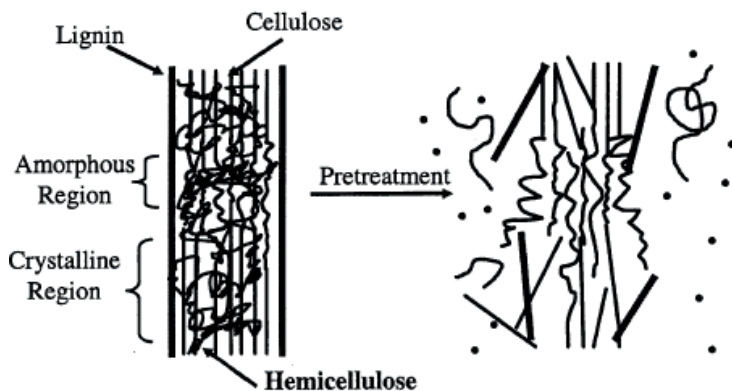

Figure 7. Simplified representation of pretreatment effects on the plant cell wall. This picture was taken from Hsu, Ladish & Tsao 1980.

1.3.2 Formation of inhibitors and toxicity effects

During pretreatments compounds that inhibit subsequent enzymatic hydrolysis and fermentation processes may be formed as degradation products from either released soluble monosaccharides or lignin fragments (Figure 8). Depending on the type of lignocellulosic biomass used and the type and intensity of the pretreatment, the generation of inhibitors will vary, in terms of both the nature and the amounts of generated compounds (Ko et al. 2015). On the one hand, pretreatment processes need to be adapted to minimize inhibitor formation and a possible need for additional processing steps, such as washing after solid/liquid separation, detoxification of hydrolysates, and development of genetically engineered microbes tolerant to inhibitors (Jung & Kim 2015). On the other hand, inhibitor tolerance may be a prerequisite for enzymatic and microbial conversion of certain types of lignocellulosic biomass (Hahn-Hägerdal et al. 2007).

The main potentially inhibitory compounds formed are weak acids, phenolics and furans (Figures 8,9). The weak acids, such as acetic, levulinic and formic acid, are derived from the acetyl groups present in the hemicellulose (acetic acid) and from hexoses (formic acid and levulinic acid; Figure 8). When it comes to microbial fermentation, formic acid has been reported as the acid with the highest inhibition effect, while usually more abundant acetic acid is thought to give the lowest inhibition (Larsson et al. 1999). These acids can diffuse across the plasma membrane into the cytosol and decrease the intracellular pH. If the concentration of acids is very high, the proton pumping capacity becomes critical and the ATP reservoir of the cell may be depleted while trying to maintain the intracellular pH, which can result in cell death through lethal intracellular acidification (Palmqvist & Hahn-Hägerdal 2000).

Phenolic compounds result from breakdown of lignin (Almeida, Modig & Petersson 2007) and these low molecular weight aromatic compounds are known to have considerably inhibitory effects on microbes (Rahikainen et al. 2013). In fact, these phenolic compounds are more toxic to microorganisms than the usually more abundant sugar-derived compounds furfural and 5-hydroxymethyl-2-furaldehyde (HMF) that are discussed below (Carrión-Prieto et al. 2018).
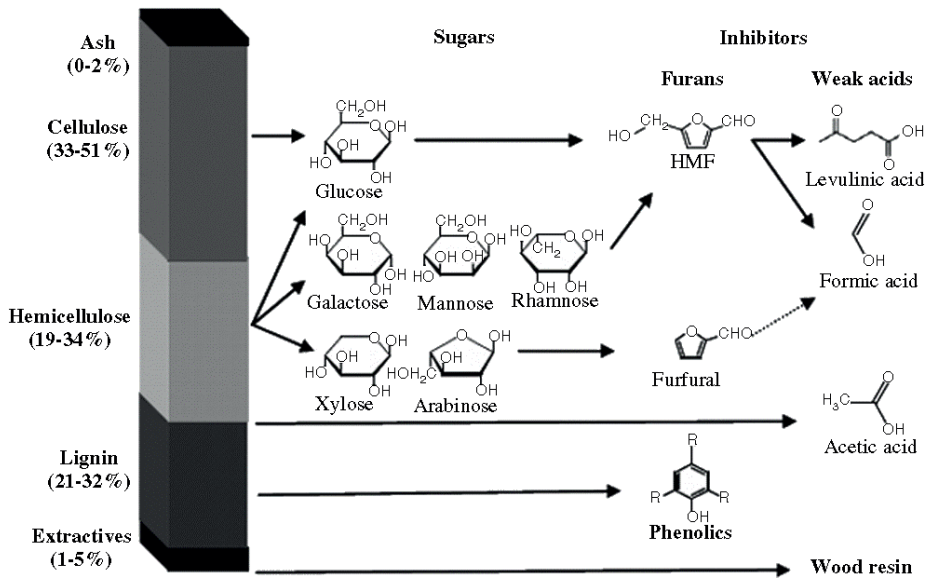
Figure 8. Average composition of lignocellulosic biomass (left), major monosugars (middle), and the most prominent products that may have inhibitory effects (right). This Figure was taken from Almeida, Modig & Petersson 2007.

Degradation of hexoses and pentoses generates the furan compounds 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde (also known as furfural), respectively. These furans, whose levels depend on the type of material and pretreatment method, can inhibit cell growth and will thus affect growth rates during SCP production (Palmqvist & Hahn-Hägerdal 2000). Both furan compounds cause a longer lag phase, but HMF is considered less toxic than furfural (Mussatto & Roberto 2004). In yeasts, furans interfere with redox-cofactor dependent enzymes, which are able to reduce furans to less toxic compounds (Sa et al. 2003). Therefore, the intracellular redox cofactor equilibrium can be disturbed, resulting into an inhibition of metabolic activity (Figure 9).

Of note, synergistic effects by the combined actions of various inhibitory compounds have been observed. Zaldivar and Ingram, 1999 demonstrated that the toxicity of hemicellulosic hydrolysates is due to the synergistic effect of combining several toxic compounds, including furfural, aldehydes and acetic acid.

One option to avoid problems generated by inhibitory compounds is to remove the inhibitors by hydrolysate detoxification. This may be achieved by, for example, physical (evaporation and membrane separation), chemical (ion exchange, neutralization and organic solvent extraction) or biological (treatment with laccase or peroxidase) methods (Liu et al. 2016). However, these processing steps come with costs (Almeida, Modig & Petersson 2007) and may lead to loss of fermentable sugars (Rivard et al. 1996), which may hamper the development of economically feasible large-scale fermentation processes.
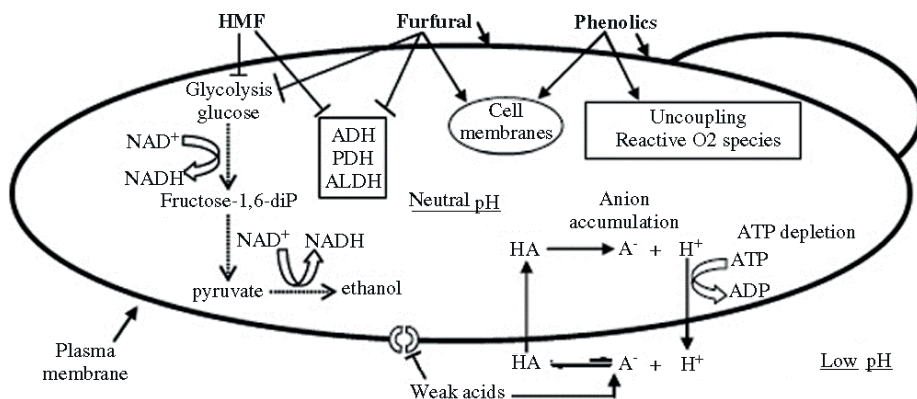
Figure 9. Schematic view of known inhibition mechanisms of furans, weak acids and phenolic compounds in *S. cerevisiae*. This Figure was taken from Almeida, Modig & Petersson 2007.

BALI^{TM} hydrolysates, produced by using a pretreatment based on a sulphite cooking step utilizing calcium and sodium as counter ions, are used as carbon source for production of single cell protein in the form of yeast in the studies described in Papers II and III. These hydrolysates contain low concentrations of fermentation inhibitors (Rødsrud, Lersch & Sjöde 2012).

1.3.3   Enzymatic saccharification of lignocellulose

The pretreatment step produces a solid fraction where cellulose is the main component and where the amounts of hemicellulose and lignin vary depending on the pretreatment used. BALI^{TM} pretreatment, which was used in the experimental studies described in Papers II and III of this thesis results in relatively "clean" cellulose, with a glycan content

in the order of 88.3 % of DM (Chylenski et al. 2017). Subsequent enzymatic hydrolysis of cellulose and remaining hemicellulose results in the production of monosugars, primarily glucose, which may be used in subsequent fermentations. Due to the crystalline nature of cellulose and the complex nature of hemicellulose, a single enzyme cannot carry out complete enzymatic hydrolysis; instead, multiple enzymes are required to deconstruct and depolymerize the cellulose and degrade the other polysaccharides (Figure 10).

Cellulases are produced by fungi, bacteria and protozoans and hydrolyse cellulose by cleaving ß-(1-4)-glycosidic bonds through hydrolysis. It is generally accepted that three kinds of cellulases are needed to convert cellulose to glucose monomers, namely exo-ß(1,4)-glucanases, EC 3.2.1.91 and EC 3.2.1.176 (also known as cellobiohydrolases), endo-ß(1,4)-glucanases, EC 3.2.1.4, and ß-glucosidases, EC 3.2.1.21 (also known as cellobiases). Endoglucanases (EC 3.2.1.4) hydrolyse internal ß(1,4)-glucosidic linkages randomly, primarily at amorphous sites in the cellulose. Exoglucanases (EC 3.2.1.91) are thought to attack the chain ends generated by the activity of endoglucanases, releasing short oligosaccharides, primarily dimers (cellobiose). Several well-known exoglucanases display processivity, which means that they catalyse multiple consecutive releases of cellobiose without fully dissociating from the cellulose chain in between catalytic cycles (Zhang et al. 2018). Finally, cellobiases (EC 3.2.1.21) hydrolyse the glycosidic bonds of cellobiose and cello-oligosaccharides and produce glucose.

Importantly, in 2010, Vaaje-Kolstad et al discovered a novel class of enzymes involved in cellulose conversion that are called lytic polysaccharide monooxygenases (LPMOs) (Vaaje-Kolstad et al. 2010). These enzymes cleave the glyosidic bond in the presence of an oxygen co-substrate (O_2 or H_2O_2) and reducing agent. However, the reaction mechanism of LPMO is still not well understood and it has been claimed that the full potential of LPMOs, which are present in modern commercial cellulase cocktails (Müller et al. 2015) has not yet been fully harnessed (Müller et al. 2018). The recent finding that LPMOs may use H_2O_2 rather than O_2 (Bissaro et al. 2017, 2018) may help in harnessing the full potential of these enzymes in an industrial setting, as recently shown by Müller et al. 2018.

Introduction

Due to the heterogeneous nature of hemicellulose, multiple enzymes are required for efficient hydrolysis. Xylans are the main hemicelluloses in hardwoods and herbaceous crops whereas mannans are common and often dominating in softwood, including spruce. Xylans, with a backbone of the pentose sugar xylose, may be degraded by xylanases, although additional enzymes may be needed to remove decorations (branches) that may inhibit the xylanases. Xylanases convert the xylan to shorter oligosaccharides, which are converted to xylose monomers by ß-xylosidases. Likewise, β-mannans may be degraded by mannanases and ß-mannosidases. An in-depth discussion of all the enzymes involved in hydrolysis of the polysaccharides in lignocellulosic substrates, and of the different synergic interactions between such enzymes, is not within the scope of this thesis and several reviews are available on the topic (Dyk & Pletschke 2012; Juturu & Wu 2014; Passos, Jr & Castro 2018; Srivastava et al. 2018). It is important to note though that this enzymatic step may represent a major cost (Stichnothe et al. 2016) and that the resulting sugar solutions will vary depending on not only the raw material but also the enzymes used. One major variable among cellulase-rich enzyme cocktails, which are needed to convert the main component, cellulose, is the degree to which these cocktails also depolymerize hemicelluloses.
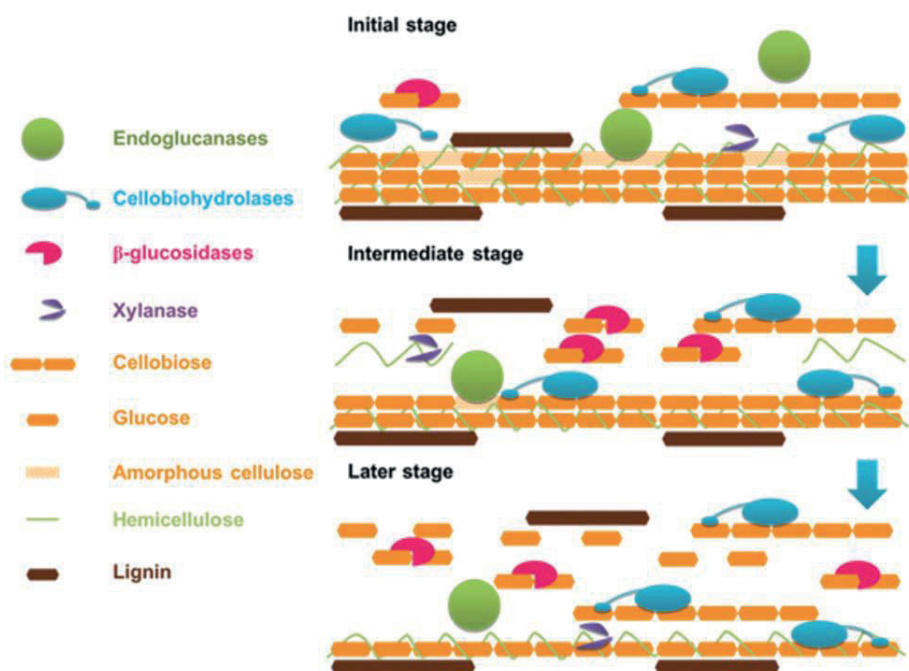
Figure 10. Schematic model of enzymatic degradation of polysaccharides in lignocellulosic biomass. Note that LPMOs are lacking from this picture; see main text for more details. This picture was taken from Du et al. 2013.

1.3.4 Spruce derived BALI™ sugars as a carbon source

Borregaard in Sarpsborg Norway is one of the most advanced biorefineries in operation today. Borregaard has developed the BALI™ process, which is based on sulfite-pulping and generates multiple high-value process streams, including (soluble) lignosulfonates and a relatively clean cellulose fraction. Figure 11 provides a schematic overview of the BALI™ process. The first, chemical pretreatment and fractionation step is crucial; it makes the lignin water soluble, and disrupts the lignocellulosic structure, making the cellulose and hemicellulose more accessible for further enzymatic hydrolysis. Process conditions can be tailored to vary the degree to which the hemicellulose ends up in the cellulose or the lignin fraction. In the first case, it will be part of the subsequent enzymatic hydrolysis process and yield monosugars that are eventually used for SCP production. In the latter case, the soluble lignin fraction will contain more sugars, which, in the BALI™ process, are removed by fermentation, before the lignin is further refined. The BALI™ process is attractive because the resulting lignin fraction, containing lignosulfonates, has a wide range of commercially attractive applications, e.g. as dispersing agents, binders and complexing agents.

Figure 11. Schematic description of the BALI™ process. See text for further details. Note that in the work described in this thesis, the hexoses and pentoses resulting from enzymatic hydrolysis were not used for production of ethanol or other chemicals, but for production of SCP. This picture was taken from Rødsrud, Lersch & Sjöde 2012.

The cellulose fraction resulting from the BALI[TM] pretreatment substrate contains only very low amounts of inhibitory compounds. Indeed, several yeasts, such as *Saccharomyces cerevisiae*, *Kluyveromyces max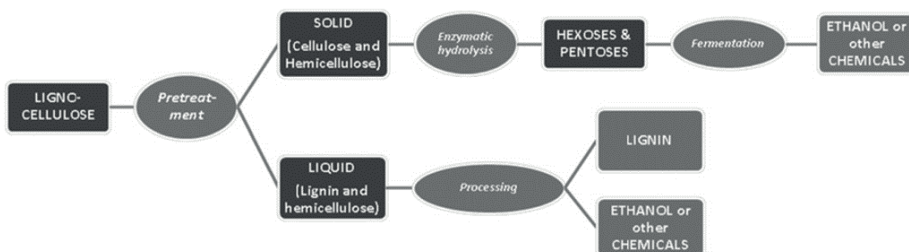ianus* and *Pichia jadinii* have been successfully grown using a BALI[TM] hydrolysates as a carbon source (Rødsrud, Lersch & Sjöde 2012). The BALI[TM] hydrolysates have been used as an alternative to commercial glucose for production of SCP in the studies described in this thesis. Papers II and III describe studies concerning the performance of BALI[TM] hydrolysates in fermentation of four different yeast strains: *Cyberlindnera jadinii* (anamorph name *Candida utilis*), *Wickerhamomyces anomalus*, *Blastobotrys adeninivorans* (synonym *Arxula adeninivorans*) and Thermosacc® Dry (*Saccharomyces cerevisiae*).

1.4 Protein hydrolysates as rich nitrogen source

Industrial fermentations are normally operated using semi-defined and complex media, including yeast extracts, peptones and growth factors, which can easily raise the price of the fermentations. Hydrolysates of different animal by-products such as fractions from salmon, chicken, pork and beef, can be used as a nitrogen source for production of SCP. These hydrolysates are often highly complex mixtures, containing minerals, carbohydrates, lipids, peptides and free amino acids derived from the original substrates, and may thus supply the medium with multiple important nutrients, besides nitrogen (Kampen 2014). By definition, protein hydrolysates are the amino acids and peptides of various sizes that result from the use of proteolytic enzymes or chemicals that break down and hydrolyse proteins (Paraskevopoulou et al. 2003). During the hydrolysis of proteins, protein solubility increases, allowing the proteins, peptides and free amino acids to dissolve into an aqueous phase which can easily be separated from lipids and sediments.

1.4.1 Pretreatment

Prior to hydrolysis, different pretreatments can be applied to the raw material depending on the starting material and the desired final product. In general, it is recommended to homogenize the meat and fish by-products in order to achieve a homogeneous starting raw material with good accessibility for enzymes. After mincing, some dilution of the raw material with water may be needed to allow good mixing and enzyme diffusibility (Kristinsson 2007).

Inactivation of endogenous enzymes by a short heat treatment has been applied in various studies in order to stabilize the material and control enzymatic reactions in processes with exogenous enzymes (Opheim et al. 2015). Such a heat treatment is not necessarily beneficial since it may lead to protein denaturation and precipitation, which may reduce hydrolysis yield (Slizyte & Nguyen 2004). Furthermore, there is no a priori reason to not exploit endogenic proteolytic activity when producing hydrolysates. One strategy, originally developed in the 1970ies (Mohr & Hanto 1973) is to use a slow temperature gradient when heating up the reaction mixture to the optimal temperature for the selected exogenous enzyme (typically around 50 ºC), in order to take advantage of endogenous enzyme activity at lower temperatures (Aspmo, Horn & G.H. Eijsink 2005; Lapeña et al. 2018). Of note, heating steps improve the microbial stability of the raw material. Thus, if heating steps are not applied, one needs to work with fresh raw materials and processes need to be fast.

1.4.2   Enzymatic hydrolysis

Enzymatic hydrolysis will alter the chemical, functional and sensory properties of the protein-rich by products (Paraskevopoulou et al. 2003). Proteolytic enzymes act by cleaving the peptide bonds of proteins and peptides, thus generating smaller peptides and amino acids. Several factors, such as the type of raw material, the type and amount of endogenous enzymes present in the substrate, process conditions (pH, temperature and time, as well as dry matter concentration), and the type and dosage of exogenous enzymes, will affect the efficiency of the hydrolysis process and the properties and composition of the resulting protein hydrolysate.

Proteolytic enzymes responsible for cleaving the peptide bonds can be divided into groups depending on different criteria. Several types of proteases are known and can be classified depending on several criteria, including their origin (plant, animal, microbial), pH preferences (acid, neutral or alkaline), catalytic mechanism (serine-, thiol-, carboxyl- and metallo-proteases), site of action (endoproteases and exoproteases) and substrate sequence preferences, which may be broad, as in e.g. pepsin, or narrow, as in e.g. trypsin.

Table 5 shows an overview of some known industrial proteases and their key properties, including the proteases used in the work described in Paper I of this thesis.

Table 5. Commercial proteases. The table was taken from Aspmo, Horn & G.H. Eijsink 2005.

Name	Enzyme Commission #	Source	Type	pH-range	Temperature range	Activity	Price[a] (Euro/kg)
Actinidin	3.4.22.14	Kiwi fruit (*Actinidia chinensis*)	Thiol protease	4–7	15–40	~100 TU/mg[b]	30
Alcalase® 2.4L	Subtilisin 3.4.21.62, Glutamyl endopeptidase 3.4.21.19, Ananain 3.4.22.31	*Bacillus licheniformis*	Serine protease	6–10	55–70	2.4 AU/mg[c]	25[d]
Bromelain	Bromelain A, B 3.4.22.32	Pine apple stem (*Ananas comosus*)	Thiol protease	5–8	20–65	~100 TU/mg[b]	20
Neutrase® 0.8L	3.4.24.28	*Bacillus amyloliquefaciens*	Metalloproteinase (Zn)	5.5–7.5	45–55	0.8 AU/mg[c]	15[e]
Papain	Papaya peptidase I, Papain 3.4.22.2, Papaya peptidase II, ChymoPapain 3.4.22.6, Papaya peptidase III, Caricain 3.4.22.30, Papaya peptidase IV, Glycyl endopeptidase 3.4.22.25	Papaya stem (*Carica papaya*)	Thiol proteases	5–9	40–80	100 TU/mg[b]	7
Protamex™	Subtilisin 3.4.21.62, Neutral proteinase 3.4.24.28	*Bacillus subtilis*	Thiol protease and metalloprotease	5.5–7.5	35–60	1.5 AU/g[c]	42
Supermix of Actinidin, Bromelain, Papain and Ficin	See above + 3.4.22.3	Kiwi fruit, pine apple stem, papaya stem and Fig latex (*Ficus elastica*)	See above + thiol protease	4–10	15–80	~100 TU/mg[b]	25

Sources: http://brenda.bc.uni-koeln.de/ and datasheets provided by manufacturers.
[a] As specified by suppliers. Prices may vary depending on purchase order quantity.
[b] One Tyrosine unit (TU) is defined as the amount of enzyme which releases the equivalent of 1 µg of tyrosine/min from a specified casein substrate under the conditions of the assay.
[c] One Anson unit (AU) is defined as the amount of enzyme that will release 1.0 mEq of tyrosine from urea–denatured hemoglobin/min at 25 °C, pH 7.5.
[d] 12.3% dry matter.
[e] 50% dry matter.

1.4.3 Autolysis

The use of endogenous enzymes for hydrolysis of proteins is usually referred to as autolytic processes, where the potential of natural enzymes present in the tissues is exploited. The use of endogenous enzymes may save costs, but the applicability of this strategy depends on the type and source of the raw material, and complicates process control. As an example, in fish by-products, the highest endogenous activity is normally found in viscera and liver, compared to muscle tissues (Opheim et al. 2015). Bower, Malemute and Bechtel, 2010 found different proteolytic activities in pink salmon tissues, and revealed differences between male and female fish and variation related to different levels of spawning maturity. It is worth noting that enzymes from seafood species are more adaptable to low temperatures than the ones from terrestrial animals, which means enzymatic activity is less depressed during refrigerated storage (Nielsen & Nielsen 2012).

The most relevant enzymes present in meat and fish that contribute to endogenous proteolytic reactions include both endopeptidases and exopeptidases. Endo-peptidases, such as calpains, cathepsins and caspases, are able to cleavage internal peptide bonds away from termini of the polypeptide chains. Calpains are neutral cysteine endopeptidases found in the sarcoplasm of muscles and they are most active at neutral pH (6.5-7.5) and 30 °C (Kowdziejska, Smorski & Universiry 1997). They degrade myofibrillar proteins, except myosin and actin, and their stability is poor (Koohmaraie 1994). Cathepsins are cysteine endopeptidases with an optimal pH of about 6 – 7 for Cathepsin H and pH 4.5 for Cathepsin D. The optimum temperature for activity is reported to be about 40 – 50 °C and activity decreases at lower temperatures. These enzymes are very stable, with reported activities lasting for many months (Toldra 1998). Caspases are cysteine-aspartate proteases involved in postmortem changes in proteins (Yuan & Horvitz 2004). Exopeptidases, such as tripeptidylpeptidases and dipeptidylpeptidases, cleave peptide bonds near the N- or C-terminus of the polypeptide chains (Reig & Toldra 2015). Tripeptidylpeptidases I and II are able to release tripeptides from the amino termini of peptides, giving an optimal pH of 4.5 and 6.5, respectively. Dipeptidylpeptidases I, II, III and IV are able to release dipeptides and have optimal pH values of 5.5 for types I and II and between 7.5 and 8 for types III and IV (Huff-Lonergan 2010).

1.4.4 Hydrolysis with exogenous enzymes

The use of exogenous enzymes is considered a good option in order to make the proteolytic hydrolysis more controllable and reproducible. In contrast to chemical hydrolysis, enzymatic hydrolysis can be achieved under mild conditions, and by playing with the conditions and using enzymes with varying specificities, scientists and manufacturers are usually able to tailor the process according to the specifications of the desired end product. Exogenous enzymes commonly used to produce protein hydrolysates for applications in biotechnology (Table 5) may be obtained from plants, animals and microbes. The most common proteases from animal source are pepsin, pancreatin and trypsin. Well known proteases from plant sources include papain and bromelain, whereas the most commonly used microbial proteases include products such as Alcalase, Protamex and Neutrase. Different commercial enzymes have been tested in the hydrolysis of a wide range of protein-rich raw materials derived from salmon (Idowu et al. 2019), tuna (Guerard, Guimas & Binet 2002), cod (Godinho et al. 2016), animal blood (Bah et al. 2016), chicken (Jin et al. 2014), and pork and beef (Meinert et al. 2015). Judged from the scientific literature, the most commonly used commercial enzymes are Alcalase (Seo et al. 2015), Protamex (Palupi, Windrati & Tamtarini 2010) and Neutrase (Pokora et al. 2013), from bacterial sources, and Papain (Seniman, Yusop & Babji 2014) and Bromelain (Slizyte et al. 2016), from plant sources.

Comparing existing studies on the use of exogenous enzymes presents several challenges. Firstly, there are several methods and assays to evaluate the hydrolysis performance, such as the determination of the degree of hydrolysis by evaluating the proportion (%) of α-amino nitrogen with respect to the total N in the sample, determination of the increase in trichloroacetic (TCA) soluble proteins, or determination of total nitrogen solubilisation using the Kjeldahl method. Secondly, exogenous enzymes vary in terms of optimal process conditions and such conditions, i.e., pH, temperature, dry matter loading, tend to vary between independent studies. Finally, the application of the protein hydrolysates will vary between different studies, which means that comparative assessment of the functionality (and value) of produced hydrolysates is complicated. For example, hydrolysates may be assessed as feed or food ingredients, or as a nitrogen source for SCP production, as in the studies described in this thesis. The choice of exogenous enzyme and process conditions, resulting in a certain final hydrolysate that is optimal according

to some standards and for some applications, is not necessarily optimal for other applications.

Looking at literature data, it is difficult to shortlist certain commercially available proteases as being the best or most promising, especially since the choice of protease also affects the functional qualities of the end product. It would seem though that Alcalase, which has been used to obtain peptides with antioxidant activity (Hamzeh, Benjakul & Senphan 2016), anticoagulant activity (Ren et al. 2014), calcium-binding peptides (Wang et al. 2018) and to produce peptones for microbial fermentation (Aspmo, Svein Jarle Horn & Eijsink 2005) is one of the most promising proteases.

1.4.5 Process conditions

The efficiency of a proteolytic reaction depends on several factors which have to be taken into account when producing protein hydrolysates. These factors are usually related to the choice of proteolytic enzyme as well as to the proteolytic susceptibility of the substrate. The pH and temperature are the most critical parameters, since they will directly affect the stability and activity of the exogenous enzymes, and may also affect proteolytic susceptibility of the substrate (although the latter is neglected in most studies, perhaps because it is difficult to assess). Optimal temperature and pH are normally determined based on experiments carried out by manufactures using well-defined substrates such as casein or haemoglobin in buffered solutions, i.e., conditions that are quite different from conditions during industrial bioprocessing of more complex substrates. Optimization of reaction temperature is straightforward. On the other hand, pH optimization can be more problematic for some applications, since the pH will strongly affect solubility of both substrates and (longer) products, and since pH management may lead to increased salt and ash contents, which can increase wear and tear on process equipment and be detrimental for the growth of microbes, while being costly to remove (White & Munns 1951).

Other important process parameters relate to mechanical and physical factors, such as the volume of the vessel, stirring of the enzyme-substrate mix, the amount of water used in the reaction (i.e., the dry matter, or DM, content of the reaction mixture), enzyme loadings and particle size of the raw material. Depending on the manufacturer and type of product,

the reactor size can vary greatly and the proteins are normally solubilized in water to anywhere between 8 – 20 % solids (Pasupuleti & Braun 2008). Obviously, several of these factors are related, for example through known or expected trade-offs between the enzyme dosage and DM content on the one hand, and processing time and time-space yields on the other hand. For example, studying hydrolysis of tongue sole, Sylla et al. 2008 showed that lower DM contents and higher enzyme dosages give faster processes but not necessarily higher space-time yields (due to the low DM content) or costs (due to high enzyme loadings). In addition, Diniz & Martin 1996 showed that increasing the enzyme:substrate ratio (E/S) above a certain limit can decrease the yield due to enzyme inhibition because the enzymes hydrolyse each other. Of note, when working with fresh materials, the processing time will be limited by the need to limit microbial growth as alluded to in section 1.4.1.

Another process variable concerns the possible inactivation of the proteases at the end of the reaction. At laboratory scale, and in order to get reproducible results, enzymes are commonly inactivated by immersion of the samples in water baths usually using temperatures ranging from 80 °C to 90 °C, for short period of times (10 - 20 min) (Whitehurst & Wiley 2010). Importantly, heat treatments may lead to heat-induced denaturation of proteins and one may need to optimize conditions, for example by using a decreased temperature for a longer time (Conesa & Fitzgerald 2013). When protein hydrolysates are going to be used as a microbial growth media, the temperature – time conditions employed during inactivation need to be carefully chosen to minimize protein and peptide aggregation, precipitation or gelation (Maux et al. 2017). Precipitation of metal ions that may be limited by incorporating low concentrations of chelating agents such as ethylene diamine tetraacetic acid (EDTA), citric acid or plyphosphates (Hughes & Poole 1991). Maillard reactions may also occur during heat treatments which entails that amino groups from amines and amino acids react with carbonyl groups from reducing sugars, aldehydes and ketones, possibly resulting in substances that can inhibit growth of some microorganisms (Kim & Lee 2003).

Finally, following the hydrolysis process and the inactivation of the enzymes, a separation step needs to be executed. Unless the lipid fraction has been removed before the enzymatic hydrolysis took place, the hydrolysate will be made up of three different

phases: an oil layer, a water phase (protein hydrolysate) and the sludge phase, which is composed of non-hydrolysed components and sediments. The different phases can be separated by centrifugation and then the water phase, which constitutes the water-soluble protein hydrolysate, may be filtered through different membranes depending on the application. Additionally, evaporation processes in order to concentrate the hydrolysate, as well as spray drying, may be carried out. Concentration is used to reduce the water content of the protein hydrolysates before drying because it reduces the energy cost during the drying step. The temperatures used for evaporation and evaporation rate vary for different protein hydrolysates depending on the composition of the mixture and other properties influencing the boiling point such as degree of hydrolysis, protein source or the choice of enzyme (Petrova, Tolstorebrov & Magne 2018). The protein hydrolysate solution can usually be concentrated up to 50 % solids (Pasupuleti, Holmes & Demain 2008). Subsequent drying of the hydrolysates ensures a stable shelf-life and allows easy transportation of the protein hydrolysates due to the reduced mass and volume of the final product (Crosby 2016).

1.5 Production of SCP (fermentation)

Efficient production of SCP requires the use of efficient fermentation technology. The main objectives for the design and process optimization of SCP production is to find culture conditions that give high biomass productivity and yield while at the same time efficiently utilizing the carbon source. During aerobic respiration processes, the maximum microbial biomass yield relative to the amount of glucose consumed is typically around 50% (w/w) (Verduyn et al. 1991).

Heterotrophic production of microbial biomass requires the supply of an organic source of carbon and energy, as well as sources of nitrogen, sulphur, phosphorous and other elements in smaller amounts (Ugalde & Castrillo 2002). For yeasts, potential carbon sources include a wide variety of substrates, such as glucose, xylose or a mixture of the two (Liu et al. 1995), diesel oil (Ashy & Zeid 1982), sulphite liquor (Sestakova 1979), hydrocarbons (Singh et al. 1990), ethanol (Prokop et al. 1978), methanol (Cereghino & Cregg 2000), starch (Spencer-Martins & Uden 1977), waste water (Lemmel, Heimsch & Edwards 1979), molasses (Gao, Li & Liu 2012), cheese whey (Schultz, Chang & Hauck 2006) and, in recent years, sugars from lignocellulosic biomass (Bajpai 2017). Nitrogen

may be provided in the form of protein hydrolysates or inorganic compounds such as ammonia, urea or nitrates, which are cheap and readily assimilated by most microorganisms (Leslie & Lampitt 1919). Important process parameters next to medium composition include pH, temperature and dissolved oxygen concentration, which need to be addressed in the development of an efficient bioprocess.

1.5.1 Screening of microorganisms for SCP

Process development for SCP production starts with screening for suitable microorganisms and screening of cultivation conditions. The main objective of these screening efforts is to select and identify microorganisms that are suitable for SCP production, which implies that they are able to efficiently utilize medium substrates, while producing high amounts of cells with high protein content. Screening studies are usually carried out in small volumes, for example using microtiter plate-based systems providing 96 wells of 200 µL (Sharma et al. 2018), 48 wells of 2 mL (Wehrs et al. 2019) and 24 wells of 11 mL (Kosa et al. 2018), or using shake flasks (Klöckner & Büchs 2011). It may be advisable to screen microorganisms using both methods. Shake flask cultivations can be more laborious than microtiter plate-based systems since bigger volumes are used, but may give more realistic data. Microtiter plate-based systems are particularly convenient when extensive screening studies are needed. The disadvantages of both methods are that only temperature and media composition can be controlled, whereas several potentially crucial fermentation parameters remain uncontrolled and unknown, such as water evaporation, pH, and lack of aeration (which may lead to anaerobic metabolism and production of fermentation products such as ethanol and acetate) (Anderlei et al. 2007). Screening studies with shake flasks and simple microtiter plates can yield useful qualitative and some quantitative data in the early stage of process development, but further optimization work using fermenters, where several factors can be better controlled, will usually be needed.

1.5.2 Process design and control in fermenters

A fermenter provides a controlled, aseptic environment for the growth of microorganisms or animal cells for prolonged periods of time. Fermenters can greatly vary in size and volume, from laboratory scale (usually 1 – 3 L) to pilot plant scale (from a few hundred

to a thousand liters), to industrial scale, where fermenters may reach sizes up to hundreds or even thousands of cubic meters. Fermenters are equipped with aeration systems that provide microorganisms in submerged cultures with the levels of oxygen that are needed to satisfy metabolic requirements. Agitation keeps oxygen levels and the concentration of the different components of the medium homogenous throughout the fermenter. The most important components of the fermenter involved in aeration and agitation are: the agitator (impeller), stirrer glands and bearings, baffles and the aeration system (sparger). The type of aeration-agitation system directly relates to the characteristics of the fermentation process and the final product. For example, Quorn^{TM}, which remains the only source of myco-protein for human consumption on the market today, is produced using a special airlift (pressure cycle) fermenter (Wiebe 2004).

The oxygen concentration, often quantified as dissolved oxygen (DO), is especially relevant for SCP production and must be subject to monitoring and control (Ugalde & Castrillo 2002). The degree of agitation, oxygen partial pressure in the supplied gas mixture and the gas flow rate are important determinants of the DO during fermentation (Hu, Zheng & Shen 2010). DO levels vary during fermentation and the typical set points for DO control range between 17 and 20 % air saturation for production of biomass for most yeasts and filamentous fungi (Bailey & Ollis 1986). Common problems in industrial fermentations may relate to both aeration and stirring and to the associated production of foam in the head space of the fermenter, which can cause overpressure, spillages and contamination problems (Hagman & Pi 2015). Thus, foam control must be a part of process design and the use of antifoam or mechanical foam breakers are important, as are pressure devices to sense, indicate, record and control the pressure.

Fermenters should also provide a system of temperature and pH control. A thermostat and a pH meter are usually coupled to the fermenter and are used to control the supply of heat and acid/base respectively. The pH of processes for production of SCP using yeasts and filamentous fungi is usually in the pH 4.5 – 5.5 range, and this acidic range is convenient since it reduces bacterial contamination (Peña et al. 2015). Of note, in some SCP production processes cooling may be needed due to heat production by the oxygen consuming microbe (Cooney, Wang & Mateles 1968) and mechanical agitation. When working at laboratory scale, it is usually sufficient to control the heat by placing the fermenter in a thermostatically controlled bath or by using a fermenter with a cooling

jacket where water is circulated. However, at industrial scale temperature control is not trivial and typically involves use of large volumes of cooling water. For example, the consumption of cooling water in a 55 m^3 fermenter can range from 0.5 to 2 m^{3}h^{-1} and 2 to 10 m^{3}h^{-1} for bacterial and fungi fermentations, respectively (Müller & Kieslich 1966).

In general, for yeast and fungal fermentations, it is recommended that carbon is the limiting factor in the fermentation, while oxygen levels are kept adequate for a balanced growth through oxidative metabolism (Nasseri et al. 2011). After the carbon source, the nitrogen source is usually the next most plentiful and important substance in the fermentation medium (Kampen 2014). Importantly, microbial growth in a fermenter will lead to continuous modification of the conditions within the fermenter, which may lead to time-dependent (i.e., fermentation stage-dependent) effects on microbial physiology, in particular in batch fermentation processes. For example, starvation resulting from substrate depletion and oxygen limitation issues due to increasing concentrations of microbial cells may come into play. Consequently, an adequate fermentation strategy needs to aim at having an appropriate amount of carbon, nitrogen and DO for a prolonged period of time. Fermentation strategies can be classified into batch & fed batch fermentations, continuous fermentations, or repeated fed-batch fermentations.

1.5.2.1 Batch/Fed-batch fermentation

During a batch and fed-batch fermentation a seed culture is used to inoculate the medium in the fermenter containing fixed starting concentrations of substrates. Batch fermentations will end after a short period of time because of depletion of at least one of the crucial medium components. Therefore, while batch fermentations are inadequate for the purpose of large-scale industrial biomass production (Fiechter, Kappeli & Meussdoerffer 1987; Oura 1983), batch fermentations can be useful for small scale production and research purposes.

The term fed-batch fermentation refers to processes where batch fermentations are initially established in batch mode after which the fermenter is continuously, or sequentially fed, with fresh medium without removal of culture (Yoshida & Yamane 1973). Thus, the liquid volume of the fermenter will increase during the fermentation

period. Fed-batch fermentation is better suited for biomass production as it can run for a longer time and avoid very high initial concentrations of substrates and nutrients that may be inhibiting for the microorganisms (Monika, Ian & Marison 2012). One main disadvantage of batch and fed-batch processes relates to the increasing oxygen demand throughout the fermentation process; at the beginning the oxygen demand is very low while at the end is very high due to the high biomass concentration, while this high concentration increases the viscosity of the broth, reducing the diffusion of oxygen. Increasing viscosity will also affect the diffusion of other components and put strain on stirrer and cooling systems (Stanbury, Whitaker & Hall 2017b).

1.5.2.2 *Continuous fermentation*

During continuous fermentations, fresh media is constantly fed into the fermenters while medium containing microbial biomass is continuously harvested. By doing so, medium conditions, including cell densities, are rather stable, oxygen levels are easy to control and inhibitors, if any, will not accumulate. In principle, continuous fermentation makes it possible to keep the microbes in their exponential growth phase during the whole cultivation under steady-state conditions, by controlling the dilution rate, which has to be below the maximum specific growth rate. The main advantage of continuous fermentations over batch and fed-batch fermentations is stable process conditions and high biomass productivity. The total biomass productivity during batch and fed-batch fermentations comprises an average over the fermentation period, where initial and late phases have growth rates below maximum. On the other hand, productivity during continuous fermentations operating at their optimum dilution rate will be constant and always close to the maximum (López-Gómez et al. 2019). Nevertheless, continuous fermentation also has some weaknesses. In order to achieve maximum growth rate the microbe needs a certain substrate concentration. Thus, in continuous fermentation there is a trade-off between maximum growth rate and waste of sugars and nutrients, and the dilution rate will determine the substrate concentration. Additionally, the lower concentration of the continuously harvested product, can lead to higher costs and a need for more equipment in order to concentrate the biomass (Rushton & Khoo 1977). Continuous fermentations can operate for many weeks (Vasey & Powell 1984), and that is sometimes used as an argument against continuous fermentations because there might be a greater possibility for contamination (Stanbury, Whitaker & Hall 2017b). However,

the majority of SCP production processes that have been established are based on continuous fermentation (Drejer et al. 2017; Rose 1980; Westlake 1986; Wiebe 2004).

1.5.2.3 *Repeated fed-batch fermentation*

Repeated fed-batch (also called repeated-batch) fermentation is an interesting semi-continuous system of operation for production of SCP, since it combines features of batch/fed-batch fermentations and continuous fermentations (Pirt 1974). The process can be divided into a first stage, which resembles a batch fermentation, followed by multiple consecutive second steps. After substrate depletion in the first step, the microorganisms will stop growing, as reflected in an (easily measurable) reduction in oxygen consumption (Huang, Chen & Chen 2008). When growth slows down, a percentage of the medium, which can range from 25% to 75% of the fermenter volume, is withdrawn from the fermenter, while fresh medium is added to the remaining microbial suspension in the reactor acting as an inoculum for the next batch culture (Bajpai & Bajpai 1988). In contrast to batch fermentation, production of biomass can be prolonged over time, there is no inoculum requirement because of the reuse of microbial cells for subsequent fermentation runs, and fermenter conditions remain favourable since microbial biomass is withdrawn and replaced with fresh medium before issues related to the increase of microbial biomass, such as problems related to oxygen diffusion, arise (Anastassiadis & Rehm 2006). Thus, repeated batch fermentation usually increases productivity (Radmann, Reinehr & Costa 2007). As another advantage, the harvested medium contains high concentrations of microbial biomass, which is beneficial for the efficiency and costs of subsequent downstream processing, compared to the continuous fermentation. The repeated fed-batch fermentation strategy has been presented as an alternative to batch and fed-batch fermentation modes not only in SCP production (Bajpai & Bajpai 1988), but also in fermentative production of lipids, docosahexaenoic acid and penicillin (Dashti 2016; Li, Zhao & Yuan 2005; Qu et al. 2013).

1.6 Downstream processing of SCP

Prior to the formulation of the final SCP product, several downstream processes are required depending on the type of microorganism used in the fermentation, and the application of the final product. After fermentation, the microbial biomass needs to be

separated from the fermentation broth and, subsequently, several downstream processes, such as washing, cell disruption, and drying may be carried out. The different downstream processes have an effect on the nutritional value of the final product (Jamal, Alam & Salleh 2008).

The extraction, recovery and purification of fermentation products might be difficult and costly. Therefore, efficient recovery of a high-quality product, at minimal cost and maximum speed is required (Stanbury, Whitaker & Hall 2017c). It has been estimated that the costs of downstream processing (DSP) in SCP production may vary from 15 % to as high as 70% of the final cost of the product (Straathof 2011). The DSP costs for yeast SCP are estimated to be between 20 – 30 % of the total production cost (Hacking 1986; Van't 1984). Separation of yeasts from the fermentation broth by centrifugation techniques is relatively easy due to the big size of the cells and their flocculation properties (Soares 2010). Centrifugation is commonly applied in the recovery of baker's yeast, SCP, fodder yeast and algae. In general, DSP requires a multi-stage system that combines washing and separating in order to reach an adequate product yield and cleanliness, i.e., with adequate removal of undesired compounds originating from the medium or generated during the fermentation (Gelines 2017).

A typical DSP strategy for treating baker's yeast would be multi- stage separation using a series of centrifuges, while water recycled from the process is recirculated and used to clean the yeasts. It has been demonstrated that this configuration gives high production capacity, while water consumption is reduced (Wielen & Ottens 2016). It is important to remove as much water as possible in order to minimize the heating costs in the subsequent drying process. The need for washing depends on the medium used but is clearly needed if the medium contains e.g., side products derived from lignocellulosic materials and/or undigested compounds from hydrolysates of meat and fish. The yeasts may be used as whole cells, or the cells may be first disrupted to increase the bioavailability of the protein, remove nucleic acids and/or release (non-proteinaceous) yeast cell wall components (YCW) for which different applications exist (Maeng et al. 1975; Nasseri et al. 2011; Schiavone et al. 2015), as outlined in section 1.7, below.

After processing, a drying treatment is needed that removes water while ensuring that there is a minimum loss of nutritional value. The drying treatment can affect the

digestibility of the microbial biomass and also affect the quality of the protein and other valuable cell components (Pradelles et al. 2008). Logistically, a dried product is easier to handle and package, can be stored more conveniently and can reduces costs in transport. A detailed review of the theory and practice of drying can be found in Perry & Green 1984. Freeze-drying (also known as lyophylization or cryodesiccation) can be used in laboratory scale, but, in an industrial scale, freeze-drying is only used in the production of biological and pharmaceutical products with higher selling prices than SCP (Day & Stacey 2007). Spray-driers are the most economical alternative for drying large volumes of cell suspensions such as in the production of brewer's yeast and SCP (Luna-Solano et al. 2005).

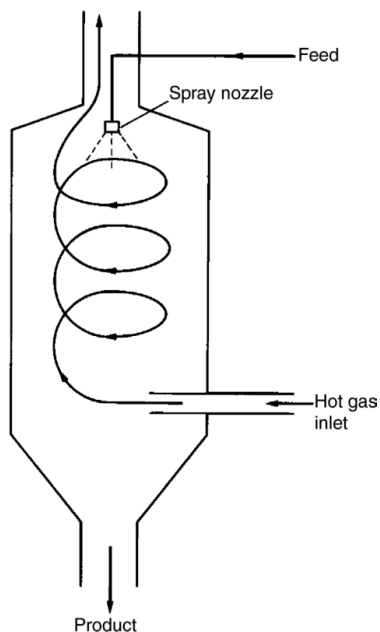Figure 12. Spray-drier. This picture was taken from Stanbury et al., 2017b.

Figure 12 shows the principle of spray-drying. The material to be dried does not come into contact with heating surfaces, but is sprayed in small droplets through a nozzle into a spiral stream of hot gas at 150 – 250 °C. Due to the high surface area of the droplets, the microbial biomass dries in a few seconds, while heat exposure remains limited.

Finally, the spray-dried microbial biomass needs to be blended into a feed preparation, usually using an extruder. This is an essential step that should be optimized to preserve the nutritional value of the ingredients and promote digestibility. A feed paste, including microbial biomass, is conditioned by mixing and addition of water, oil, and steam, and the temperature is increased to 80–90 ° C during extrusion. Apart from heat, the feed is also subjected to strong shear forces and pressure during extrusion. The resulting pellets are then dried, usually by using hot air, and cooled, usually by using cold air (Storebakken et al. 2004). Extrusion technology is widely employed in the production of aquaculture feeds even though the effect of extrusion processing (mainly heat) is a major concern regarding the nutritional value of the ingredients (Camire, Camire & Krumhar 1990). Still, it has been shown that extruded diets containing microbial biomass may outperform benchmark commercial diets during feeding trials with tilapia fish (Simon et al. 2019).

1.6.1 Cell wall degradation and isolation of single cell protein products

In recent years, yeasts have been receiving increased attention in health and animal nutrition, due to their antimicrobial, biosorption and probiotic activities (Øverland & Skrede 2016; Wang & Chen 2006; Younis et al. 2017). Even though the mechanisms and causes underlying these health- and/or growth-promoting activities are still being studied and remain largely unclear (Hatoum, Labrie & Fliss 2012), it is generally assumed that they are connected to cell wall components. Cell walls are crucial structural components that give the yeast its shape and rigidity, and correspond to 26 – 32 % of the dry weight of *Saccharomyces* and several other yeast species (Nguyen, Fleet & Rogers 1998). Electron microscopy studies indicate an ultrastructure with two distinct layers (Osumi 1998), an outer layer consisting of mannoproteins (30 – 50% of cell wall dry mass), and an inner layer made of chitin (2 – 5% of cell wall dry mass) and β-glucans (40 – 60% of cell wall dry mass), see Figure 13.
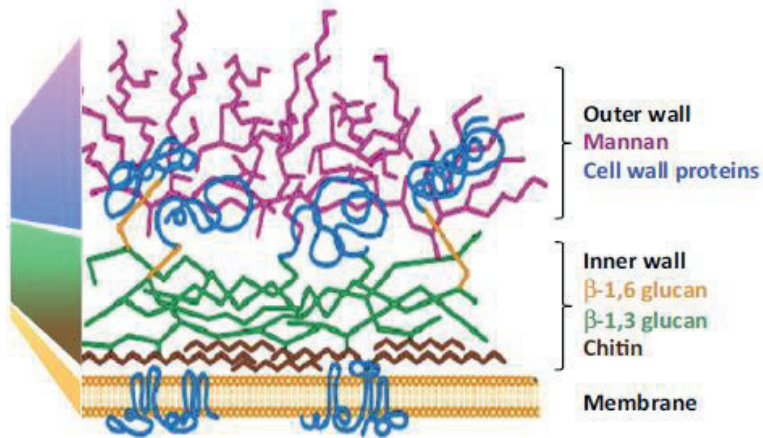
Figure 13. Structure of the *C.albicans* cell wall. This picture was taken from Gow et al. 2013.

Yeast mannoproteins have been extensively studied in the fields of animal and fish nutrition because of their prebiotic activities (Ganner & Schatzmayr 2012; Kyu et al. 2014), whereas β-glucans have been reported to promote health in both animals and humans (Du, Bian & Xu 2014). The extent to which a yeast cell wall exerts biological functions depends of the abundance and biological availability of different cell wall components, which again depends on growth conditions and downstream processing methods (Aguilar-Uscanga & François 2003; Nguyen, Fleet & Rogers 1998). Several methods can be used to disrupt yeast cell walls and isolate cell wall components, including the use of mechanical forces (crushing, pressure homogenization and ultra-sonification), chemical treatments (acid or alkaline), physical treatments (thermolysis and osmotic shock) and endogenous or exogenous enzymes (Bzducha-Wrobel et al. 2014).

Chemical and physical methods can be very harsh and may destroy the cell wall components if they are not well controlled in terms of their harshness (e.g., high temperature or extreme pH) and duration (Raschmanová et al. 2018; Schiavone et al. 2018). Enzymatic treatments are interesting because they are delicate, specific and can be based on using only endogenous enzymes in a process called autolysis. The later process, autolysis, is considered an adaptive response triggered by starvation conditions that allows the survival of the cells due to recycling of the building blocks resulting from self-digestion (Takeshige et al. 1992). Autolysis involves disturbance of internal membrane

systems and release of vacuolar hydrolases into the cytoplasm (Connew 1992). Thus, undesired autolysis can occurred when carbon sources are exhausted during fermentation and yeasts are kept under stationary phase for a prolonged period of time (Cebollero & Gonzalez 2006). From a process design perspective, uncontrolled autolysis can be prevented by withdrawal of yeast biomass, and addition of fresh media by semi continuous and continuous fermentation strategies, as discussed above. On the other hand, autolysis is a well-known industrial method to produce yeast extract, which can be controlled by temperature, pH, duration and concentration of yeasts (Peppler 1982). Of these factors, temperature is the most important, and temperatures around 50 ºC are known to promote autolysis (Tangüler & Erten 2009). Alternatively, exogenous enzymes such as proteases, β-glucanases and chitinases can be used to remove yeast cell walls and/or to generate cell wall-derived products (Krysan et al. 2005). Another possibility is the combination of mechanical and enzymatic treatments (Baldwin & Robinson 1994; Damodaran & Kinsella 1983).

1.6.2   Nucleic acids and their removal

Yeast and bacteria generally have high nucleic acid contents, in particular RNA. High nucleic acid contents can be problematic when the end product is going to be used as a feed ingredient for animals, or used as a food ingredient for human consumption (Anupama & Ravindra 2000). Intake of SCP with high content of nucleic acids can provoke the production of high concentrations of uric acid, which can lead to health disorders such as gout and kidney stone in humans (Moe 2014). Similarly, the inclusion of SCP with high nucleic acid levels (i.e., more than appr. 8 - 12 % of dry weight) into animal feed is not recommended for animals with a long-life span. However, SCP with high levels of nucleic acids can be used for animals with a short life span, such as fish (Øverland & Skrede 2016) or broiler chickens (Esteve-Garcia et al. 2007; Jacob & Pescatore 2014). The amount of nucleic acids depends on growth conditions, growth rate and C/N ratios in the medium (Trevelyan 1976). There are different methods to reduce nucleic acid levels in SCP, including chemical treatments by alkaline extraction at high temperatures, which results in high protein yields, but may cause the formation of toxic compounds such as lysinoalanine and, generally, reduce product quality (Damodaran & Kinsella 1983). Enzymatic treatments form an alternative and may be based on using

endogenous RNA degrading enzymes (ribonucleases), as is done in the production of Quorn[TM] (Trinei 1992), or by using exogenous ribonucleases (Hames 2015).

1.7 Functional assessment of SCP

The term "functional feeds" refers to feeds that have positive effects on health and/or growth, by supplying additional compounds beyond basic nutrients (Tacchi et al. 2011). Since SCP in not necessarily cheaper than conventional protein sources such as soybean meal, additional "functional" effects are of major importance to judge the overall potential of SCP production (Øverland et al. 2010). For example, there is increased interest in replacing antibiotics in aquaculture and animal farming, and using yeasts as feed additives has been suggested as one way to reduce the dependency on antibiotics since yeast could improve the health and robustness of fish and animals (Gao et al. 2008; Li et al. 2006; Nayak 2010). These positive effects of yeasts are often attributed to the structural components of the yeast cell described in section 1.6.1, above. Therefore, it is important to investigate these yeast cell wall components (YCW) in *in vitro* and *in vivo* trials.

1.7.1 *In-vitro* evaluation

The screening of the health promoting effects of functional feed ingredients in animals or fish is a difficult task due to several factors such as long periods of growth, lack of controlled experimental facilities, the need for larger amounts of test material and high costs (Caruffo et al. 2015). These difficulties have led researchers to find alternative, inexpensive and relatively simple models that allow screening of many potentially health-promoting ingredients in a controllable manner by the use of appropriate cell lines. Such *in vitro* screening methods can produce results within weeks to months, rather than within months to years, as often is the case for *in vivo* experiments. A cell line is a population of cells from a multicellular animal or plant that can be propagated outside the organism through the serial transfer (sub-cultivation) of cells from one culture vessel to another (Schaeffer 1990). In the case of aquaculture research, rainbow trout (RT) cell lines were the first models used, starting with the establishment of the rainbow gonadal cell line (Wolf & Quimby 1962), and to this day, RT cell lines continue to be developed (Bussolaro et al. 2019). Recently, J. Wang et al. 2019, suggested that RTgutGC cell lines

possess characteristic features of functional intestinal epithelial cells, indicating that these cells could be used as an *in vitro* model to evaluate effects of feed ingredients on gut epithelial function, including intestinal absorption of nutrients, immunity and barrier function.

Indeed, the literature contains several claims concerning feed functionalities that are based on experiments with many types of cells, and also *in vitro* assays mimicking the gastrointestinal tract (Moyano et al. 2014). For example, Tomas et al. 2017 described the immunostimulatory effects of three fungal glucans on innate immune responses in Pacific red snapper, using leukocytes, whereas probiotic properties of the yeast *K. marxianus* were demonstrated using cell cultures derived from the human colon (Maccaferri et al. 2012). The use of *in vitro* approaches is required in order to facilitate further research on the use of whole yeast cells or derived products containing various fractions of the yeast cells or the yeast cell wall. The goal is to map as much as possible related to nutritional value, digestibility, immune responses and effects on disease resistance, before a potential functional feed ingredient is tested in full animal trials.

1.7.2   SCP in animal and fish diets

Evaluation of a novel protein ingredient implies performing *in vivo* experiments with different animals and/or fish. In general, the evaluation starts with short-term experiments to determine the nutrient digestibility (i.e., the proportion of a nutrient in an ingested feedstuff/diet that has been absorbed from the digestive tract), metabolic utilization (i.e., balance and respiration experiments) and/or the palatability of the ingredient (Glencross, Booth & Allan 2007; Kong & Adeola 2014). To evaluate growth performance or retention of nutrients in the body, longer-term experiments are usually needed (months). In addition, a range of different experiments can be performed to evaluate potential positive or negative health effects in the animals (or fish). Finally, to determine long-term effects of a novel feed ingredient, field trials are often used for verification. The same applies to testing of SCP (Bajpai 2017). These parameters, and, generally, the availability of nutrients provided by a given SCP, are affected by several factors including the yeast species, the fermentation medium used for yeast production, growth conditions during fermentation, and downstream processing (Kargi et al. 1980).

Yeast and other microbial feed ingredients have received considerable attention in aquaculture diets in recent years (Gamboa-Delgado & Márquez-Reyes 2018; Øverland & Skrede 2016). Digestibility and amino acid profiles of one of the most commonly used yeasts, *Cyberlindnera jadinii* (anamorph name *Candida utilis*), are comparable to fish meal and soybean meal (Øverland & Skrede 2016), thus *C. jadinii* may be added to fish and animal diets as a partial replacement of other protein sources, as has been confirmed in feeding trials with e.g., Atlantic salmon (Øverland et al. 2013), and pigs (Cruz et al. 2019). Furthermore, yeast and yeast-derived components such as β-glucans, mannan oligosaccharides and nucleic acids, may have health beneficial effects such as enhanced performance and immune responses and increased disease resistance in both farm animals such as pigs and broiler chicken diets and fish (Bass et al. 2019; Grammes et al. 2013; Morales-Lopez & Brufau 2013).

Of note, nucleic acid levels, toxins that can be produced by some microbes, potential allergic reactions, and potential harmful effects caused by traces from the feedstocks used in the fermentation medium, all have to be considered and, if possible, tested before using SCP in animal and fish diets (Ritala et al. 2017). In Europe, only yeast species that are GRAS (generally-regarded-as-safe), and included in the *Commission Regulation (EU) 2017/1017 of 15 June 2017 amending Regulation (EU) No 68/2013 on the Catalogue of feed materials* 2017 are approved for use in farm animal and fish diets. A further discussion of the use of yeast in aquaculture is not within the scope of this thesis and several reviews on this topic are available (Mohan, Ravichandran & Muralisankar 2019; Øverland & Skrede 2016; A. Wang et al. 2019). Suffice to say that yeasts are promising feed ingredients in fish feed.

2   Outline and aim of the thesis

The main objective of the work described in this thesis was to demonstrate that protein hydrolysates produced from by-products generated during meat and fish production and sugars produced through enzymatic hydrolysis of lignocellulosic biomass, can be used to efficiently produce microbial proteins in the form of yeast biomass. In order to achieve this objective, the following sub-goals were set: 1) Production and detailed characterization of protein hydrolysates from pork and beef, chicken and salmon, using endogenous and commercial exogenous enzymes; 2) Screening, production and characterization of yeasts grown on media composed of spruce-derived sugars and protein hydrolysates; 3) Optimization, production and upscaling of yeast production using a semi continuous fermentation strategy (repeated fed-batch fermentation), and use of different cell disruption methods to generate samples for future *in vitro* and *in vivo* trials. Enzymatically prepared lignocellulose-derived sugar solutions were produced in pilot facilities at Borregaard AS. Of the multiple protein hydrolysates produced and tested in the initial phase of the study (Paper I), only hydrolysates derived from poultry by-products were used in subsequent fermentation studies (Papers II and III).

Paper I describes studies aimed at the efficient conversion of different protein-rich industrial by-products, from pork and beef, chicken and salmon, to protein hydrolysates by using endogenous enzymes alone (autolysis) or in combination with different exogenous commercial proteases. The efficiency of the enzymatic hydrolysis was evaluated based on the minimum enzyme loading required to generate high nitrogen solubilisation in an (industrially relevant) short period of time at the natural pH of the substrate. A comparative analysis of the protein hydrolysates, with focus on molecular weight distribution and amino acid composition, was also conducted. Finally, the resulting hydrolysates were tested for their ability to replace commercial nitrogen sources commonly used in fermentation media for *C. utilis*.

In the study described in paper II, a medium consisting of sugars produced through enzymatic hydrolysis of lignocellulosic biomass and enzymatically hydrolyzed chicken by-products was produced for cultivation of SCP in the form of yeast. Four different yeast strains were tested: *Cyberlindnera jadinii* (anamorph name *Candida utilis*), *Wickerhamomyces anomalus*, *Blastobotrys adeninivorans* (synonym *Arxula*

adeninivorans and Thermosacc® Dry. We carried out a preliminary screening of growth in microtiter plates, where ten different growth media were tested. The most promising medium was then tested in batch fermentations using benchtop fermenters, where concentrations of cells, substrates, side-products and yeast protein were monitored. We also characterized and compared the four different yeast biomasses generated after the batch fermentations, assessing properties such as amino acid composition, and the content of nucleic acids, minerals, lipids, carbohydrates and ash. Finally, we assessed the composition of the yeast biomass with Fourier transform infrared spectroscopy (FTIR).

Based on the results described in Paper II, three different yeast strains were selected for further studies on the optimization of the fermentation: *Cyberlindnera jadinii* (anamorph name *Candida utilis*), *Wickerhamomyces anomalus* and *Blastobotrys adeninivorans* (synonym *Arxula adeninivorans*). The purpose of the study described in Paper III was to produce SCP in a semi-continuous mode (repeated fed-batch fermentation) using a medium composed of sugars from enzymatically hydrolysed lignocellulosic biomass, enzymatically hydrolyzed poultry by-products and urea. Repeated fed-batch fermentations at 1.5 L scale were carried out using benchtop fermenters, where concentrations of cells, substrates, side-products and yeast protein were monitored. Production of the best performing yeast strain, *W. anomalus*, was scaled up to 25 L, and the resulting yeast biomass was analyzed for protein and amino acid content. Finally, the effects of several cell disruptive methods were investigated using autolysis, exogenous enzymes and mechanical force.

3 Main results and discussion

Paper I

Paper I describes a study aimed at using hydrolysis to upgrade three different protein-rich industrial by-products from the animal and fish industry: mixed pork-beef, chicken and salmon viscera. The objective was to develop a process based on using endogenous enzymes alone (autolysis), or in combination with one of two exogenous enzymes (Alcalase® 2.4L or Papain), to produce hydrolysates with high nitrogen yields that could be used as an alternative nitrogen source for cultivation of single cell protein in the form of yeast (**Paper II**). The processes were based on using short hydrolysis times and minimum enzyme doses.

To ensure homogenous distribution of the initial substrates in the hydrolysis reaction tubes, raw materials were minced with a manual knife mill and subsequently kept in aliquots in the freezer until further use. Proteolytic reactions were carried out using the natural pH of the substrate in order to avoid the addition of chemicals which could hamper microbial growth when utilizing the hydrolysates in fermentation media, and to minimize costs. As a consequence, the exogenous enzymes used in this study, especially Alcalase® 2.4L, were utilized under suboptimal pH conditions. A temperature gradient of about 30 min followed by incubation at 60 °C was utilized in order to exploit the activity of the endogenous enzymes present in the different raw materials (Aspmo, Horn & G.H. Eijsink 2005; Mohr & Hanto 1973; Reig & Toldra 2015). During the optimization of the hydrolysis process, several exogenous enzyme loadings (w/w) (weight of enzyme power or liquid/weight of wet raw material) of Alcalase® 2.4L and Papain were tested, from 0 % (Autolysis) to 0.02, 0.05, 0.1, 0.2, and 0.5 % (w/w), using incubation times of 1 h or 2 h only. After the hydrolysis reactions, centrifugation and filtration techniques were used to separate the protein hydrolysates from lipids and residual particles (for details, see the Materials and methods section of the **Paper I**). The protein hydrolysates were characterized with respect to soluble dry matter (Tables S1-3 in the supplementary information), total recovery of protein (Figures 1-5), the peptide molecular-weight distribution (Figure 6) and the composition of total and free amino acids (Tables 2-3 and Figure 7).

For pork-beef by-products, nitrogen solubilisation was about 60 % without any incubation (0h) and 70 % for autolysis. The highest solubilisation yields were obtained by adding 0.05 % of Alcalase® 2.4L or Papain, which resulted in solubilisation yields of 90 % after 2 h. There was no clear dose-response for both commercial proteases (Figure 1, Paper I), and this encouraged us to shorten the incubation time to 1 h, and carry out a hydrolysis experiment using the highest enzyme loading (0.5 %), the best enzyme loading from the previous experiment (0.05 %), and autolysis. The results were similar to hydrolysis using 2 h, except for the minimal 0.05 % (w/w) enzyme loading, where the yield after 1 h was only about 70 %. Papain performed slightly better than Alcalase® 2.4L.

The dosing of commercial enzymes was more important in hydrolysis of chicken by-products. The non-hydrolysed chicken by-products (0h) showed only 20 % nitrogen solublization, and autolysis yielded 50 % protein solubilisation after 2 h. Enzyme doses of 0.02 % (w/w) did not improve solubilisation compared to autolysis, but a clear enzyme-dosage effect was observed from 0.05 % to 0.5 % (w/w) for both Alcalase and papain, reaching maximum nitrogen solubilization yields of 77 and 69 %, respectively. Higher enzyme dosages, 1 % (w/w), did not improve the yields any further, which could be due to a combination of the substrate becoming limiting and increased self-destruction (auto-hydrolysis) of the proteases (Diniz & Martin 1996). For chicken by-products the reduction of hydrolysis time from 2 h to 1 h led to a reduction of solubilisation yields.

Addition of Alcalase® 2.4L or Papain did not improve nitrogen solubilisation yields for salmon viscera, which varied from 80 to 85 %. The solubilisation yield for non-hydrolyzed viscera (0 h) was as high as 70 %. The final pH of the hydrolysis reaction after 2 h (6.3-6.4) was almost identical to the initial pH (6.5), and similar solubilisation yields were achieved after 1 h (results not shown in Paper I). Apparently, the fresh salmon viscera used in this study contained a high amount of endogenous enzymes, and some autolysis had likely taken place somewhere between slaughtering, freezing, and subsequent handling. Similar observations have been reported previously (Opheim et al. 2015). The highest endogenous activity in seafood species is normally found in viscera and liver, compared to muscle tissues (Aspmo, Horn & G.H. Eijsink 2005; Opheim et al. 2015). These enzymes are likely adapted to low temperatures, compared to, e.g., enzymes from terrestrial animals, which means that their enzymatic activity is less depressed

during refrigerated storage (Nielsen & Nielsen 2012) or when running reactions at low initial temperatures, as done here.

For all three raw materials, the protein content in the hydrolysates, calculated from Kjeldahl nitrogen, represented 85-90% of the solubilized dry matter. The peptide molecular weight distribution showed that use of Alcalase® 2.4L and Papain had a clear effect on the peptide composition of the meat-based hydrolysates, even though their use had a modest effect on the solubilization of nitrogen. On the other hand, and in line with the lack of effects on nitrogen solubilization yields, commercial enzymes did not have any effect on the peptide distribution in the hydrolysates of salmon viscera.

The amino acid contents of the various hydrolysates were determined and compared with the amino acid content of commercial YP medium, which was used for bench-marking. The amino acid compositions varied substantially between the different substrates, and statistical analysis showed that, with the exception of chicken hydrolysates, the variations in the amino acid compositions are defined by the raw material and not by the hydrolysis method. Peptone in YP is industrially produced by hydrolysing animal tissues and that could explain the similarities with the amino acid composition of pork-beef hydrolysates. The content of free amino acids in the different hydrolysates was between 6 and 12%, being lower than the free amino acid content of YP.

Finally, growth of the yeast *C. utilis* (today referred to as *Cyberlindnera jadinii*) on media based on the protein hydrolysates was compared with growth on commercial YP medium. All three protein hydrolysates outperformed commercial YP, and there were no significant differences between the three in terms of the final dry cell weight (g/L) of *C.utilis* after 24 h. Additional work was needed in order to further evaluate the potential of these protein hydrolysates, and such work, done for chicken hydrolysates only, is described in **Paper II**.

Overall, **Paper I** demonstrates that it is technically possible, and perhaps of commercial interest, to produce useful hydrolysates by enzymatic hydrolysis of different protein-rich by-products. This was achieved through the combined use of endogenous enzymes and small amounts of commercial enzymes, with minimal hydrolysis times and without pH adjustment. The preliminary growth experiments of **Paper I** showed that these different protein-rich hydrolysates could be promising ingredients for growth media for yeasts.

Paper II

On the basis of the results described in **Paper I**, chicken by-products were the selected agroindustrial substrate to be enzymatically hydrolysed by using 0.5 % (weight of the enzyme powder/weight of wet chicken by-products) Papain at 60 ºC, with agitation at 50 rpm, without pH adjustment, using slow heating (temperature gradient) to 60 ºC and a total incubation time of 2 h. In order to produce enough fermentation media, enzymatic hydrolysis was done using 15 kg batches wet chicken by-products that were mixed with 15 L of water in a 30 L reactor. The main aim of the study described in **Paper II** was to further study the possibility to produce SCP in the form of yeast on a medium consisting of enzymatically hydrolysed sulphite-pulped spruce wood (BALI^{TM} hydrolysate), mainly providing glucose, and enzymatically hydrolysed chicken by-products, mainly providing nitrogen. Experiments were conducted with four different yeast strains, selected because of their high potential for biotechnological and especially food-related applications: *Cyberlindnera jadinii* (anamorph name *Candida utilis*), *Wickerhamomyces anomalus*, *Blastobotrys adeninivorans* (synonym *Arxula adeninivorans*) and Thermosacc$^{®}$ Dry. Fermentations with a selected spruce-chicken medium and a commercial reference medium were carried out after preliminary growth tests in microtiter plates consisting of 24-square deep wells plates of 11 mL, in 2.5 L fermenters. The four different yeast biomasses generated after batch fermentations were subjected to detailed compositional analysis.

We carried out a preliminary screening of the four yeasts with ten different fermentation media in microtiter plates: yeast nitrogen base without amino acids and with ammonium sulfate plus glucose (YNBAS+G), yeast nitrogen base without amino acids and with ammonium sulfate plus BALI^{TM} hydrolysate (YNBAS+B), yeast nitrogen base without amino acids and with urea plus BALI^{TM} hydrolysate (YNBU+B), yeast extract and meat peptone plus glucose (YP+G), yeast extract and meat peptone plus BALI^{TM} hydrolysate (YP+B), chicken by-products hydrolysate plus glucose (CH+G), chicken by-products hydrolysate plus BALI^{TM} hydrolysate (CH+B), chicken by-products hydrolysate (CH) and BALI^{TM} hydrolysate (B). The nitrogen content (5.86 g/L; based on the nitrogen content of standard YP, containing 20 g/L yeast extract and 30 g/L meat peptone, as measured by Kjeldahl) and glucose content (50 g/L) were identical in all media, except in the control media containing only sugar (B) or only protein (CH). The microtiter plates

were filled with 2.5 mL sterile liquid medium. The plates were incubated at 30 °C and 450 rpm and the initial pH was 5.0. Samples were taken at 8h, 16h and 24 h, for the measurement of cell dry weight (CDW), free amino nitrogen and pH. The results showed that yeasts are able to grow equally well on the chicken by-product hydrolysate (CH) and commercial YP, and that they also performed equally well on glucose (G) and BALI^{TM} sugar (B) in most cases. In a perfectly balanced aerobic fermentation process, the maximum biomass yield relative to the amount of carbon source would be 50% (w/w) (biomass / initial substrate) (Akinyemi, Betiku & Solomon 2004), which in this case would be 25 g/L. Figure 1 of **Paper II** shows that the by far highest CDW values were reached by *B. adeninivorans* (33-39 g/L), and this could be explained because *B. adeninivorans* is known for being able to use a large variety of substrates as carbon and nitrogen source (Malak, Baronian & Kunze 2016; Middelhoven, Jong & Winter 1991),including purines, which are abundant in chicken by-products (Spalvins, Ivanovs & Blumberga 2018). *C. jadinii* reached lower CDW values (18 -28 g/L) but reached values above the theoretical maximum of 25 g/L when grown on a combination of chicken by-product hydrolysate and BALI^{TM} hydrolysate (CH+B), which could be a consequence of either the consumption of other sugars present in the BALI^{TM}, which contained 0.32 gram of other sugars for each gram of glucose, and/or the utilization of some peptides as a carbon source (Freese et al. 2011). *W. anomalus* reached CDW values between 16-20 g/L, while Thermosacc® Dry reached 18-20 g/L CDW on YP+G or CH+G, but its growth was lower when using BALI^{TM} as a sugar source (10-14 g/L). In conclusion, even though several potentially crucial fermentation parameters remained uncontrolled such as pH (see Figure S2 in **Paper II**), and aeration (which, if insufficient, may lead to production of ethanol instead of biomass) (Anderlei et al. 2007), these initial growth experiments demonstrated that the combination medium consisting of enzymatically hydrolysed sulphite-pulped spruce wood (BALI^{TM} hydrolysate), and enzymatically hydrolysed chicken by-products constitutes a promising growth medium for multiple yeasts.

Encouraged by the results obtained in the microtiter plates, the commercial rich standard medium (YP+G) and the rich medium derived from spruce and chicken by-products (CH+B) were used in upscaled experiments in 2.5 L fermenters where parameters such as pH and dissolved oxygen (DO) were fully controlled. Overall, the four yeast candidates grew better and produced larger amounts of protein when the medium composed of spruce and chicken by-products hydrolysates (CH+B) was used. As mentioned above, the

BALI^{TM} sugar comes with the potential benefit of containing an additional 16 g of sugar per 50 g of glucose, and the chicken hydrolysates may contain additional products which can be consumed by the yeasts, which could explain why CH+B performed so well. *B. adeninivorans* and *W. anomalus* were the best candidates in terms of biomass production using YP+G and CH+B, yielding 39.8 g/L (YP+G) or 54.5 g/L (CH+B) and 28.0 g/L (YP+G) or 36.1 g/L (CH+B), respectively. Similarly, the biomass and protein yields per glucose showed similar trends with *B. adeninivorans* giving $Y_{x/glucose}$ values of 0.76 and 1.15 g/g for YP+G and CH+B, respectively, and *W. anomalus* giving $Y_{x/glucose}$ values of 0.58 and 0.77 g/g for YP+G and CH+B, whereas yields for *C. jadinii* and Thermosacc$^{®}$ Dry were 0.46 and 0.65 g/g and 0.42 and 0.55 g/g for YP+G and CH+B, respectively. All four yeasts candidates were able to consume all the sugars, but compared to *C. jadinii* and Thermosacc$^{®}$ Dry, *B. adeninivorans* and *W. anomalus* produced lower amounts of ethanol. The protein content of the yeast cells was 50 % or higher in all but one case (Thermosacc$^{®}$ Dry), which is known as a strain used for ethanol production. Thus, the protein values obtained in the 2.5 L fermenters were in the range that is desired for yeasts used for SCP production, which is considered to be 45 – 55 % (Bajpai 2017; Øverland & Skrede 2016; Ritala et al. 2017). Protein yields ranged from 0.20 g to 0.28g per g of glucose (YP+G) or from 0.30 g to 0.39 g per g of total sugar (CH+B), with the exception of the *B. adeninivorans* fermentations which yielded approximately 0.38 g per g of glucose (YP+G) and 0.6 g per g of sugar (CH+B). These yields are similar to those described in the literature for *C. jadinii* (Lee & Kim 2001; Nigam 1998). The superior growth and protein yields observed for *B. adeninivorans* can be explained by the ability of this yeast to consume a wide range of compounds as explained above, but may also be due in part to an experimentally observed higher consumption of free amino acids compared to the other three yeast strains.

The chemical composition of freeze-dried yeast biomass obtained from the bioreactor cultivations after 24 h of fermentation showed lipid contents (0.4 – 1.8 %) and nucleic acid contents (2.5 – 5.8 %) lower than the values found in literature (2 - 6% and 5 - 12%, respectively (Bajpai 2017)). Lipid accumulation is usually provoked by nitrogen starvation (Chopra & Sen 2018). The remaining nitrogen content in the culture supernatants after 24 h fermentation was not calculated, but it was shown (Figure S3 in **Paper III**) that there was no depletion of free amino acids for all the yeast strains, and

that, thus, carbon was the only limited substrate. The low nucleic acid content can be explained by the decrease of RNA that occurs in yeasts when they enter stationary phase (Gottlieb & Etten 1964), which, depending on the strain, started between 4 and 10 h before sampling. The mineral composition results revealed that yeast produced on the chicken hydrolysate and BALI^{TM} sugars (CH+B) generally had a slightly higher mineral content. A similar trend was found for the amino acid contents (Table 4), where the total amounts of amino acids were slightly higher when using CH+B as a medium, except for *W. anomalus*. Analyses of amino acid compositions showed that the measured sums of amino acids varied between 393.6 and 475.2 g/kg dry matter for the four yeast strains. Since yeast may potentially be used as an ingredient in fish feed (Øverland et al. 2013), we compared the amino acid compositions of the four yeast strains with the amino acid compositions of fish- and soybean meals, with total amino acid contents of 526.4 and 497.8 g/kg dry matter, respectively. ANOVA analysis of the amino acid compositions showed differences at the individual amino acid level between the yeasts and fish- and soybean meals. PCA analysis showed that the fermentation medium did not significantly affect the amino acid composition for each specific yeast strain, and showed that the amino acid compositions of *C. jadinii* and *W. anomalus* were most similar to the composition of fish meal. Finally, FTIR spectra showed similar features for all yeasts, independently of the medium used (Figure 3 and S5 in **Paper II**).

In conclusion, **Paper II** demonstrates that CH+B is a promising fermentation medium for production of yeast-based single cell protein. The yeasts produced using CH+B contained high levels of protein, and the amino acid compositions of the yeast biomass were similar to the compositions of traditional fish and soybean meals, except for sulphur-containing amino acids. In conclusion, this study showed a feasible path towards the production yeast-based feed-protein derived from non-edible, local and cheap by-products. Further optimization of the processes described in **Paper II** is described in **Paper III**.

Paper III

The study described in **Paper II** yielded promising results regarding the production of SCP in the form of yeast (*C. jadinii, W. anomalus* and *B. adeninivorans)* on a medium consisting of enzymatically hydrolysed sulphite-pulped spruce wood (BALI^{TM} hydrolysate) and in-house enzymatically hydrolysed chicken by-products. In the follow up study described in **Paper III**, the in-house chicken hydrolysates were replaced by enzymatic hydrolysates from chicken and turkey cut-offs produced at BIOCO AS. The poultry hydrolysates contained 50.37 ± 0.03 % dry matter and 44.40 ± 0.10 protein %, but information regarding the enzymatic hydrolysis process, such as the type and dosage of exogenous enzymes, as well as process conditions (pH, temperature, time, and dry matter concentration during processing) could not be disclosed. Therefore, initially, 24 hours batch fermentations, as described in **Paper II**, using the poultry hydrolysate were conducted to compare its fermentation performance with the performance of the in-house prepared chicken hydrolysate.

In general, biomass yields and protein contents were lower when using the poultry hydrolysate. The CDW (g/L) and the protein content (%) for *C. jadinii* decreased by around 40 % and 20 % after 24 h, respectively. The decrease was less for *W. anomalus* and *B. adeninivorans* showing a 15 % decrease in CDW (g/L) and 8 – 14 % decrease in protein content (%). As before, *B. adeninivorans* showed superior growth performance, but showed the lowest protein content after 24 h (41.9 %). The differences in growth performance and protein content of the yeast biomass could be attributed to variation in the raw material and/or in the hydrolysis process. The in-house protein-rich hydrolysates used in **Paper II** were produced using a broader range of by-products from chicken (heart, liver and digestive tract), while the poultry hydrolysate used in **Paper III** was derived purer chicken fractions (cut offs) eligible for human consumption. The choice of exogenous enzyme and the hydrolysis conditions, including the potentially crucial temperature regime, were probably different, which will lead to variation in the hydrolysates, not only in terms of amino acid content and peptide length but also, perhaps, in terms of the presence of other growth-promoting factors such as certain lipids or vitamins. Regardless, the poultry hydrolysate was selected for the subsequent fermentation experiments because these preliminary batch experiments still gave reasonably good results, in particular for *W. anomalus* and *B. adeninivorans*.

Importantly, BIOCO AS could provide a large standardized batch of hydrolysate with a protein concentration almost seven times higher compared to the in-house produced chicken hydrolysates, facilitating the up-scaling process to larger fermenters and avoiding batch-to-batch variations in the production of the hydrolysate.

Subsequently, a screening of media containing different amounts of poultry hydrolysate and urea was performed in order to evaluate the importance of the hydrolysate for growth. These experiments were conducted in shake flasks and the amount of nitrogen provided by the poultry hydrolysate varied from 0 – 100 %, with the rest being supplied by urea. In cultivations with only urea as a nitrogen source, there was barely any growth, while after 24h, the growth based on cell dry weight was between 9.8 – 13.2 g/L for all yeasts and all fermentations containing at least 20 % poultry hydrolysate. The growth yields achieved were below the maximum theoretical growth yield (biomass / initial substrate) for yeasts under perfectly balanced aerobic conditions, which is considered to be 50% (in this case 25 g/L). The protein contents of the cells were nevertheless high, amounting to around 50 % for *C. jadinii* and *W. anomalus* and 43 % for *B.adeninivorans*. Analysis of the culture supernatants showed that the shake flask cultures were clearly limited regarding oxygen supply, causing high ethanol concentrations and medium acidification, which explains the low productivity of the shake flaks cultures. Comparison of the fermentations with varying amounts of poultry hydrolysate showed a declining trend in biomass production, when reducing the amount of poultry hydrolysate. This indicates that the yeasts need some of the components present in the poultry hydrolysate, although buffering effects of the hydrolysate may also play a role. It is well known that salts containing nitrogen can help meeting nitrogen requirements, while organic nitrogen sources, in addition, supply trace elements and vitamins required for growth (Atkinson & Mavituna 1991; Batistote, Helena Da Cruz & Ernandes 2006; Zeng & Jibin Sun 2010).

It is known that batch fermentations are not ideal for the purpose of SCP production, and the shake flasks experiments suffered from a lack of pH control, sub-optimal aeration (pO_2) and stirring. Therefore, in the next step, *C. jadinii*, *W. anomalus* and *B. adeninivorans* were cultivated in 2.5 L fermenters in repeated fed-batch mode. The main objectives were to identify the best ratios of poultry hydrolysate and urea to support high biomass productivity (Q_x), while producing yeast biomass containing around 50 % protein. In contrast to the shake flask experiments, in the repeated fed-batch

fermentations, substituting poultry hydrolysates with urea resulted in an increase of biomass production, but this effect sometimes came at the cost of protein content. For example, when using 100 % poultry hydrolysates and 40 % poultry hydrolysates with 60 % urea, *W. anomalus* increased the cell biomass production from 20.9 to 28.9 g/L, but the protein content decreased from 48.4 to 37.7 %. The enhancement in cell biomass production and higher growth rates could be due to the synergistic effect that can occur with some yeasts when non-peptide nitrogen sources (i.e. ammonium sulfate or urea) and peptides are combined (Patterson & Ingledew 2018). A similar trend was observed for *B. adeninivorans*, which, notably, did not perform very well in this repeated fed-batch set-up (see below). While the interplay between protein production and cell growth is still not clear (Kafri et al. 2016), the trade-off between cell biomass and protein content found here for *W. anomalus* was also observed by Sharma et al. 2018 in a study on production of *C. jadinii* by fed-batch fermentation. On the other hand, in the present study, *C. jadinii* reached similar values in terms of biomass production at 100 % and 40 % poultry hydrolysate (20.1 g/L and 21.1 g/L), respectively, while, unexpectedly, the protein content increased from 40.0 % with 100 % poultry hydrolysate to 44.9 % with 40 % poultry hydrolysate. Overall, the results from the repeated fed-batch experiments with 100 % poultry hydrolysate and 40 % poultry hydrolysate showed that *W. anomalus* had the highest productivity regarding both biomass (Q_x in the range of 2.61-3.61 g/L/h) and protein (Q_P in the range of 1.26-1.36 g/L/h) production. It is important to mention that the higher protein productivity when only 40 % poultry hydrolysates was used is due to the much higher biomass productivity, while the protein content of the cells was considerably reduced.

In the batch fermentations described in **Paper II**, B. *adeninivorans* outperformed *C. jadinii* and *W.anomalus*. However, in the first repeated fed-batch experiments, *B. adeninivorans* did not deplete neither glucose nor xylose during the 8 h cycles and the growth performance was not good. Therefore, in a next series of experiments, with 60 % poultry hydrolysate, the cycle time for *B. adeninivorans* was increased to 12 h, in an effort to increase sugars assimilation. Despite improved sugar utilization, and improved process parameters, *B. adeninivorans* still performed less well than *W. anomalus* and the latter was selected for further experiments described below. For *W. anomalus* the change from 40 % poultry hydrolysate to 60 % poultry hydrolysate resulted in an increased protein

content (from 37.7 % to 41.2 %) and higher productivity of both cell biomass (from 3.61 to 3.72 g/L/h) and protein (from 1.36 to 1.53 g/L/h).

Some yeasts need biotin as a cofactor for urea amidolyase (Roon & Levenberg 1972). To elucidate if the capacity of *W. anomalus* to assimilate urea could be manipulated to improve protein its content, we carried out an additional experiment with the medium composed of 60 % poultry hydrolysate and 40 % urea supplemented with 0.4 mg of biotin per gram of urea. It turned out that the addition of biotin had no effect of productivity and protein content, indicating that the concentration of biotin was adequate. Biomass productivity was still much higher than in the fermentations with 100 % poultry hydrolysate (3.75 g/L/h vs 2.61 g/L/h), whereas the protein content remained lower (40.6 % vs 48.4 %).

Since the results so far indicated that both high amounts of poultry hydrolysate and the presence of some urea had beneficial effect of fermentation parameter, a final repeated fed-batch fermentation was run using a medium composed of 80 % poultry hydrolysate and 20 % urea, without addition of biotin. When using these conditions, the protein content increased to 45.0 %, while biomass and protein productivity stayed as high 3.38 g/L/h and 1.52 g/L/h, respectively. Thus, these conditions were concluded to be close to optimal.

Based on the results described above, *W. anomalus* was selected for scale-up to 25 L since it exhibited the best growth performance and protein productivities compared to *C. jadinii* and *B. adeninivorans*. The nitrogen source in this upscaled repeated fed-batch fermentation consisted of 80 % poultry hydrolysate and 20 % urea. This larger scale fermentation gave results similar to the 1.5 L fermentation under the same conditions, but with somewhat higher Q_x (3.92 g/L/h) and Q_P (1.87 g/L/h), as well as a higher protein content (47.8 %) (see Figure 3 and Table 5 in **Paper III**). These minor improvements could be explained by differences in dissolved oxygen between the reactors. During the 8 h cycles in the 1.5 L reactors the DO dropped to 3.5 – 7 % (data not shown) and therefore around 3 g/L of ethanol was produced. On the other hand, the DO was always within the 30 % dissolved oxygen set-point for the cultivation in the 25 L reactor (data not shown). This value is above the 17 – 20 % range most yeasts and filamentous fungi need for production of biomass (Bailey & Ollis 1986). Indeed, ethanol production was very low

in the 25 L fermentation, the concentration not exceeding 0.5 g/L. Based on the low ethanol concentration and the high dissolved oxygen values, it seems that the oxygen transfer rate was higher for the 42 L fermenter. This rate is influenced by multiple parameters, including the spargers and the geometrical parameters of the reactor (Garcia-Ochoa & Gomez 2009). The spargers differed between the two fermenters as well as the dimensions of the vessels; the height over width ratio of the 42 L fermenter was larger than that of the 2.5 L fermenters.

Importantly, to the best of our knowledge, **Paper III** describes the first study of production of *W. anomalus* using repeated fed-batch fermentation as a bioprocess strategy for SCP production, and the   results obtained with *W. anomalus* seem favourable compared to the results found in literature for SCP production based on *C. jadinii* (anamorph name *Candida utilis*) (Bajpai & Bajpai 1988; Gao, Li & Liu 2012; Lee & Kim 2001). Of note, **Paper III** also shows that the amino acid composition for *W. anomalus* is similar to *C. jadinii* grown on other lignocellulosic substrates (Rajoka et al. 2004; Sharma et al. 2018; Yunus, Nadeem & Rashid 2015). It is thus very possible that the potential of *W. anomalus* as a feed ingredient is similar to that of *C. jadinii*.

In conclusion, **Paper III** shows it is possible to effectively produce protein-rich *W. anomalus* biomass using a medium composed of spruce derived sugar (BALI^{TM} hydrolysates), poultry hydrolysates and urea. Calculations presented in this paper show that, when using the conditions used in the 42 L fermenter, it would take 25 industrial (300 m^3) continuously operated fermenters to replace 10 % of the fish feed protein used in Norway by yeast.

Of note, assessment of the suitability of *W. anomalus* as a feed source requires further studies, addressing down-stream processing technology, feed formulation and functional testing in feed trials. Preliminary studies included in **Paper III** showed that *W. anomalus* is susceptible to disruptive methods such as homogenization and partial enzymatic hydrolysis, which presumably would affect yeast digestibility. The effects of these downstream processing steps and the performance of *W. anomalus* as a feed ingredient will be investigated in follow-up studies.

4   Conclusions and future perspectives

The research described in this thesis provides a proof-of-concept for efficient production of microbial proteins, in the form of yeast biomass, from protein hydrolysates produced from by-products generated during meat and fish production and from sugars produced through enzymatic hydrolysis of lignocellulosic biomass. During the development of any fermentation-based product, usually the main focus is on the development and optimization of the fermentation medium and on fermentation processes conditions, in order to minimize the costs of the final product. Therefore, the research presented in this thesis did not only focus on producing results for publication in respected scientific journals, but also on the development of industrially relevant processes.

The first goal of this study, addressed in Paper I, was to generate rich nitrogen sources from by-products of the meat and fish industries by harnessing the power of endogenous enzymes, while minimizing the amount of added commercial enzymes and minimizing processing times. The preliminary growth studies carried out in paper I showed that the generated protein-rich hydrolysates are a promising alternative for commercial nitrogen sources that are commonly used for fermenting yeasts, and the usefulness of poultry hydrolysates is supported by the results presented on Paper II and III. Several issues need to be addressed in more detail when scaling up the hydrolytic process. While the endogenous enzymes showed to be very useful, alone or in combination with exogenous enzymes, likely because we used a temperature gradient during the hydrolysis process, their presence creates a certain lack of control, especially when the raw materials are not transported and stored under the correct conditions. More generally, the processes described in Paper I require freshness of the by-products, which potentially creates logistic issues.

Further process optimization is also possible, including, possibly, further shortening of processing times, and optimization of pH and temperature profile, in order to reduce production costs without reducing the quality of the final product. For instance, the final temperature of 60 º C used in our study can be beneficial to some exogenous enzymes and decrease risks related to microbial contamination, but can increase costs in terms of power usage and may lead to suboptimal use of endogenous enzymes. Another issue that requires further attention concerns the heat treatments in between the end of the

hydrolysis and fermentation. In the study of Paper I, enzymes were inactivated by incubation at 100 °C, and the media containing the hydrolysates were autoclaved. It is possible to simply this procedure, e.g. by immediately preparing and sterilizing the media, which will also inactivate the enzymes. The application of several heat treatments likely reduces the quality of the hydrolysates and increases production costs. Ultimately, it would be interesting to explore the possibility of applying processes that do not require sterilization of fermentation media (Li et al. 2014).

Another important issue concerns the downstream processing of the protein hydrolysates which are is made up of three different phases: an oil layer, a water phase (protein hydrolysate) and a sludge phase, which is composed of non-hydrolysed components and sediments. The complete separation of soluble lipids from the water phase may be difficult by centrifugation and some filtration methods may need to be applied for the removal of small particles. The state of the final product either in liquid or dried form also needs to be addressed. Preservation of the hydrolysates in liquid form might require addition of a preservative, which is definitely not feasible when the hydrolysate is to be used for the production of growth media. Drying of the protein hydrolysates facilitate storage and transportation and increase shelf-life (Crosby 2016). However, drying methods increase production costs and the drying process may affect product quality and solubility (Surowka & Fik 1992).

The second goal of the present study was to evaluate the meat and fish hydrolysates, as a source of nitrogen, and spruce-derived sugar solutions, as a source of carbon, for production of yeasts. Papers II and III show that the production of yeast biomass yeast protein production using the BALI^{TM} spruce hydrolysate in combination with in-house produced hydrolysates of chicken by-products or commercially produced poultry hydrolysates were similar or better than when using traditional glucose in combination with commercial yeast extract and peptones. The compositions of the produced yeasts were favourable for SCP, with protein contents amounting to 50 % and amino acid profiles similar to the profile of fishmeal. *B.adeninivorans* gave the highest biomass yields in the batch fermentations described in Paper II, likely due to its exceptional ability to utilize a wide range of compounds for growth, without producing ethanol, On the other hand *W.anomalus* was the clearly better performing yeast in the repeated fed-batch

experiments described in Paper III, where the in-house produced chicken hydrolysate was replaced by a mixture of poultry hydrolysate and inorganic nitrogen (urea). The results presented in Papers II and III show that hydrolysates of lignocellulosic and protein-rich biomass are promising medium ingredients for SCP production.

Although Papers II and III show a route towards improved yeast production, additional research efforts are needed to further optimize the yeast production process. For example, we have not yet optimized nitrogen consumption. While glucose consumption was monitored throughout the fermentations described in Paper III, nitrogen consumption was not and it is very possible that we used more of the nitrogen source than strictly needed. Other organic and inorganic nitrogen sources, and combinations thereof, also need further attention. Of note, of the three types of protein hydrolysates produced in Paper I, only one type has been tested in the fermentations described in papers II and III. It is important to note that food-grade protein-rich co-products may have higher value when used in food directly, rather than being converted to food via feed via yeast.

As to the fermentation strategy, it seems that continuous fermentation has been the preferred strategy to produce SCP industrially (Rose 1979; Ward 1992; Wiebe 2004). While the repeated fed-batch process described in Paper III seems promising, comparisons with alternative strategies, such as continuous fermentation, remain to be done. The process economy of continuous processes, and of the fermentation process in general, may be optimized by looking into recycling of supernatants and water obtained after cell harvesting and cell washing, respectively, which will reduce the need for effluent treatments and reduce water consumption (Vasey & Powell 1984). If a continuous process is selected, fine adjustments of the aeration levels to obtain pure respiratory growth under aerobic conditions at optimal dilution rate will reduce issues related to aeration, as well as reduce foaming and evaporation issues (Hagman & Pi 2015).

Although Paper III concludes that production of SCP for replacing 10 % of the fish feed currently used in Norway seems technically feasible, we have not addressed economic aspects. In order to make SCP production economically feasible, efforts have to be made to find microorganisms that, in addition to containing high levels of protein, have health-promoting effects, thus creating additional value (Bajpai 2017). Besides, it would be

interesting to assess in more detail new biorefinery models where several processes are integrated, and multiple value-added products from agri-food residues are produced simultaneously (see Figure 4 of the introduction). These strategies could improve the economic feasibility of biorefineries by increasing value creation as such, but also by generating a flexibility that allows the biorefinery to vary (prioritize) the production of each value added product depending on the supply-demand situation and fluctuations of commodity prices (Anderson 2010; Dube et al. 2014). For instance, in the late 1980's and 1990's the world market price of cheap protein from plants was 50% lower than the production costs of SCP (Ugalde & Castrillo 2002).

It has been claimed that further research in the SCP field should include the identification and production of nonconventional microorganisms, which may have hitherto non-recognized SCP potential (Rai, Pandey & Sahoo 2019). The use of genetically modified organisms (GMO) tailored to have optimal SCP properties is also an option but the use of such organisms in food and feed has not found public acceptance yet, especially in Europe. However, new techniques such as those based on the use of Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR) that allow specific editing of the genome without introduction of foreign DNA can open new opportunities (Raschmanová et al. 2018). Genetic engineering could be used to enhance the capacity of microorganisms to metabolize a broad spectrum of substrates and/or to make the utilization of these substrates more efficient (Campbell, Xia & Nielsen 2017). For example, in a review by Hou *et al.*, 2017, several strategies and challenges regarding progress in engineering xylose fermenting strains are discussed. Genetic engineering could also be used to increase the nutraceutical value of SCP by tailoring amino acid profiles or other physiological aspects of the microbes (Ritala et al. 2017).

It is clear that a thorough economic assessment of the protocols described in this study should be part of follow-up studies and that such assessments should include the potential (economic) impact of using improved strains, the benefits of the yeasts beyond being a protein source, and the possibility to generate side products during downstream processing, such as bio-active cell wall fractions. Finally, creation of public awareness and consumer acceptance regarding the use of microorganisms grown on by-products as

a replacement of traditional feed and food ingredients, will be a key factor in determining the success of commercial implementation of SCP production.

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Paper I

Comparative Assessment of Enzymatic Hydrolysis for Valorization of Different Protein-Rich Industrial Byproducts

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Ⓢ *Supporting Information*

ABSTRACT: Hydrolyzed protein-rich byproducts from food production may find a variety of applications, for example, as rich ingredients of fermentation media. We have conducted a study of the enzymatic hydrolysis of three byproducts from Norwegian food industries: chicken byproducts, mixed pork and beef byproducts, and salmon viscera. The efficiency and optimization of the enzymatic hydrolysis were evaluated using endogenous enzymes alone and in combination with commercial proteases. Hydrolysis reactions were conducted with freshly thawed raw materials using short incubation times and including an initial temperature gradient from 4 to 60 °C to both harness the power of endogenous enzymes and minimize microbial contamination. Subsequently, hydrolysates were characterized by analyzing the total recovery of protein, the peptide molecular-weight distribution, and the composition of total and free amino acids. The action of endogenous enzymes played an important role in raw-material hydrolysis, particularly when hydrolyzing salmon viscera but less so when hydrolyzing chicken byproducts. For pork−beef and chicken byproducts, the addition of Alcalase or Papain improved protein recovery, reaching levels up to 90%. Next to showing efficient hydrolysis protocols, the present data also provide a comparison of the amino acid compositions of hydrolysates derived from these three different protein-rich byproducts. Growth studies showed that the obtained protein-rich hydrolysates from meat and fish industries are a promising alternative for expensive nitrogen sources that are commonly used for fermenting yeasts.

KEYWORDS: proteolysis, hydrolysates, meat, fish, peptides, yeast

INTRODUCTION

The agrifood and aquaculture industries generate immense amounts of byproducts, which need to be properly treated because of environmental concerns and may be a source of value-added products. Currently, byproducts derived from slaughter and processing of cattle, pigs, and chickens represent around 49, 44, and 37% of the total live weight of these animals, respectively.[1] Traditionally, many of these byproducts have been used as either animal feed or fertilizer and in the production of biogas,[2] with a low economic value to the industry. The fish-processing industry also produces enormous amounts of byproducts, and approximately 57% (w/w) of the total wild catch is not directly utilized for human consumption.[1] In Norway, where aquaculture is a large and fast-growing industry, the production of salmon and trout reached 1.25 million tons in 2016.[3] In 2016, Norwegian salmon production generated 401 000 tons of byproducts, of which viscera accounted for about 145 000 tons, thus representing the major constituent of the byproducts.[3] Similarly to byproducts from the meat industry, the byproducts from aquaculture are also used in products such as animal feed and fertilizers.[4]

Animal and fish hydrolysates have a wide range of promising applications, such as being a source of natural antioxidants[5−8] and higher-value feed[9,10] or flavoring ingredients,[11] as well as in nutraceutical applications for pets[12,13] and humans.[14] Enzymatic hydrolysis will alter the chemical, functional, and sensory properties of the protein-rich byproducts.[15] Several factors influence the properties and components of the final protein hydrolysate, such as the composition and seasonal variation of the raw materials, the amount of endogenous enzymes present in the substrate, and the processing conditions, including the dosage of exogenous enzymes. The use of endogenous enzymes (autolysis) to more or less "spontaneously" hydrolyze the proteins is a low-cost approach, but the applicability of this strategy depends on the type and source of raw material. For instance, fish viscera normally have higher amounts of endogenous protease activity than other byproduct fractions, such as cutoffs and liver fractions.[16,17] Endogenous enzymes may be vulnerable to inactivation, which could take place, for example, during preprocessing steps. These factors can limit reproducibility and controllability of the hydrolysis process. The use of exogenous enzymes is considered a good option in order to make the hydrolytic process more controllable and reproducible. However, commercial proteases add significant costs to the hydrolysis process.

One possible application of fish hydrolysates is to use them as a nitrogen source for microbial biomass production.[18−24] However, studies evaluating hydrolysates from meat and salmon industries as potential nitrogen sources for microbial growth are still limited, in particular when it comes to comparing various byproducts. Comparative studies based on literature data present several challenges. First, commercial enzymes have different specific activities, optimal working conditions, and

sensitivities for inhibitory compounds. Second, there is variability in the methods used to assess hydrolysis performance, which include determination of protein recovery based on total nitrogen solubilization, direct determination of the degree of hydrolysis, or determination of trichloroacetic acid (TCA)-soluble peptides. Third, the contribution of endogenous enzymes is dealt with in different manners. Finally, the proteolytic process of choice and its efficiency is usually application-dependent.

The aim of this study was the enzymatic upgrading of different protein-rich industrial byproducts from pork and beef, chicken, and salmon by using endogenous enzymes alone (autolysis) or in combination with different exogenous commercial proteases. We used short processing times with heat gradients to maximize the contribution of endogenous enzymes and minimize microbial contamination. The efficiency of the enzymatic hydrolysis was evaluated on the basis of the minimum enzyme loading required to generate high nitrogen solubilization in an (industrially relevant) short period of time at the natural pH of the substrate. We present a comparative analysis of the resulting hydrolysates, with focus on the molecular-weight distributions and amino acid compositions, and we demonstrate the performance of these hydrolysates in a fermentation medium.

■ MATERIAL AND METHODS

Raw Materials. Three different raw materials were obtained from Norwegian meat- and fish-processing facilities: a mixture of pork and beef residues, which comprised 70−75% pig byproducts (intestines, spleen, fat, liver, and tails) and 25−30% cattle intestines; chicken byproducts (heart, liver, and digestive tract); and salmon viscera. Nortura SA (Oslo, Norway) delivered the meat byproducts, whereas Nutrimar AS (Kverva, Norway) provided the salmon viscera. The different meat byproducts were sent in iceboxes the same day they were collected from the slaughterhouses and immediately frozen in aliquots. The salmon viscera were sent frozen in aliquots. All samples were kept at −20 °C until further use. Prior to the enzymatic-hydrolysis reactions, samples were thawed on ice at 4 °C and minced with a manual knife mill (Retsch GM300) for 40 s at 4000 rpm. Then, the different batches were immediately frozen at −20 °C in aliquots until further use.

Enzymes and Other Chemicals. Alcalase 2.4L (Novozymes A/S, Bagsvaerd, Denmark) is a commercial alkaline bacterial endoprotease of the serine type that can hydrolyze most peptide bonds within a protein molecule and has previously been found to be exceptionally efficient in hydrolyzing fish viscera.[18] Alcalase 2.4L has a declared activity of 2.4 AU/mg and a temperature optimum of 60 °C. It is active in a wide, slightly alkaline pH range and has highest activity at pH 6.5−8.5. Papain from *Carica papaya* powder, ≥3 U/mg (Merck, Darmstadt, Germany), is a commercial cysteine protease with a wide specificity, primarily cleaving peptide bonds next to basic amino acids, leucine, or glycine, and is used as a meat tenderizer. The optimal working conditions for Papain are reported to be at pH 6.0−7.0 and 65 °C. Both enzymes fulfill recommended purity specifications for food-grade enzymes of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC).[25,26] All other chemicals and reagents used in the experiments were of high purity and analytical grade. For comparison with the ingredients of YP medium, used for growing yeast, peptone from meat (Merck, Darmstadt, Germany) and yeast extract (Becton Dickinson, Miami, FL), both in powder form, were mixed in a 2:1 (w/w) ratio.

Hydrolysis Reactions. The minced and frozen substrates of pork−beef byproducts, chicken byproducts, and salmon viscera were thawed to 4 °C, and 50 g samples (wet weight) were mixed with 50 mL of Milli-Q-purified water, resulting in dry-matter concentrations in the range of 12−15%, as detailed below. Reactions were carried out in 250 mL polycarbonate centrifuge bottles with sealing caps from Nalgene, which were kept on ice until hydrolysis was

started. Hydrolysis experiments were carried out in triplicate, to test three different enzymatic conditions: autolysis (no enzyme addition), addition of Alcalase, and addition of Papain. The hydrolysis was carried out in a shaking incubator (New Brunswick Scientific, Edison, NJ) at 60 °C and 225 rpm without pH adjustment. Experiments with pH control were set up using 100 mM Na_2HPO_4/NaH_2PO_4, pH 8.0, instead of water. Because of the low buffer capacity ($pK_a = 6.86$) of the phosphate buffer at pH 8.0, the initial pH dropped to 7.5. Importantly, the tubes were placed directly from the ice into the incubator, and it was determined that it took approximately 30 min for the reaction mixtures to reach 60 °C. Thus, a temperature gradient from 0 to 60 °C was applied, allowing the process to benefit from endogenous-enzyme activity as much as possible.[27]

Final pH values were measured with a Sentron SI400 pH meter (Sentron Europe BV, Leek, The Netherlands), and hydrolysis was terminated by boiling the samples in a water bath at 100 °C for 30 min. The hydrolysates were cooled down on ice and centrifuged in a Beckman Coulter Avanti J-25 refrigerated centrifuge (Beckman, Brea, CA) at 4 °C and 20 000g for 15 min. After centrifugation, the pellet (consisting of sludge and lipids) was removed, and the supernatant (the hydrolysate) was collected. The hydrolysate had different layers: an oil layer on top, a protein-hydrolysate solution in the middle, and sediment on the bottom with particles that were not attached to the pellet during centrifugation. The oil layer was especially noticeable with salmon viscera. The liquid phases of the hydrolysis samples were separated by pipetting. Finally, the hydrolysates were filtered through two sieves coupled in series of 0.850 mm and 75 µm Ø, followed by filtering with a T-1500 depth (Pall Corporation, New York, NY) in order to remove residual particles. Finally, the hydrolysates were stored at −20 °C until analysis. All hydrolysis experiments were performed in triplicate.

Composition Analysis of Raw Materials and Hydrolysates. Dry matter content was calculated by weighing the samples before and after drying for 24 h at 105 °C, and ash content was determined according to a protocol from the National Renewable Energy Laboratory.[28] The total nitrogen content was determined using the Kjeldahl method according to ISO 5983-2.[29] The crude protein content was estimated by multiplying total nitrogen by a factor of 6.25.[30] The method of Bligh and Dyer[31] was used for the extraction of lipids, and the total lipid content was determined gravimetrically.

Total Protein Recovery. The protein contents of the starting reaction mixture (grams per 100 g of dry matter) and the hydrolysates (grams per 100 g of dry matter) were determined by the Kjeldahl method, and these values, combined with values for the dry-matter contents, were used to determine the protein recovery in percent. We determined true protein recoveries by comparing the total amount of protein in the obtained hydrolysate with the amount of protein entering the hydrolysis reaction. The total amount of protein in the hydrolysates equaled the total amount of recovered hydrolysate, in grams, multiplied by the protein concentration in the hydrolysate, expressed in grams per gram of wet hydrolysate.

Molecular-Weight Distribution. Molecular-weight distributions of peptides in the supernatants were determined by gel-filtration chromatography on a Superdex Peptide HR 10/300 column (10 mm i.d., 300 mm length; GE Healthcare, Little Chalfont, United Kingdom), which separated peptides in a molecular-weight range from 7000 to 100 Da. The column was coupled to an ÄKTA Pure Chromatography System (GE Healthcare, Little Chalfont, United Kingdom). The mobile phase consisted of 70% (v/v) Milli-Q-purified water and 30% (v/v) acetonitrile (Merck, Darmstadt, Germany) with 0.1% trifluoroacetic acid (TFA; Merck, Darmstadt, Germany). The flow rate was 0.5 mL/min, and compounds were detected by recording absorbance at 214 nm. Sterile, filtered (0.2 µm, Millex GP, Millipore, Molsheim, France) hydrolysate sample (50 µL) was diluted 10 times with the mobile phase and injected onto the column. The column was calibrated using cytochrome C (12 400 Da), aprotinin (6500 Da), vitamin B12 (1355 Da), and uridine (244 Da; all from Merck, Darmstadt, Germany).

Determination of Total Amino Acid Content and Tryptophan. Analysis of total amino acids and tryptophan was performed in

triplicate on the basis of the Official EU Methods of Analysis, 71/393/EEC.[32] The sample size was 2 mL of sterile filtered hydrolysate (0.45 μm, Millex GP, Millipore, Molsheim, France). The analytical setup consisted of a Biochrom 30+ Amino Acid Analyzer (BioChrom, Cambridge, United Kingdom) equipped with an ion-exchange column and postcolumn derivatization with ninhydrin. All amino acids were detected at 570 nm, except proline, which was detected at 440 nm. This method is suitable for cysteine, methionine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, and arginine.

For determination of total tryptophan, the samples were hydrolyzed with a saturated barium hydroxide solution by heating at 100 °C for 20 h. Tryptophan was determined by liquid chromatography with fluorescent detection using an Ultimate 3000 HPLC with an autoinjector (Thermo Fisher Scientific, Waltham, MA) and a Shimadzu fluorescence detector (Shimadzu Corporation, Kyoto, Japan).

Determination of Free Amino Acid Content. Analysis of free amino acids was performed in triplicate as described by Moe et al.,[33] with some modifications related to preparation of the samples. Free amino acids were measured by adding 5 mL of an internal-standard solution (0.1 M HCl, 0.4 μmol/mL L-Norvalin; Merck, Darmstadt, Germany) to 5 g of hydrolysate. The samples were homogenized using an Ultra-Turrax homogenizer for 5 min at 20 000 rpm and then sonicated for 30 min. The samples were then centrifuged (Thermo Scientific, Heraeus Multifuge X3 Centrifuge Series, Karlsruhe, Germany) at 2500g for 40 min at 4 °C. The supernatant (1 mL) was mixed with 1 mL of 4% TCA (Merck, Darmstadt, Germany) and then incubated on ice for 30 min. After centrifugation at 15 600g for 5 min at 4 °C, the samples were filtered (0.2 μm cellulose acetate filter; Advantec, Dublin, CA) and stored in a freezer (−20 °C) until analysis.

Before separation, 350 μL of borate buffer (0.4 M, pH 10.2, Agilent Technologies, Singapore) was added to 50 μL of sample. Separation of amino acids was performed using an Agilent series 1200 pump (Agilent Technologies, Singapore), an Agilent 1200 series autosampler, an Agilent 1200 series column oven, an Agilent 1200 series thermostat, and an Agilent 1200 series fluorescence detector. The system was equipped with an XTerra RP 18 column (150 × 4.6 mm; Waters, Milford, MA) operated at 42 °C and controlled by OpenLAB CDS (Agilent Technologies) software.

Microbial Growth on Hydrolysates. *Candida utilis* (LYCC 7549, Lallemand Yeast Culture Collection) was provided by Lallemand Inc. (Salutaguse, Estonia) and stored in a medium containing 15% (v/v) glycerol at −80 °C. A commercial medium composed of yeast extract and meat peptone (Sigma-Aldrich, St. Louis, MO) was prepared with an initial concentration of 10 g/L yeast extract and 20 g/L meat peptone (YP medium). Media based on the hydrolysates were prepared by mixing an amount of hydrolysate that would yield the same nitrogen content (measured by Kjeldahl) as that in YP medium. All media were sterilized by autoclaving at 121 °C for 20 min and then supplemented with 20 g/L glucose. Overnight precultures were prepared by adding 200 μL of seed culture, prepared on YP, to 25 mL of the medium to be tested in a 250 mL baffled shake flask; the cultures were incubated at 30 °C, 220 rpm, for approximately 16 h, prior to growth experiments in the microtiter plates.

C. utilis was grown in the Duetz-microtiter plate system (Enzyscreen, Heemstede, The Netherlands), consisting of 24 square polypropylene deep-well plates, sandwich covers, and cover clamps, which were mounted in a shaker (Infors HT Shaker Minitron, Bottmingen, Switzerland). Autoclaved and dried microtiter plates were filled in with 2.5 mL of sterile liquid medium containing either YP or the different meat- and fish-hydrolysate-based media, using 20 g/L glucose as a carbon source. The initial pH was adjusted to 5.0 using 1 M NaOH or HCl. Media were inoculated with the precultures to obtain initial ODs of 0.5 measured at 595 nm with a UV−vis spectrophotometer (Hitachi U1900, Tokyo, Japan). The plates were incubated at 30 °C and 300 rpm for 24 h, after which the dry cell mass was determined by centrifuging 2 mL culture samples at 10 000g,

4 °C, for 5 min (Centrifuge 5415R, Eppendorf, Westbury, NY). The cell pellets were resuspended in distilled water and washed two times prior to drying. Dry matter (g/L) was determined after drying at 105 °C for 24 h. These experiments were performed in triplicate.

Statistical Analysis. All hydrolysis reactions were carried out in triplicate, and all resulting hydrolysates were analyzed. The presented results are the means of the replicates, and the standard deviations are shown as error bars in the figures. Data handling and statistics were performed using the Excel software package (Microsoft Excel 2013, Microsoft Corporation, Redmond, WA). Single-factor ANOVA was used to analyze the influences of different enzymatic treatments on amino acid composition using the Analysis ToolPak in Excel (α = 0.05). Principal-component analysis (PCA) was performed using the Unscrambler X, V10.5 (CAMO, Oslo, Norway).

■ RESULTS AND DISCUSSION

Basic Compositional Analysis. The goal of this study was to compare and optimize the hydrolysis of three different industrial byproducts: pork−beef and chicken byproducts and salmon viscera. Table 1 shows the approximate compositions of these materials.

Table 1. Compositions of the Different Byproducts[a]

	pork−beef byproducts	chicken byproducts	salmon viscera
dry material	24.32 ± 0.83	30.12 ± 0.50	27.90 ± 0.50
protein	6.84 ± 0.26	15.10 ± 1.20	10.23 ± 0.03
ash	2.79 ± 0.54	4.47 ± 0.29	3.24 ± 0.36
lipids	12.48 ± 0.93	6.91 ± 0.55	14.20 ± 1.29

[a]The values represent percents of wet weights and are mean values ± SD (n = 3).

Optimization of Hydrolysis. Hydrolysis reactions were performed over short time periods using a 0−60 °C temperature gradient of approximately 30 min followed by incubation at 60 °C for the rest of a reaction period of a maximum of 2 h in total. This procedure was designed to exploit endogenous enzymes, while at the same time minimizing problems associated with microbial contamination. The temperature of 60 °C is within the optimal temperature range of the commercial enzymes used in this study. Hydrolysis reactions were performed both with and without pH control. Reactions
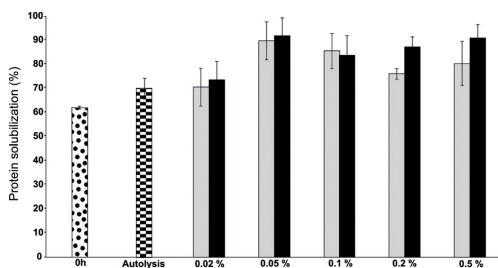

Figure 1. Protein solubilization upon hydrolysis of pork−beef byproducts for 2 h. The dotted bar represents the 0 h incubation with no enzymes added, the checkered bar represents the 2 h incubation with no enzymes added (i.e., autolysis), and the gray bars (Alcalase) and black bars (Papain) represent 2 h incubations with enzymes added at the indicated dosages. Protein recoveries are presented as percentages of the theoretical maximum values. Table S1 shows the underlying data.
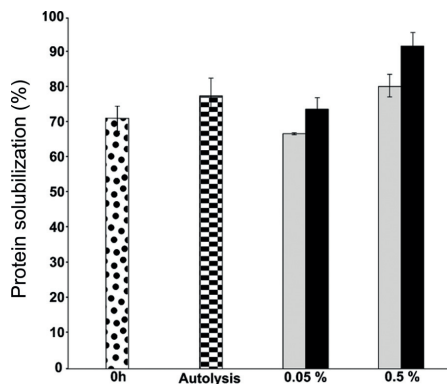
Figure 2. Protein solubilization upon hydrolysis of pork−beef byproducts for 1 h. The dotted bar represents the 0 h incubation with no enzymes added, the checkered bar represents the 1 h incubation with no enzymes added (i.e., autolysis), and the gray bars (Alcalase) and black bars (Papain) represent 1 h incubations with enzymes added at the indicated dosages. Protein recoveries are presented as percentages of the theoretical maximum values. Table S1 shows the underlying data.

Figure 4. Protein solubilization upon hydrolysis of chicken byproducts for 1 or 2 h. The dotted bar represents the 0 h incubation with no enzymes added, the striped bar represents the 1 h incubation with no enzymes added (i.e., autolysis), the checkered bar represents the 2 h incubation with no enzymes added (i.e., autolysis), and the gray bars (Alcalase) and black bars (Papain) represent 1 and 2 h incubations with 0.5% enzymes. Recoveries are presented as percentages of the theoretical maximum values. Table S2 shows the underlying data.

without the addition of buffers and pH adjustment are of interest because they are potentially the cheapest and thus more industrially relevant.

Initially, several enzyme loadings (weight of the enzyme divided by the weight of the wet biomass, expressed in %) and incubation times were tested, which led to the conclusion that maximum incubation times of 2 h and maximum enzyme dosages of 0.5% (w/w) were to be used. Autolytic hydrolysis was assessed by running reactions without the addition of enzymes.

Pork−Beef Byproducts. Figure 1 shows protein recoveries obtained after treating pork−beef byproducts in various manners. The initial pH of the substrate was around 6.6 at 4 °C, and the final pH was within the range of 5.9−6.1 after 2 h of hydrolysis. Protein solubilization was about 60% without any
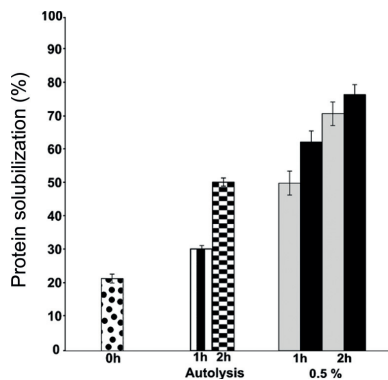
incubation (0 h) and about 70% when autolysis was allowed to happen (see Table S1 for exact numbers). The highest protein recoveries were obtained by adding 0.05% protease, which led to solubilization yields slightly above 90% for both enzymes. It is interesting to note that there is no clear dose−response curve and that for Alcalase, the yield at a 0.5% dosage is actually lower than that at a 0.05% dosage. This may indicate that there is a trade-off between increased activity of the exogenous enzyme and increased degradation of endogenous proteases, which are likely less stable and thus prone to proteolysis. Solubilized protein constituted around 90% of the dry matter in most of the hydrolysates (Table S1).

Use of a shorter incubation time (1 h, Figure 2) showed that the reactions based on autolysis and those carried out at the highest enzyme loadings (0.5%) were essentially finished after
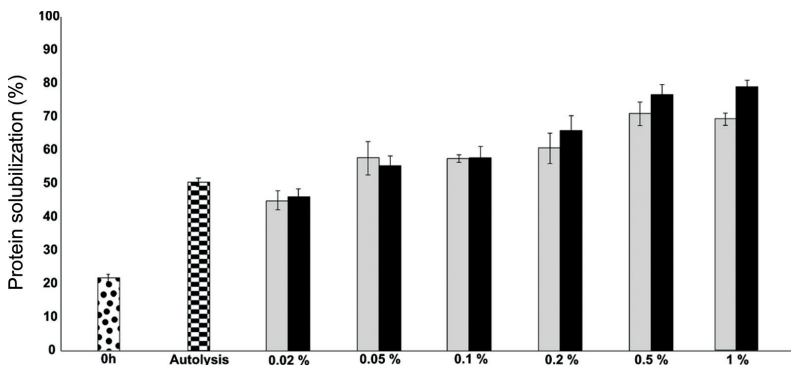

Figure 3. Protein solubilization upon hydrolysis of chicken byproducts for 2 h. The dotted bar represents the 0 h incubation with no enzymes added, the checkered bar represents the 2 h incubation with no enzymes added (i.e., autolysis), and the gray bars (Alcalase) and black bars (Papain) represent 2 h incubations with enzymes added at the indicated dosages. Protein recoveries are presented as percentages of the theoretical maximum values. Table S2 shows the underlying data.
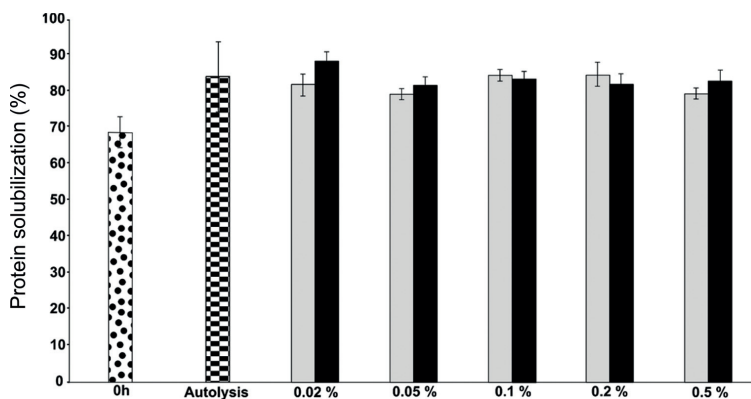
Figure 5. Protein solubilization upon hydrolysis of salmon viscera for 2 h. The dotted bar represents the 0 h incubation with no enzymes added, the checkered bar represents the 2 h incubation with no enzymes added (i.e., autolysis), and the gray bars (Alcalase) and black bars (Papain) represent 2 h incubations with enzymes added at the indicated dosages. Protein recoveries are presented as percentages of the theoretical maximum values. Table S3 shows the underlying data.

1 h (for these reactions, the values shown in Figures 1 and 2 are the same). On the other hand, when using the lower dosage of 0.05%, the yields after 1 h were lower than those after 2 h (approximately 70 and 90% solubilization, respectively). The endogenous enzymes are likely no longer active after 1 h because of the temperature reaching 60 °C after approximately 30 min. All in all, slightly better results were achieved by using Papain, but it is important to take into account that Alcalase was used at a suboptimal pH value (to avoid additional processing steps connected to pH control and adjustment).

Chicken Byproducts. Similar experiments with chicken byproducts showed that compared with beef−pork byproducts, proteolytic activity was more important for protein solubilization. The protein recovery for the nonhydrolyzed chicken byproducts was around 20% (compared with around 60% for pork−beef). Autolysis led to solubilization of around 50% of the protein, indicating the presence of relevant endogenous-enzyme activity. Solubilization yields increased upon adding Papain or Alcalase at a minimum dosage of 0.05%, and there were dose−response effects up to enzyme dosages of 0.5%, resulting in solubilization yields of 77 and 69%, respectively. Higher enzyme dosages did not further improve the yields (Figure 3). Notably, low dosages (0.02%) of externally added enzyme led to reduced yields compared with that from autolysis, which was likely due to the negative effect of the externally added enzymes on the endogenous enzymes, as discussed above. The protein content in the hydrolysates represented 85−90% of the dry matter in all samples (Table S2).

Addition of external enzymes did not yield similarly high recoveries for chicken byproducts compared with for pork−beef byproducts in reactions that were run at the natural pH of the substrates (i.e., close to 6). In an attempt to further increase the yields for chicken byproducts, Alcalase reactions, at dosages of both 0.05 and 0.5%, were repeated using 100 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 8.0, instead of water, thus creating more favorable conditions for Alcalase. Because of the low buffer capacity ($pK_a = 6.86$) of the phosphate buffer at pH 8.0, the initial pH dropped to 7.5, and the final pH values were 6.37 and 6.15 for 0.05 and 0.5% Alcalase loading, respectively.

The solubilization yields obtained using these conditions were essentially identical to those found in the absence of buffer (data not shown).

Figure 4 shows that reduction of the reaction time from 2 to 1 h led to clearly reduced yields. This reduction was also observed for the reactions without added enzyme, showing that solubilization due to endogenous-enzyme activity continued, at least to some extent, after the temperature of the reaction had reached 60 °C. Fonkwe et al.[34] hydrolyzed mechanically deboned turkey residues and reported 90% protein solubilization after a 2 h reaction using 0.4% (w/w, wet weight) Papain at 60 °C and natural pH. Thus, using conditions similar to the ones used in the present study and a similar, but certainly not identical, substrate, Fonkwe et al. reached higher solubilization yields than those we report here. Nikolaev et al.[35] obtained maximum protein recovery of 75% from poultry leftovers (chicken necks) after incubation for 2.5 h with a multienzyme cocktail (Alcalase, Neutrase, Flavourzyme, and Protamex) at dosages of 0.20−0.25% (weight of the enzyme solution divided by the weight of the wet biomass).

Salmon Viscera. Solubilization yields obtained with salmon viscera varied from 80 to 85% and were not improved by the addition of Alcalase or Papain (Figure 5). The protein recovery for the nonhydrolyzed substrate was 70%. There were no significant changes in pH during the hydrolysis reactions; the initial pH of the substrate was 6.5 at 4 °C, and the final pH after 2 h of incubation was 6.3−6.4. In all cases, the protein content in the hydrolysates composed 85−90% of the dry matter (Table S3). Thus, for the salmon viscera used in this study, the presence and dominating influence of endogenous enzymes is obvious. Measurements of hydrolysis yields after 1 h showed that maximum yields were obtained after 1 h (results not shown), which was expected as the endogenous enzymes are likely cold-adapted and become inactivated as the reaction temperature reaches 60 °C. The finding that short hydrolysis times are sufficient is in line with earlier work on defatted salmon backbones.[36]

The dominating role of endogenous enzymes is supported by literature data. Slizyte et al.[36] used defatted salmon backbones as a substrate and showed that 74% protein solubilization
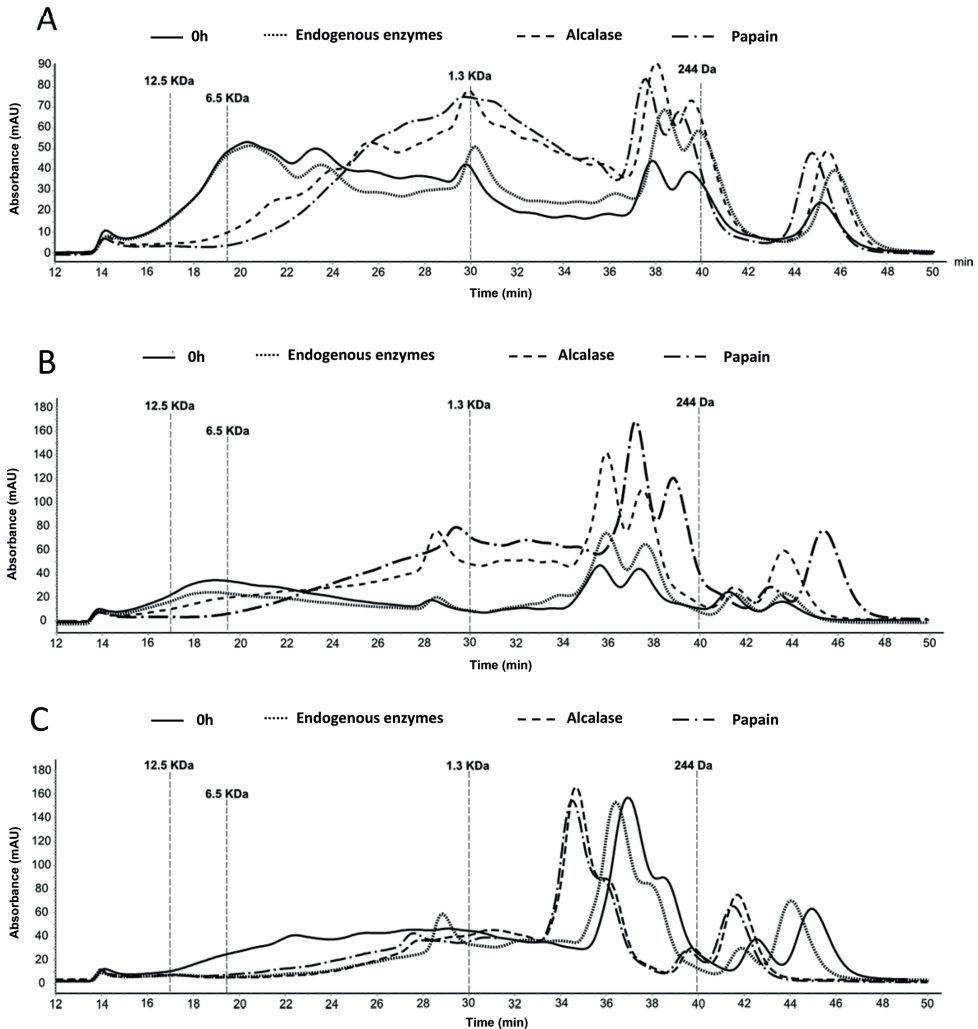
Figure 6. Size-exclusion chromatography of supernatants obtained upon hydrolyzing various raw materials: (A) pork–beef byproducts, (B) chicken byproducts, and (C) salmon viscera. The black lines represent the 0 h incubation with no enzymes added, the dotted lines represent the 1 h incubation with no enzymes added, the dashed lines represent the 1 h incubation with 0.5% Alcalase added, and the dashed-dotted lines represent the 1 h incubation with 0.5% Papain added. The approximate positions of the 12.5 kDa, 6.5 kDa, 1.3 kDa, and 244 Da standards in the elution profile are indicated. Note that the overlaid chromatograms are shifted relative to each other due to minor variations in the flow rate during sample loading. Because these shifts increase the visibility of the individual chromatograms, they were not corrected for.

could be obtained without adding external enzymes. Opheim et al.[37] did experiments with Protamex and Papain to hydrolyze salmon viscera and concluded that addition of commercial enzymes hardly affected the content of crude protein in the hydrolysates. However, others authors have found beneficial effects of adding external enzymes, and Alcalase is often found as the best candidate among the commercial enzymes. An increase in protein solubilization due to the use of Alcalase has been observed for a wide range of different fish byproducts, such as Atlantic salmon muscle proteins,[38] Atlantic cod

viscera,[19] Persian sturgeon viscera,[39] or Yellowfin tuna viscera.[40]

Although the occurrence of strong endogenous proteolytic activity in salmon viscera is well-known,[17] French et al.[41] observed that endogenous proteases varied for pink salmon depending on a wide range of factors, such as gender, harvesting time and place, maturity, and tissue type. Similar results were reported by Sovik et al.[16] for cod species caught at three different fishing grounds. These differences in proteolytic activities could explain the variation in literature data when it

Table 2. Total Amino Acid Compositions of Hydrolysates and Commercial YP[a]

	pork–beef byproducts				chicken byproducts				salmon viscera				
	raw material	autolysis	Alcalase	Papain	raw material	autolysis	Alcalase	Papain	raw material	autolysis	Alcalase	Papain	YP
							EAA[b]						
arginine	8.01 ± 0.66	7.08 ± 0.12	7.05 ± 0.09	7.13 ± 0.04	4.30 ± 0.08	6.58 ± 0.10	6.49 ± 0.02	6.54 ± 0.01	6.74 ± 0.04	4.19 ± 0.15	4.18 ± 0.08	4.13 ± 0.01	7.69 ± 0.03
histidine	2.20 ± 0.06	2.04 ± 0.02	2.01 ± 0.03	1.97 ± 0.02	2.56 ± 0.01	2.07 ± 0.03	2.26 ± 0.03	2.40 ± 0.01	2.48 ± 0.02	2.49 ± 0.06	2.53 ± 0.04	2.50 ± 0.00	1.46 ± 0.00
isoleucine	3.89 ± 0.14	3.96 ± 0.06	3.89 ± 0.05	3.84 ± 0.05	5.75 ± 0.08	4.00 ± 0.09	4.61 ± 0.03	4.75 ± 0.05	4.73 ± 0.07	5.35 ± 0.12	5.44 ± 0.04	5.35 ± 0.04	3.14 ± 0.02
leucine	7.28 ± 0.22	6.88 ± 0.07	6.84 ± 0.07	6.74 ± 0.12	8.89 ± 0.09	7.23 ± 0.11	7.83 ± 0.11	8.09 ± 0.02	8.42 ± 0.03	8.32 ± 0.17	8.39 ± 0.07	8.16 ± 0.11	4.99 ± 0.02
lysine	6.87 ± 0.17	6.78 ± 0.16	6.43 ± 0.06	6.38 ± 0.07	3.46 ± 0.04	7.59 ± 0.09	7.47 ± 0.06	7.59 ± 0.01	7.42 ± 0.08	4.43 ± 0.03	4.52 ± 0.04	4.72 ± 0.06	5.58 ± 0.03
methionine	1.99 ± 0.06	1.82 ± 0.02	1.89 ± 0.00	1.84 ± 0.00	3.14 ± 0.01	2.03 ± 0.02	2.23 ± 0.03	1.24 ± 0.02	2.28 ± 0.02	2.90 ± 0.05	2.87 ± 0.04	2.84 ± 0.05	1.42 ± 0.00
phenylalanine	3.90 ± 0.10	3.56 ± 0.06	3.62 ± 0.02	3.60 ± 0.04	4.52 ± 0.03	3.43 ± 0.03	3.96 ± 0.08	4.15 ± 0.05	4.50 ± 0.01	3.99 ± 0.06	4.08 ± 0.04	3.92 ± 0.03	3.00 ± 0.00
threonine	4.38 ± 0.16	4.16 ± 0.03	4.09 ± 0.04	4.00 ± 0.03	5.98 ± 0.05	4.86 ± 0.03	5.10 ± 0.06	5.15 ± 0.03	4.98 ± 0.01	5.81 ± 0.12	5.86 ± 0.06	5.86 ± 0.04	3.33 ± 0.01
tryptophan	0.77 ± 0.09	0.54 ± 0.01	0.75 ± 0.08	0.73 ± 0.15	1.17 ± 0.06	0.66 ± 0.05	0.81 ± 0.03	0.98 ± 0.02	1.12 ± 0.06	1.14 ± 0.12	1.12 ± 0.11	1.17 ± 0.02	0.43 ± 0.02
valine	5.08 ± 0.14	5.14 ± 0.06	5.21 ± 0.03	5.18 ± 0.05	7.03 ± 0.08	5.34 ± 0.11	5.70 ± 0.05	6.04 ± 0.05	5.78 ± 0.07	6.69 ± 0.17	6.84 ± 0.15	6.84 ± 0.02	4.22 ± 0.03
							NEAA[c]						
alanine	6.84 ± 0.17	7.21 ± 0.06	7.25 ± 0.08	7.21 ± 0.03	7.49 ± 0.02	6.89 ± 0.05	6.46 ± 0.04	6.41 ± 0.03	6.43 ± 0.05	7.48 ± 0.01	7.50 ± 0.07	7.51 ± 0.04	8.66 ± 0.01
aspartic acid	8.83 ± 0.11	8.68 ± 0.08	8.72 ± 0.08	8.58 ± 0.00	8.91 ± 0.03	9.33 ± 0.06	9.75 ± 0.05	9.73 ± 0.01	9.62 ± 0.01	8.81 ± 0.17	9.00 ± 0.03	9.02 ± 0.06	7.93 ± 0.03
glutamic acid	13.8 ± 0.20	14.3 ± 0.04	13.7 ± 0.12	13.5 ± 0.02	14.1 ± 0.08	16.9 ± 0.08	15.5 ± 0.09	14.9 ± 0.04	14.1 ± 0.03	14.4 ± 0.02	14.4 ± 0.06	14.6 ± 0.04	14.0 ± 0.05
glycine	10.3 ± 1.08	12.1 ± 0.18	12.2 ± 0.32	12.7 ± 0.23	8.60 ± 0.29	8.13 ± 0.21	6.91 ± 0.16	6.72 ± 0.06	6.58 ± 0.13	8.91 ± 0.34	8.66 ± 0.17	8.85 ± 0.02	17.6 ± 0.14
serine	4.93 ± 0.01	4.63 ± 0.04	4.65 ± 0.02	4.62 ± 0.02	5.43 ± 0.04	5.08 ± 0.06	5.09 ± 0.07	5.09 ± 0.04	4.93 ± 0.07	5.35 ± 0.28	5.48 ± 0.17	5.36 ± 0.04	4.66 ± 0.01
tyrosine	2.52 ± 0.10	2.31 ± 0.04	2.46 ± 0.21	2.66 ± 0.04	1.33 ± 0.02	2.28 ± 0.07	2.69 ± 0.09	3.04 ± 0.13	2.96 ± 0.09	1.55 ± 0.06	1.52 ± 0.05	1.62 ± 0.06	0.78 ± 0.00
cysteine	1.19 ± 0.05	0.96 ± 0.03	1.06 ± 0.01	1.13 ± 0.01	1.15 ± 0.00	1.11 ± 0.00	1.28 ± 0.03	1.41 ± 0.00	1.30 ± 0.02	1.21 ± 0.04	1.27 ± 0.00	1.31 ± 0.00	0.42 ± 0.00
proline	6.98 ± 0.38	7.69 ± 0.12	7.95 ± 0.11	8.06 ± 0.05	6.08 ± 0.12	6.38 ± 0.32	5.71 ± 0.14	5.62 ± 0.01	5.45 ± 0.01	6.86 ± 0.49	6.18 ± 0.16	6.15 ± 0.03	10.5 ± 0.08
							Sums						
ΣEAA	44.41	42.01	41.84	41.48	46.85	43.85	46.51	47.00	48.51	45.38	45.88	45.54	35.30
ΣNEAA	55.59	57.99	58.16	58.52	53.15	56.15	53.49	53.00	51.49	54.62	54.12	54.46	64.70
ΣEAA/ΣNEAA	0.80	0.72	0.72	0.71	0.88	0.78	0.87	0.89	0.94	0.83	0.85	0.84	0.55

[a]Amounts of amino acids are given as weight percentages of the total amounts of amino acids. Values are means ± SD (n = 3). [b]EAA, essential amino acids. [c]NEAA, nonessential amino acids.
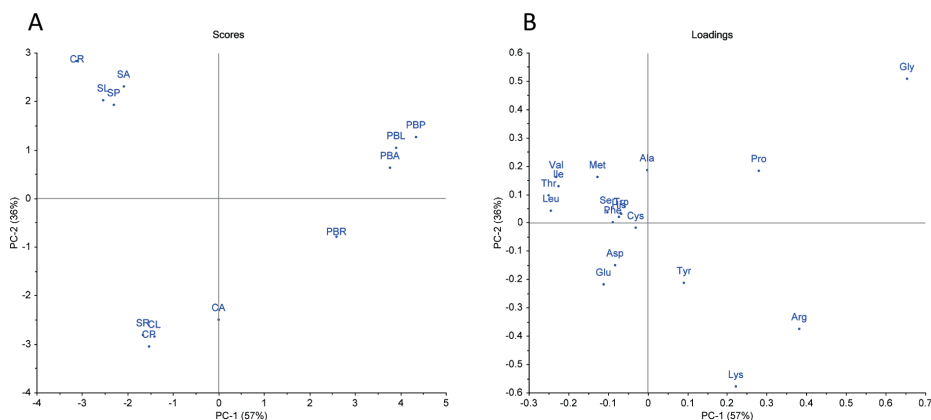
Figure 7. Principal-component analysis (PCA) of total amino acid compositions: (A) scores plot and (B) loadings plot. P, pork—beef byproducts; C, chicken byproducts; S, salmon viscera; R, raw material; A, autolysis; L, Alcalase; P, Papain. Note that chicken hydrolysates group together with the salmon-viscera raw material, whereas the salmon-viscera hydrolysates group with the chicken raw material; this is primarily because chicken hydrolysates are enriched in Arg and Lys and low in Gly, relative to the raw material, whereas the situation is opposite for salmon viscera, as is visible here (B) as well as in Figure S1.

comes to estimating the need for adding external enzymes to solubilize fish viscera. Clearly, even at equal solubilization levels, the externally added proteases may have impacts on hydrolysate functionality by modulating properties such as peptide length. This is further explored below.

Characterization of the Hydrolysates. Peptide composition influences the functional properties and, therefore, the usability of the hydrolysates. To obtain insight into peptide-length distributions and possible variation therein, hydrolysates generated after 1 h of hydrolysis (using conditions that generate maximum yields after 1 h) were analyzed by size-exclusion chromatography, and the results are shown in Figure 6. The chromatograms show that for the meat-based byproducts, Alcalase and Papain considerably reduce average peptide lengths compared with those of hydrolysates obtained using endogenous activity only. Thus, although the exogenous proteases have a modest effect on nitrogen solubilization, they have a clear effect on hydrolysate characteristics. On the other hand, the chromatograms for the salmon-viscera samples seem almost unaffected by the presence of external proteases, underpinning the dominant effect of the endogenous proteases, which are apparently capable of drastically reducing peptide size without help from externally added enzymes. It is worth noting that the viscera hydrolysates gave higher signals in the lower-molecular-weight range, including peaks eluting later than the 244 Da standard (Uridine) that likely represent free amino acids.[36] This suggests that the endogenous enzymes in the salmon viscera are indeed very active and have broad proteolytic potential.

Because the amino acid content is a major determinant of the functionality and value of the hydrolysates (e.g., when used as fermentation ingredients), the amino acid profiles of the various hydrolysates were determined and compared (Table 2). Because we had a special interest in using the hydrolysates for producing yeast, the amino acid content of a commercial YP medium, prepared by mixing peptone from meat and yeast extract in a 2:1 ratio, was used for bench-marking. Table 2 shows that the amino acid compositions, summarized by the

ratios between essential and nonessential amino acids, vary considerably among the raw materials. There is much less variation due to the enzyme treatment, and this limited variation is largest among the various chicken hydrolysates. Statistical analyses confirmed that the differences in the amino acid compositions of the hydrolysates are primarily determined by the raw material and not by the hydrolysis method (Figure 7) and that the choice of enzyme has a slightly larger effect on chicken hydrolysates, compared with on hydrolysates derived from the other two raw materials (Figures 7 and S1).

The sulfur-containing amino acids, cysteine and methionine, as well as tryptophan occur in low concentrations in all hydrolysates, whereas glutamic acid (representing Glu and Gln) generally is the most abundant, as has been described previously (e.g., for hydrolyzed poultry byproducts).[42] The ratio between essential and nonessential amino acids varies from 0.71 to 0.89 for the hydrolysates, whereas this ratio was low as 0.55 for YP (Table 2). The peptone in YP is industrially manufactured by controlled enzymatic hydrolysis of animal tissues, which could explain why the amino acid composition of YP is most similar to the composition of the pork—beef hydrolysates. Both pork—beef hydrolysates and YP are relatively rich in glycine and proline (Table 2 and Figure 7) which could be due to the presence of collagen from the cartilage, blood vessels, and guts.[37]

Table 3 shows that free amino acids constitute about 6—8, 8—10, and 11—12%, of the total amino acids in the pork—beef, chicken, and viscera hydrolysates, respectively, as opposed to 30% for commercial YP. Others have reported higher levels of free amino acids in hydrolysates, which is likely due to the use of more powerful proteolytic conditions (more enzyme, longer incubation times) during the hydrolysis reactions. Lazzi et al.[42] found 30% free amino acids after hydrolysis of poultry-processing leftovers, whereas Opheim et al.[37] reported 27—53% of free amino acids after hydrolysis of salmon viscera. On the other hand, Liaset et al.[43] reported only 7% of free amino acids after hydrolyzing salmon frames with Protamex.

Table 3. Composition of Free Amino Acids in Hydrolysates and Commercial YP[a]

	pork–beef byproducts			chicken byproducts			salmon viscera			YP
	autolysis	Alcalase	Papain	autolysis	Alcalase	Papain	autolysis	Alcalase	Papain	
arginine	13.8 ± 1.09	14.8 ± 0.19	15.7 ± 0.19	7.78 ± 0.23	7.84 ± 0.06	8.86 ± 0.03	5.25 ± 0.14	5.03 ± 0.09	5.37 ± 0.03	13.3 ± 0.29
histidine	2.09 ± 0.06	1.92 ± 0.02	1.93 ± 0.02	1.98 ± 0.03	1.88 ± 0.02	1.97 ± 0.02	3.42 ± 0.10	3.49 ± 0.01	3.43 ± 0.06	1.62 ± 0.00
isoleucine	4.63 ± 0.25	4.18 ± 0.02	4.48 ± 0.01	4.56 ± 0.07	5.02 ± 0.04	5.07 ± 0.00	6.97 ± 0.01	6.95 ± 0.00	6.96 ± 0.05	5.19 ± 0.04
leucine	12.8 ± 0.17	12.9 ± 0.05	12.4 ± 0.03	10.7 ± 0.30	12.8 ± 0.27	13.0 ± 0.05	12.5 ± 0.20	12.6 ± 0.07	12.5 ± 0.02	10.6 ± 0.05
lysine	14.0 ± 1.05	13.3 ± 0.25	13.5 ± 0.26	9.01 ± 0.17	9.23 ± 0.05	9.70 ± 0.04	5.05 ± 0.13	4.95 ± 0.09	5.52 ± 0.11	10.5 ± 0.06
methionine	3.32 ± 0.02	3.36 ± 0.01	3.20 ± 0.01	3.00 ± 0.01	2.72 ± 1.90	3.71 ± 0.01	4.34 ± 0.02	4.43 ± 0.03	4.36 ± 0.01	2.23 ± 0.01
phenylalanine	7.35 ± 0.14	7.63 ± 0.01	7.39 ± 0.03	5.70 ± 0.13	6.97 ± 0.13	6.74 ± 0.05	6.24 ± 0.03	6.31 ± 0.05	6.26 ± 0.02	7.11 ± 0.05
threonine	3.32 ± 0.18	3.23 ± 0.02	3.22 ± 0.02	4.59 ± 0.15	4.32 ± 0.09	4.18 ± 0.01	5.89 ± 0.04	5.90 ± 0.05	5.91 ± 0.03	3.86 ± 0.02
tryptophan	2.04 ± 0.06	2.06 ± 0.01	0.97 ± 0.03	1.73 ± 0.07	1.95 ± 0.05	1.83 ± 0.01	1.97 ± 0.06	2.03 ± 0.02	1.93 ± 0.03	1.60 ± 0.02
valine	4.52 ± 0.25	4.31 ± 0.02	4.53 ± 0.06	5.29 ± 0.03	5.31 ± 0.09	5.24 ± 0.01	8.01 ± 0.04	8.09 ± 0.04	8.13 ± 0.01	5.80 ± 0.05
alanine	6.18 ± 0.19	6.11 ± 0.01	6.20 ± 0.04	7.54 ± 0.06	7.50 ± 0.08	7.74 ± 0.02	9.28 ± 0.00	9.34 ± 0.01	9.34 ± 0.03	10.0 ± 0.08
aspartic acid	2.42 ± 0.44	2.17 ± 0.00	2.25 ± 0.02	7.53 ± 0.25	5.89 ± 0.06	5.31 ± 0.09	4.56 ± 0.14	4.31 ± 0.03	4.48 ± 0.01	4.28 ± 0.00
glutamic acid	6.28 ± 0.77	6.54 ± 0.04	6.35 ± 0.06	13.3 ± 0.42	11.1 ± 0.35	9.92 ± 0.07	10.5 ± 0.27	10.4 ± 0.13	10.7 ± 0.10	11.8 ± 0.06
glycine	2.64 ± 0.08	2.61 ± 0.00	3.14 ± 0.04	4.43 ± 0.14	3.30 ± 0.07	3.71 ± 0.02	4.88 ± 0.13	4.87 ± 0.04	4.88 ± 0.02	4.34 ± 0.01
serine	4.10 ± 0.19	3.90 ± 0.01	3.87 ± 0.04	5.42 ± 0.13	5.13 ± 0.11	4.80 ± 0.01	5.65 ± 0.02	5.85 ± 0.06	5.73 ± 0.03	4.70 ± 0.04
tyrosine	7.04 ± 0.09	7.42 ± 0.03	6.92 ± 0.01	4.96 ± 0.17	6.59 ± 0.23	6.20 ± 0.04	3.01 ± 0.06	3.01 ± 0.11	2.99 ± 0.08	2.25 ± 0.09
glutamine	3.43 ± 0.03	3.34 ± 0.02	2.63 ± 0.07	2.66 ± 0.09	2.48 ± 0.35	1.82 ± 0.15	2.27 ± 0.00	2.30 ± 0.03	1.31 ± 0.03	0.58 ± 0.04
$\sum$(free AA)/$\sum$AA	0.06	0.08	0.08	0.09	0.08	0.08	0.12	0.11	0.11	0.26

[a]The amounts of amino acids are given as weight percentages of the total amounts of free amino acids. The table also shows the ratios between free amino acids and total amino acids. Values are means ± SD ($n = 3$).

Leucine, lysine, phenylalanine, alanine, arginine, and glutamic acid are among the most abundant free amino acids in all hydrolysates (Table 3). Lysine and arginine are higher in Alcalase- and Papain-treated pork−beef hydrolysates compared with in chicken-byproduct and salmon-viscera hydrolysates. Leucine and glutamic acid are the most prominent free amino acids in the hydrolysates obtained from chicken byproducts. The hydrolysates from salmon viscera have higher contents of leucine, alanine, and glutamic acid compared with those of the other hydrolysates.

Microbial Growth on Protein-Rich Hydrolysates. Growth of the yeast *Candida utilis* on media based on the hydrolysates was compared with growth on commercial YP medium. Growth data revealed similar growth rates and lag phases for all cultures (data not shown), and assessment of dry-cell-mass yields after 24 h (Table 4) showed that the

Table 4. Growth of *C. utilis* in 2.5 mL Well Microtiter Plates Using Commercial Yeast Extract and Meat Peptone (YP) and Nine Different Hydrolysates as Nitrogen Sources[a]

	enzymatic treatment		
raw material	autolysis	Alcalase	Papain
pork−beef byproducts	13.43 ± 0.66	13.73 ± 0.42	12.81 ± 0.62
chicken byproducts	13.28 ± 0.60	14.45 ± 0.43	13.03 ± 0.73
salmon viscera	13.65 ± 0.79	14.38 ± 0.36	13.86 ± 0.76
YP		11.08 ± 0.56	

[a]All media contained 20 g/L glucose as a carbon source. Dry cell weight (g/L) was measured after washing and drying the microbial mass. Values are means ± SD ($n = 3$).

hydrolysates outperform commercial YP. Further work is needed to assess the underlying causes of this promising result. Using cod-viscera hydrolysates, Aspmo et al. obtained similarly promising results for *Saccharomyces cerevisiae*,[18] whereas Klompong et al.[23] did not observe differences between the performances of fish hydrolysates and commercial peptone when studying the growth of *S. cerevisiae* and *Candida albicans*. Notably, as pointed out in Møretrø et al.,[44] hydrolysate performance in fermentations may relate to factors other than nitrogen and amino acid supply, such as the presence of other nutritionally important compounds that are released and preserved in the mild hydrolysis processes used in this study. Table 4 shows that cell-mass yields relative to the amount of glucose added were above 50%, which is generally considered a maximum for pure oxidative growth on sugar.[45] However, when the primary carbon source becomes limiting, other compounds, such as amides, amino acids, and peptides can be used as carbon sources,[46] which could explain the high cell-mass yields obtained in this study.

In conclusion, the present study shows how various protein-rich byproducts may be hydrolyzed efficiently, harnessing the power of endogenous enzymes, limiting the amounts of externally added commercial enzymes, and minimizing processing times. Process efficiency was likely due in part to the use of a temperature gradient, which allowed endogenous enzymes to work before the commercial enzymes were activated at higher temperatures. The contribution of autolysis was considerable but varied among the three raw materials. For pork−beef byproducts, 70−77% solubilization was achieved without the addition of commercial enzymes, whereas endogenous-enzyme activity was less powerful for the chicken byproducts. For salmon viscera, exogenous enzymes were not needed to reach maximum

protein solubilization. Importantly, the gel-filtration data showed that although they have little effect on protein solubilization, the exogenous enzymes have a considerable effect on the average molecular weights of the generated peptides. This may be of importance because, for example, reduced peptide size is considered to reduce risks of allergenicity and increase safety related to protein-transmitted diseases.[47]

The large effect of endogenous enzymes should be taken into account when devising strategies for treating and storing raw materials. The salmon viscera used in this study is an extreme case. The viscera hydrolysates were not influenced by the addition of commercial enzymes in terms of protein recovery, peptide-size distribution, and amino acid composition. This illustrates the power of the endogenous enzymes and implies that storage conditions from slaughtering to hydrolysis will affect the final product.

The use of short processing times, such as in this study, is beneficial for several reasons, including reduced microbial contamination and capital costs. Likewise, raising the processing temperature as high as 60 °C is favorable because this temperature is optimal for Alcalase and Papain, while also reducing undesired microbial growth. The optimal time for hydrolysis and the best-suited protease cocktail is raw-material specific, but the protocols described in this study can be easily applied on a wide range of test materials.

There is an increasing interest in the use of microbial biomass, particularly yeasts, as a sustainable ingredient in feed, because this could allow the conversion of nonedible biomass from forestry, agriculture, and aquaculture into food. Next to providing protein, yeast in feed may have positive health effects as a result of the presence of bioactive and immunostimulating compounds such as β-glucan- and mannan-oligosaccharides.[48] Our preliminary growth studies (Table 4) show that the hydrolysates produced here are promising components for growth media for yeasts. It is important to emphasize that hydrolysate functionality is likely process-dependent, not only because the process affects the presence of amino acids and peptides but also because of effects on the presence and nutritional value of other compounds that influence microbial growth.[18] Further experiments are needed in order to fully assess the impact of the use of different enzymatic processing strategies on the growth of *C. utilis*.

Depending on whether food-grade conditions can be met, other applications of the hydrolysates are also possible. Protein hydrolysates are important to the food industry, where they may be used as high-quality sources to enhance the protein contents of food, as milk-powder replacements, as oil binders in meat products, or as emulsifiers in spread-texture food.[49] Other potential applications may relate to the presence of bioactive peptides that are of interest to the pharmaceutical and health-product industries.[50,51]

■ ASSOCIATED CONTENT

ⓈSupporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b02444.

Total amino acid compositions of raw materials and hydrolysates, composition of hydrolysates of pork−beef byproducts, composition of hydrolysates of chicken byproducts, and composition of hydrolysates of salmon viscera (PDF)

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ABBREVIATIONS USED

DM, dry matter; HPLC, high-performance liquid chromatography; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; YP, commercial yeast extract and peptone from meat; AA, amino acid; EAA, essential amino acids; NEAA, nonessential amino acids

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A comparative assessment of enzymatic hydrolysis for valorization of different protein-rich industrial by-products

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Pork-Beef by-product

B

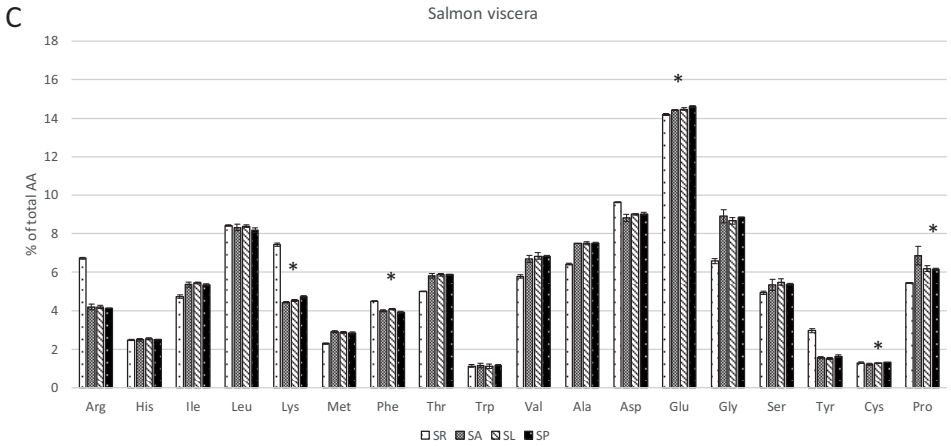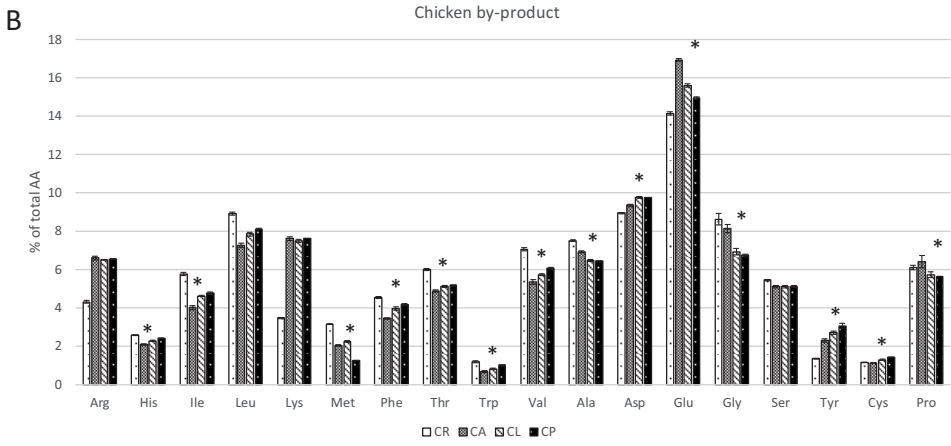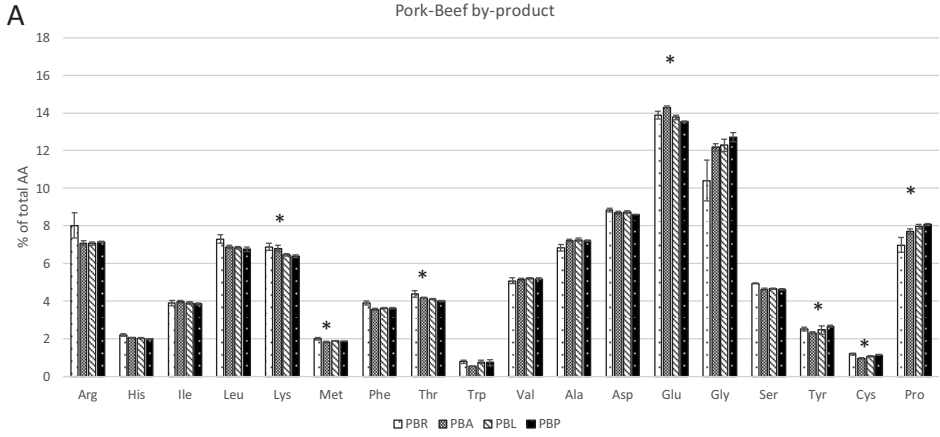Chicken by-product

C

Salmon viscera

2

Figure S1. Total amino acid composition of raw materials and hydrolysates. The amounts of amino acids are given as weight percentages of the total amount of amino acids and the bars show mean values ± SD (n = 3). ANOVA analyses were performed for the treated raw materials. Amino acids for which the amounts differ significantly (α=0.05) between the three different enzymatic treatments are marked by a * (positioned above the middle of the three values); note the relatively high number of asterisks in panel B. See main text, including Fig. 7, for further analysis and details. Abbreviations: PB, Pork-beef by-products; C, Chicken by-products; S, Salmon-viscera; R, Raw material; A, Autolysis; L, Alcalase; P, Papain.

Table S1. Composition of hydrolysates of pork-beef by-products. Mean values ± SD (n = 3).

		Pork-Beef By-Products					
Time (h)	Enzyme Loading (%)	DM Supernatant Alcalase (g/100mL)	DM Supernatant Papain (g/100mL)	Protein Content in DM Supernatant Alcalase (%)	Protein Content in DM Supernatant Papain (%)	Protein solubilization using Alcalase (%)	Protein solubilization using Papain (%)
0	None	2.99 ± 0.13		99.23 ± 5.20		62.11 ± 0.61	
1	None (Autolysis)	4.25 ± 1.34		92.62 ± 2.64		77.13 ± 5.45	
2	None (Autolysis)	3.93 ± 0.36		94.81 ± 3.95		70.24 ± 4.32	
2	0.02	4.05 ± 0.22	4.25 ± 0.29	92.20 ± 3.66	93.31 ± 1.29	70.82 ± 6.61	73.62 ± 7.60
1	0.05	3.34 ± 0.03	3.56 ± 0.09	95.52 ± 3.03	94.62 ± 5.96	67.04 ± 0.43	73.66 ± 4.13
2	0.05	4.79 ± 0.05	4.94 ± 0.24	88.80 ± 2.06	89.01 ± 0.82	89.78 ± 7.92	91.86 ± 7.36
2	0.1	4.04 ± 0.18	4.69 ± 0.07	97.22 ± 1.00	96.71 ± 0.53	85.52 ± 7.27	83.81 ± 7.97
2	0.2	3.96 ± 0.08	4.74 ± 0.13	90.25 ± 0.51	90.29 ± 0.67	76.04 ± 2.19	87.26 ± 4.11
1	0.5	3.36 ± 0.03	3.55 ± 0.07	94.63 ± 5.96	95.51 ± 3.03	81.72 ± 3.70	91.23 ± 4.25
2	0.5	4.69 ± 0.15	4.81 ± 0.02	82.82 ± 2.18	88.50 ± 0.32	80.33 ± 9.18	90.95 ± 5.46

Table S2. Composition of hydrolysates of chicken by-products. Mean values ± SD (n = 3).

		Chicken By-Products					
Time (h)	Enzyme Loading (%)	DM Supernatant Alcalase (g/100mL)	DM Supernatant Papain (g/100mL)	Protein Content in DM Supernatant Alcalase (%)	Protein Content in DM Supernatant Papain (%)	Protein solubilization using Alcalase (%)	Protein solubilization using Papain (%)
0	None	3.44 ± 0.32		89.59 ± 2.58		21.43 ± 1.43	
1	None (Autolysis)	4.54 ± 0.06		85.40 ± 0.98		30.72 ± 1.24	
2	None (Autolysis)	6.37 ± 0.11		83.81 ± 0.55		49.61 ± 1.42	
2	0.02	6.07 ± 0.08	5.96 ± 0.15	85.06 ± 2.24	87.6 ± 0.60	47.98 ± 3.20	45.50 ± 2.75
2	0.05	6.25 ± 0.27	6.53 ± 0.13	89.83 ± 2.52	87.58 ± 0.61	57.04 ± 5.70	54.61 ± 3.58
2	0.1	6.24 ± 0.20	6.52 ± 0.25	88.95 ± 1.84	88.83 ± 0.28	56.80 ± 1.37	56.72 ± 4.14
2	0.2	6.15 ± 0.07	6.90 ± 0.22	91.89 ± 5.21	88.13 ± 1.54	60.07 ± 5.16	64.95 ± 4.94
1	0.5	6.77 ± 0.25	7.85 ± 0.31	83.65 ± 1.59	87.32 ± 2.85	50.23 ± 4.00	62.02 ± 3.73
2	0.5	7.14 ± 0.36	7.85 ± 0.08	82.95 ± 0.91	84.85 ± 1.15	69.14 ± 4.00	76.64 ± 3.36
2	1	7.14 ± 0.22	7.75 ± 0.08	81.91 ± 2.06	84.85 ± 1.48	66.50 ± 2.41	79.25 ± 3.38

Table S3. Composition of hydrolysates of salmon viscera. Mean values ± SD. (n = 3).

Time (h)	Enzyme Loading (%)	DM Supernatant Alcalase (g/100mL)	DM Supernatant Papain (g/100mL)	Protein Content in DM Supernatant Alcalase (%)	Protein Content in DM Supernatant Papain (%)	Protein solubilization using Alcalase (%)	Protein solubilization using Papain (%)
		Salmon Viscera					
0	None	6.77 ± 0.09		87.11 ± 0.60		69.60 ± 4.79	
1	None (Autolysis)	6.61 ± 0.62		81.81 ± 5.79		86.81 ± 5.26	
2	None (Autolysis)	5.93 ± 0.22		87.65 ± 2.17		84.17 ± 7.49	
2	0.02	6.12 ± 0.22	6.18 ± 0.18	86.1 ± 1.16	88.12 ± 0.13	82.84 ± 3.45	87.93 ± 2.99
2	0.05	5.91 ± 0.05	5.83 ± 0.10	88.37 ± 1.25	89.31 ± 0.38	79.82 ± 1.72	81.34 ± 2.63
2	0.1	6.22 ± 0.11	6.07 ± 0.21	85.37 ± 0.90	87.35 ± 1.24	84.03 ± 1.90	83.47 ± 2.25
2	0.2	6.30 ± 0.24	5.96 ± 0.14	84.25 ± 1.37	85.51 ± 1.58	84.87 ± 3.69	82.68 ± 3.34
1	0.5	6.18 ± 0.37	6.28 ± 0.13	86.03 ± 5.98	85.67 ± 0.51	86.80 ± 3.38	86.75 ± 4.79
2	0.5	5.93 ± 0.05	6.36 ± 0.35	85.8 ± 0.49	82.39 ± 6.09	79.67 ± 1.75	83.09 ± 3.48

Paper II

Production and characterization of yeasts grown on media composed of spruce-derived sugars and protein hydrolysates from chicken by-products

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Abstract

Background: A possible future shortage of feed protein will force mankind to explore alternative protein sources that can replace conventional soymeal or fishmeal. Several large industrial organic side-streams could potentially be upgraded to feed protein using fermentation process to generate single cell protein. Yeast is the most widely accepted microorganism for production of single cell protein, because of its superior nutritional quality and acceptability among consumers. Here, we have assessed the growth of four different yeasts, *Cyberlindnera jadinii*, *Wickerhamomyces anomalus*, *Blastobotrys adeninivorans* and Thermosacc® Dry (*Saccharomyces cerevisiae*), on media composed of enzymatically saccharified sulfite-pulped spruce wood and hydrolysates of by-products from chicken and we have characterized the resulting yeast biomass.

Results: Generally, the yeast grew very well on the spruce- and chicken-based medium, with typical yields amounting to 0.4 – 0.5 g of cell dry weight and 0.2 – 0.3 g of protein per g of sugar. *B. adeninivorans* stood out as the most versatile yeast in terms of nutrient consumption and in this case yields were as high as 0.9 g cells and 0.5 g protein per g of sugar. The next best performing yeast in terms of yield was *W. anomalus* with up to 0.6 g cells and 0.3 g protein per g sugar. Comparative compositional analyses of the yeasts revealed favorable amino acid profiles that were similar to the profiles of soymeal, and even more so, fish meal, especially for essential amino acids.

Conclusions: The efficient conversion of industrial biomass streams to yeast biomass demonstrated in this study opens new avenues towards better valorization of these streams and development of sustainable feed ingredients. Furthermore, we conclude that production of *W. anomalus* or *B. adeninivorans* on this promising renewable medium may be potentially more efficient than production of the well-known feed ingredient *C. jadinii*. Further research should focus on medium optimization, development of semi-continuous and continues fermentation protocols and exploration of downstream processing methods that are beneficial for the nutritional values of the yeast for animal feed.

Keywords: microbial protein, yeast, fermentation, spruce, protein hydrolysate, feed, aquaculture, enzymatic hydrolysis

Background

The world´s population is projected to reach about 9.7 billion people in 2050 (1). Such a population would need 1,250 million tons of meat and dairy products per year to meet the demand for animal-derived protein assuming current consumption levels (2). This growing demand will force mankind to search for alternative protein sources that can replace or supplement plant proteins that are currently used as animal feed. Of note, conventional plant proteins tend to be inefficiently converted: approximately 6 kg of plant protein is needed to produce 1 kg of meat protein (3). Increasing meat production to match global demand is ultimately not sustainable (4).

Aquaculture, which currently contributes 17% of the global intake of animal protein (5), appears to be a possible solution, in part because of better feed conversion rates (6). Fish meal and plant-based proteins are the currently preferred protein sources for many aquaculture species. However, increased use of fish meal is not sustainable since it is based on catch of wild fish stocks (7). Plant-based proteins such as soybean protein require the use of arable land, raising potential ethical conflicts between food and feed production (8,9). Combined with the increasing demand for fish feed protein, these considerations show that other sources of protein must be identified and developed.

One solution for this challenge is to use microbial proteins, also known as single cell protein (SCP), produced by fungi, algae or bacteria. Yeasts are among the preferred candidates due to their rapid growth and high protein content, a low risk of contamination, and ease of harvesting due to their cell size and flocculation abilities (2,9) Yeasts are considered a well-balanced source of amino acids and can provide vitamins (mainly the B group) (10)They also contain lower amounts of nucleic acids (5-12%) than bacteria (8-14%), which is beneficial for a human food or animal feed ingredient (2,10). Additionally, it has been shown that certain yeasts may have positive health effects in pigs (11), poultry (12) and fish (13), possibly as a result of the presence of bioactive and immunostimulating compounds such as ß-glucans and α-mannan.

The production of yeast biomass as a source of SCP should be cheap and environmentally friendly in order to replace the aforementioned unsustainable feed ingredients for the production of meat. Therefore, it is important to find yeast strains with optimal properties and to develop high quality, cheap and sustainable fermentation media. It has been estimated that in yeast SCP production, 62% of the total product cost comes from the raw materials used for fermentation (14). Yeasts can convert readily available and low-cost industrial organic by-products into high quality protein and lipids for animal feed and even for human consumption (10,15) Hydrolyzed protein-rich by-products from

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food production such as meat and fish residues may be utilized as an alternative to inorganic nitrogen sources that are commonly used for fermenting yeasts Non-edible residues produced from agricultural and forestry industries, such as saw dust or straw, can be utilized as alternative carbon sources. Since such side-streams are rich in cellulose, hemicellulose and lignin and since yeasts do not have enzymes for efficiently processing these polymers, the use of these raw materials requires an enzyme pre-treatment to produce sugars that can be assimilated. Recently, the Norwegian company Borregaard developed a pretreatment technology for lignocellulosic biomass, which includes a sulfite cooking step that solubilizes lignin and washes out most of the hemicellulose, leaving a relatively clean cellulose fraction. After this process, called BALI, for Borregaard Advanced Lignin™ (16), modern cellulase cocktails can efficiently convert cellulose and hemicellulose into hexose and pentose (17,18)

The aim of this study was to use a medium consisting of sugars produced through enzymatic hydrolysis of lignocellulosic biomass (16,19) and enzymatically hydrolyzed chicken by-products (20) to produce SCP in the form of yeast. Four different yeast strains were tested: *Cyberlindnera jadinii* (anamorph name *Candida utilis*), *Wickerhamomyces anomalus*, *Blastobotrys adeninivorans* (synonym *Arxula adeninivorans* and Thermosacc® Dry. We carried out a preliminary screening of growth in microtiter plates, where ten different growth media were tested. The best two media were then tested in batch fermentations using benchtop fermenters, where concentrations of cells, substrates, side-products and yeast protein were monitored. We also characterized and compared the four different yeast biomasses generated after the batch fermentations, assessing properties such as amino acid composition, and the content of nucleic acids, minerals, lipids, carbohydrates and ash. Finally, we assessed the composition of the yeast biomass with Fourier transform infrared spectroscopy (FTIR).

Methods

Materials

Fresh chicken by-products (heart, liver and digestive tract) were provided by Nortura Hærland (Hærland, Norway) and kept at -20 °C until further use. Prior to the enzymatic hydrolysis reactions, samples were thawed and minced

with a BIRO® MODEL 6642 feed grinder (Marblehead, Ohio, USA). The composition of the chicken by-products corresponded to 30.12 ± 0.50 % dry material, 15.10 ± 1.20 % protein, 4.47 ± 0.29 % ash and 6.91 ± 0.55 % lipids (20). Yeast extract, meat peptone, yeast nitrogen base w/o amino acids and w/o ammonium sulfate, glucose, cellobiose, xylose, lactic acid, acetic acid, sulfuric acid, hydrogen chloride, sodium hydroxide, ninhydrin, glycine and stannous chloride were purchased from Sigma-Aldrich (Missouri, USA). Ammonium sulfate was purchased from VWR (Pennsylvania, USA), and urea was kindly provided by Yara International ASA (Oslo, Norway). Kjeltabs for Kjeldahl analysis were purchased from Thomson and Capper Ltd. (Cheshire, UK).

Enzymatic hydrolysis of chicken by-products and spruce

15 kg (wet weight) minced chicken by-products were mixed with 15 L of water in 30L Einar hydrolysis reactors (Belach Bioteknik, Skogås, Stockholm, Sweden), resulting in a dry-matter concentration of 15 %. The enzymatic hydrolysis of the chicken by-products was carried out using 0.5 % (weight of the enzyme powder/weight of wet chicken by-products) papain from *Carica papaya*, (≥ 3 U/mg; Merck, Darmstadt, Germany) at 60 ºC and 50 rpm without pH adjustment and using slow heating to 60 ºC, as described previously (20). The hydrolysates were removed from the hydrolysis tanks after 2 hours and were filtered through a sieve of 0.85 mm Ø in order to remove insoluble particles. Subsequently, the hydrolysates were cooled down to 4 °C and stored overnight, which led to accumulation of lipids on the top of the hydrolysate. The liquid fraction was centrifuged in a Beckman Coulter Avanti J-26S XP centrifuge (Indianapolis, Indiana, USA) at 4 °C and 10.000 g for 10 min. Finally, the chicken by-products hydrolysates (CH) were filtered using a sieve of 75 µm Ø and stored at -20 °C until use. Due to the large hydrolysis volume (15 kg raw material and 15 kg water), the inactivation of proteolytic enzymes was not carried out directly after hydrolysis but by autoclaving of specific aliquot volumes used when preparing fermentation media.

Enzymatic hydrolysates of BALI™ pretreated spruce wood (*Picea abies*) were kindly provided by Borregaard AS (Sarpsborg, Norway) (16) The raw material used in the pulping process was chipped spruce with chip sizes of up to 4.5 x 4.5 x 0.8 cm^3. The carbohydrate composition of the spruce hydrolysate is shown in Table S1.

Growth experiments

Microtiter plates

C. jadinii LYCC 7549, Thermosacc® Dry (*Saccharomyces cerevisiae*) (Lallemand Yeast Culture Collection, Montreal, Canada), *W. anomalus* CBS100487 (Strain collection of the Swedish University of Agricultural Sciences, Uppsala, Sweden, internal strain number J121) and *B. adeninivorans* LS3 (Swedish University of Agricultural Sciences, Uppsala, Sweden), were stored in cryovials containing 20 % (v/v) glycerol at -80 °C. Ten different media were tested for growth: yeast nitrogen base without amino acids and with ammonium sulfate plus glucose (YNBAS+G), yeast nitrogen base without amino acids and with ammonium sulfate plus BALI™ hydrolysate (YNBAS+B), yeast nitrogen base without amino acids and with urea plus BALI™ hydrolysate (YNBU+B), yeast extract and meat peptone plus glucose (YP+G), yeast extract and meat peptone plus BALI™ hydrolysate (YP+B), chicken by-products hydrolysate plus glucose (CH+G), chicken by-products hydrolysate plus BALI™ hydrolysate (CH+B), chicken by-products hydrolysate (CH) and BALI™ hydrolysate (B). The nitrogen content (5.86 g/L; based on the nitrogen content of standard YP, containing 20 g/L yeast extract and 30 g/L meat peptone, as measured by Kjeldahl) and glucose content (50 g/L) were identical in all media, except in the control media containing only sugar (B) or only protein (CH). All media were sterilized by autoclaving at 121 °C for 20 min. Overnight pre-cultures were prepared by adding 200 µL of a seed culture to 25 mL of the to-be-tested medium in a 250 mL baffled shake flask, followed by incubation at 30 °C, 220 rpm for approx. 16 h.

The four yeast strains were grown in the Duetz-microtiter plate system (Duetz-MTPS) (Enzyscreen, Heemstede, The Netherlands), consisting of 24-square polypropylene deep well plates (11mL), sandwich covers and cover clamps, which were mounted in a shaker (Infors HT Shaker Minitron, Bottmingen, Switzerland). Autoclaved and dried microtiter plates were filled with 2.5 mL of sterile liquid medium. The initial pH was adjusted to 5.0 using 1M NaOH or HCl. Media were inoculated with the overnight pre-cultures to obtain an initial OD of 0.5, as measured at 595 nm with a UV/VIS spectrophotometer (Hitachi U1900, Tokyo, Japan). The plates were incubated at 30 °C at 450 rpm and samples were taken at 8h, 16h and 24 h, for the measurement of cell dry weight (CDW), free amino nitrogen and pH. These experiments were performed in triplicates.

Bioreactor runs

The bioreactor cultivations were performed in 2.5 L total volume glass fermenters (Minifors, Infors, Bottmingen, Switzerland) with working volumes of 1.5 L and equipped with two 6-bladed Rushton impellers, using YP+G (i.e. a standard commercial medium) and CH+B (i.e. the "chiken+spruce" medium). YP or CH were autoclaved at 121 °C for 15 min in the bioreactors. Glucose and BALI^{TM} hydrolysate were autoclaved separately. Overnight pre-cultures were prepared by adding 1 mL of a seed culture into 250 mL of YP+G or CH+B medium in 2 L baffled shake flasks, followed by incubation at 30 °C, 220 rpm for approx. 16 h. The bioreactors were inoculated with 30 mL overnight preculture (2 % (v/v) and each fermentation was run in duplicates. The temperature for all cultivations was 30 °C. The pH was monitored with a pH probe (Mettler Toledo, Greifensee, Switzerland) and was kept at 5.0 by automatic addition of 1M NaOH or 5M H$_2$SO$_4$. Dissolved oxygen (DO) was set at 30 % saturation and regulated by manual adjustment of the stirrer speed (300 – 1250 rpm). Cultures were aerated through a sparger at an initial rate of 1.5 L/min (1 VVM), which was increased to up to 3 L/min (2 VVM) during the fermentation to maintain DO. Exhaust CO$_2$ and O$_2$ analysis was performed with a FerMac 368 off-gas analyzer (Electrolab Biotech, Tewkesbury, UK). Foam was controlled via a foam sensor with five times diluted Glanapon DB 870 antifoam (Busetti, Vienna, Austria). Fermentation data were recorded using IRIS process control software (Infors). During the fermentation, samples were aseptically taken every two hours for analysis of the culture supernatant and the yeast biomass.

Analytical Methods

Cell dry weight (CDW)

Fermentation broth samples (25 ml from bioreactors and 2 ml from microplates) were centrifuged at 4700 g (25 mL) or 10 000 g (2 mL) for 5 min at 4 °C and the supernatant was collected for further analyses (sugars, organic acids, ethanol, free amino nitrogen, protein). Then, the yeast cells were washed twice with cold distilled water, frozen at -20C and then freeze-dried using an Alpha 2-4 LDplus (Martin Christ, Osterode am Harz, Germany), at -60 °C and 0.01 mbar vacuum for 48 h. After determining their weight, to calculate CDW, the dried cells were used for analysis of protein content, amino acids, nucleic acids, lipids, fibers, minerals, ash and were also analyzed by FTIR spectroscopy.

Monosaccharides, organic acids and ethanol

Monosaccharides (D-glucose, D-xylose), organic acids (lactic acid, acetic acid) and ethanol present in the fermentation broth were analyzed by high performance liquid chromatography (HPLC) with refractive index detection. The samples were diluted 10 times with distilled water and 200 μL were vacuum-filtered through 96 well filter plates (0.45 □m). Samples were separated on a Rezex ROA-organic acid H+, 300 x 7.8 mm^2 (Phenomenex, Torrance, CA, USA) analytical column fitted with a cation-H cartridge guard column, using a column temperature of 65 °C, 5 mM H_2SO_4 as eluent and a flow rate of 0.6 mL/min. Additionally, monosaccharides were analyzed by High-Performance Anion-Exchange Chromatography (HPAEC) using a Dionex ICS 3000 system (Dionex, Sunnyvale, CA, USA) equipped with a CarboPac PA1 column operated at 30 °C, and with pulsed amperometric detection (PAD), where D-xylose, L-arabinose, D-mannose and D-galactose could be quantified. Monosaccharides were eluted isocratically at a flow rate of 0.25 ml/min using 1 mM KOH, generated with eluent generator. HPLC data were collected and analyzed using Chromeleon 7.0.

Free Amino Nitrogen

A ninhydrin-based assay was performed in order to follow the consumption of free amino nitrogen during fermentations (21). Ninhydrin reacts with free alpha amino groups resulting in a blue product that can be colorimetrically quantified by measuring the absorbance at 575 nm. Glycine was used to produce a standard curve with known free amino nitrogen content (0.04-0.4 g/L nitrogen). 10 μL (diluted, if appropriate) samples were added to 100 μL of pH 5.5 acetate buffered ninhydrin reagent (containing 25 μL/mL $SnCl_2$), mixed, and incubated at 100 °C for 10 min. The assay was performed in microplate format (two replicates) and absorbance was measured with a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, Vermont, U.S.)

Protein content

Total nitrogen was measured according to the Kjeldahl method (European Commission [EC] regulation No: 152/2009, pp 15-19) using a Kjeltec TM 8400 (FOSS, Tecator, Höganäs, Sweden) after acid digestion in an autodigestor (FOSS, Tecator, Höganäs, Sweden). The protein content of samples was estimated by multiplying total nitrogen by a factor of 6.25.

Amino acids

Analysis of the content of amino acids (except tryptophan) in freeze-dried yeast was performed according to EC regulation No: 152/2009 (pp. 23 - 32) using a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, UK). Tryptophan was analyzed according to EC regulation No: 152/2009 (pp. 32 - 37) using a Dionex Ultimate 3000 HPLC system (Dionex Softron GmbH, Germering, Germany) connected to a RF-535 fluorescence detector (Shimadzu., Kyoto, Japan). All amino acids were quantified by using external standards (Dionex Ltd., Surrey, UK).

Nucleic Acids

The nucleic acid content in yeast biomass was determined spectrophotometrically by the diphenylamine assay (DNA) and the orcinol assay (RNA) after extraction with diluted perchloric acid (22). Before extraction, 50 mg of freeze-dried yeast cells were washed with 1.5 ml 0.9% saline solution (cold), and then by 1.5 ml 0.2 M $HClO_4$ (cold). Subsequently, 1.5 ml of 0.5 M $HClO_4$ was added, and the cells were incubated at 70°C for 15 minutes, centrifuged, and the supernatant was saved. Another 1.5 ml 0.5 M $HClO_4$ was the added to the cells, followed by mixing, another incubation at 70°C for 15 minutes, and centrifugation. The supernatants were combined and diluted to 5.0 ml with 0.5 M $HClO_4$. The $HClO_4$-extracts from the yeast cells and DNA standards (calf thymus DNA; Sigma D4522) were mixed with diphenylamine reagent 1:1 (v:v) (stock solution of 1.5 g diphenylamine, 100 mL glacial acetic acid, 1.5 mL concentrated sulfuric acid and 1mL acetaldehyde solution) in 96 well plates, incubated at 30 °C over night, and absorbance was read at 600 nm on a Spectramax M2^e microplate reader (Molecular Devices, LLC, San Jose, CA, USA). $HClO_4$-extracts from the yeast cells and RNA standards (RNA from baker's yeast; Sigma R6750) were mixed with a H_2SO_4/H_2O solution (v/v; 85/15) in 96 well plates, and incubated at 40°C for 24 hours. The orcinol reagent (stock solution of 0.35 mL 6 % (w/v) orcinol to 5 mL concentrated HCl) was then added, and the plates were incubated with gentle shaking at 100°C for 30 min, after which sbsorbance at 500 nm was read on a Spectramax M2^e microplate reader (Molecular Devices).

Lipids

The total lipid content of the freeze-dried yeast biomass was determined using accelerated solvent extraction. The extraction was carried out at 125 °C and 1500 psi with a mixture of 70 % petroleum ether - 30 % acetone in a Dionex

ASE 350 accelerated solvent extractor (Dionex, Sunnyvale, CA, USA). Then, the solvent was placed in a collection glass which was submerged in a 60°C water bath for evaporation under N_2 pressure. After 10 min, only lipids remained in the collection glass, and they were placed in a vacuum drier at 70°C for 30 minutes. Finally, the samples were placed in a desiccator, and lipids were weighed.

Minerals and ash

The mineral content of freeze-dried yeast biomass was analyzed by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 8800 QQQ, Santa Clara, California, USA). Samples were decomposed by 65 % HNO_3 in a high performance microwave reactor (UltraClave, MLS, Milestone, Sorisole, Italy) (23). For halides (anions), the samples were decomposed using concentrated 25 % (w/w) tetramethylammonium hydroxide. The mineral analyses were validated using certified reference materials NCS DC73349 (National Analysis Center for Iron and Steel, Beijing, China) and CRM GBW07603 (National Research Centre for CRM, Beijing, China). The ash content of freeze-dried yeast biomass was determined according to the technical report NREL/TP-510-42622 from the National Renewable Energy Laboratory (24).

Fourier transform infrared (FTIR) spectroscopy

FTIR analysis of freeze-dried yeast biomass was performed with an Agilent 5500 Series FTIR Spectrometer (Agilent, Santa Clara, US) using a single-bounce type IIA diamond crystal attenuated total reflectance (ATR) accessory with sample press. Approximately 10 mg freeze-dried yeast samples were measured in the spectral range of 4000-650 cm^{-1} with a resolution of 8 cm^{-1} and 32 scans. Background spectra (empty crystal) were measured before each sample and used for correction. The diamond crystal of the ATR (Attenuated Total Reflection) accessory was cleaned with 70 % isopropanol and distilled water after each measurement. The obtained raw spectra were subjected to EMSC (Extended Multiplicative Signal Correction) (25). Processing of the spectra was performed with The Unscramble X 10.5 (CAMO Software, Oslo, Norway).

Statistical Analysis

The growth experiments in the Duetz-MTPS and in the 2.5 L fermenters were carried out in triplicate and duplicate, respectively. The presented results are the mean of the replicates, and the standard deviations are shown as error bars in the figures. Data handling and statistics were performed using the Excel software package (Microsoft Excel 2013, Microsoft Corp., Redmond, WA). ANOVA ($\alpha = 0.05$) was used to analyze the differences in amino acid compositions, using JPM v.14.1 (SAS, Cary, North Carolina, U.S.) and comparing all pairs using Tukey-Kramer HSD (Honest Significant Difference). The Principal Component Analysis (PCA) was performed using The Unscrambler X, V10.5 (CAMO, Oslo, Norway).

Results and Discussion

Characterization of BALI^{TM} and chicken hydrolysate and selection of yeasts

Table S1 shows that glucose is the main carbon source in the spruce derived-hydrolysate (BALI^{TM,} also abbreviated as B in this study). The production and composition of the chicken hydrolysate (CH), prepared using a commercial protease, have been described previously (20). The protein content of CH, based on the Kjeldahl method, was 65.7 g/L. In the growth experiments described below carbohydrates were dosed based on glucose, whereas the nitrogen source was dosed based on nitrogen content as determined by the Kjeldahl method.

The tested yeast strains were chosen because of their high potential for biotechnological and especially food-related applications. The Thermosacc® Dry-strain is used for industrial ethanol production and after fermentation it is a component in distillers' grain, which is used as animal feed. *C. jadinii* is known as fodder yeast (usually under its anamorph name *Candida utilis*) and can convert a variety of substrates to high- value biomass (26). *W. anomalus* is a very robust yeast. It can grow on a variety of different substrates, efficiently degrade phytate and inhibit undesirable microbes, and it has been demonstrated to improve the nutritional value of animal feed (27,28). *B. adeninivorans* is a yeast with a very broad substrate spectrum, utilizing, apart from monosaccharides, also for instance aromatic

compounds, and degrading phytate. It is also osmo- and thermotolerant and therefore promising for industrial applications (29).

Growth experiments in microtiter plates

A preliminary screening of media was performed using four different yeasts, *C. jadinii*, *W. anomalus*, *B. adeninivorans* and Thermosacc® Dry, and the Duetz-MTPS system (Enzyscreen, Heemstede, The Netherlands). Measurements of CDW and pH were performed at 0, 8, 16, and 24 h (Figures S1, S2) and Fig. 1 shows CDW levels after 24 h. Generally, growth on inorganic nitrogen sources (yeast nitrogen base with ammonium sulfate or with urea) was lower compared to organic nitrogen sources (yeast extract + meat peptone or chicken by-products hydrolysate). In addition, when using inorganic N-sources, the yeasts apparently performed better on BALI sugar than on glucose in several cases. These differences are likely related to differences in buffering capacity of both the nitrogen and the sugar source, as medium acidification occurred rapidly and was more pronounced for media showing low growth (Figure S2). Importantly, Fig. 1 shows that the chicken by-product hydrolysate (CH) functions approximately as well as the commonly used rich yeast medium YP. When using these rich, well-buffering nitrogen sources, the yeasts performed equally well on glucose (G) and BALI sugar (B) in most cases, but some conspicuous differences were observed. As to the effect of replacing G by B in an otherwise rich medium, results for *C. jadinii* did not provide a consistent picture, whereas the data for Thermosacc® Dry showed a negative impact of B, which could indicate sensitivity of this latter yeast for a compound in B. Of interest, Fig. 1 shows very low growth of *B. adeninivorans* on the urea containing medium, indicating that this yeast lacks the enzyme apparatus for urea assimilation.

We considered *B. adeninivorans* interesting as it can use a large variety of substrates as a carbon and nitrogen source (29,30). These nitrogen sources include purines, and which are abundant in chicken by-products (31). Indeed, Figure 1 shows that the by far highest CDW values were reached by *B. adeninivorans* (33-39 g/L). This translates to cell mass-yields on glucose that are considerably higher than 50 %, which in this case would be equal to 25 g/L CDW and which is generally considered as a maximum for oxidative growth on sugar (15,32). *C. jadinii* and *W. anomalus* reached lower CDW values (between 18 - 28 g/L and 16-20 g/L respectively) on the same media, showing less efficient utilization of YP or CH. Thermosacc® Dry reached 18-20 g/L CDW on YP+G or CH+G, but its growth was lower when using BALI™ as a sugar source (10-14 g/L). In conclusion, these initial growth experiments

demonstrated that the combination of the chicken hydrolysate and the BALI™ spruce hydrolysate constitutes a promising growth medium for multiple yeasts.
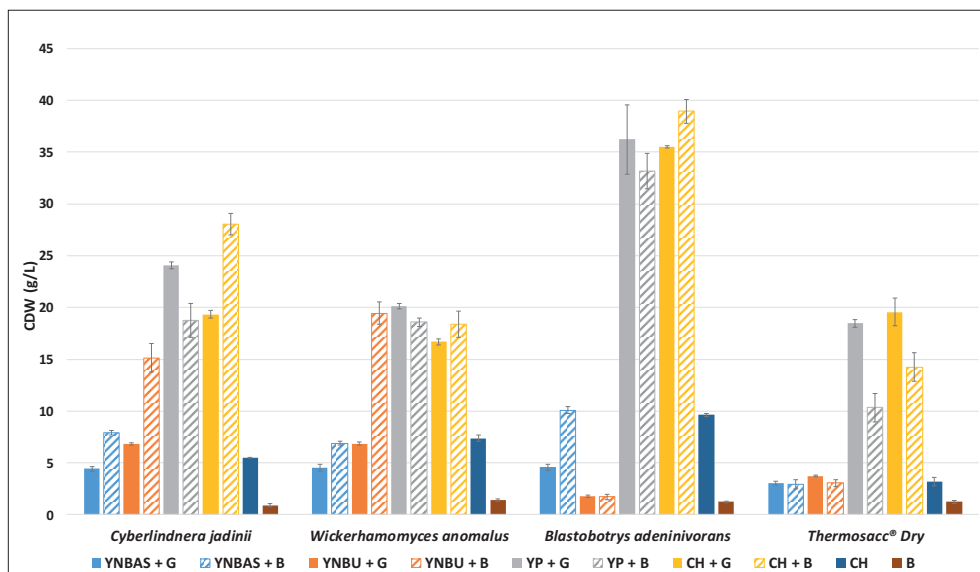

Figure 1. Growth of four yeast strains in the Duetz-MTPS system using 10 different media. The graphs show CDW (g/L) after 24h cultivation (values are means ± SD; n = 3), whereas Figs. S1 & S2 provide more complete growth curves and pH profiles. Conditions: Glucose, 50 g/L; Kjeldahl nitrogen, 5.86 g/L; $OD_{initial}$ = 0.5; volume: 2.5 mL; $pH_{initial}$ = 5.0; incubation at 30°C with 450 rpm shaking. The pH and pO_2 were not controlled and several of the apparent differences between media may be due to buffering effects.

Bioreactor runs in CH+B and YP+G media

Based on the initial experiments described above we carried out a comparative assessment of growth on a rich standard medium (YP+G) and a rich medium derived from spruce and chicken by-products (CH+B), using fully controlled (pH and pO_2) benchtop bioreactors. Figure 2 and Table 1 show growth and protein production for the four yeast strains. Table 1 also summarizes yields per gram of medium component, whereas Figure 2 also shows glucose consumption

and ethanol levels. Figure S3 shows the consumption of free amino nitrogen. Of note, the spruce hydrolysate, B, contains sugars in addition to glucose (Table S1) and would a priori be expected to enable higher biomass yields, provided that nitrogen was not limiting and depending on the ability of the yeast strains to ferment sugars other than glucose.

C. jadinii (Figure 2a) had a longer lag phase on CH+B than on YP+G, but, like all other yeasts (Fig. 2), gave a higher biomass production on the hydrolysate-based medium: 30.6 vs. 24.1 g/L CDW after 20 h (Table 1). The growth continued after glucose depletion, likely due in part to the aerobic utilization of accumulated ethanol (diauxic shift (15)). Maximal ethanol levels reached 9.7 and 15.4 g/L on CH+B and YP+G, respectively, after about 12 h-14 h. The protein content of the yeast biomass was above 50 % (w/w) throughout the cultivation (measured from 10 h-24 h) and was, at the later time points, higher for the CH+B fermentation, compared to the YP+G fermentation (e.g. 57.4 % versus 53.3 % at 20 h; Table 1).

W. anomalus growth and glucose consumption profiles for both media were very similar until the point of glucose depletion (at 12 h), whereas also in this case maximum biomass yields were higher with CH+B compared to YP+G: 36.1 and 28.0 g/L CDW after 20 h (Figure 2b, Table 1). Only minor ethanol accumulation was observed with *W. anomalus* (max. 5.6 g/L). The protein content of the yeast biomass was relatively stable over time and was higher on YP+G than on CH+B (e.g. 55.2 % versus 50.5 % after 20 hours; Table 1).

B. adeninivorans consumed glucose at similar rates for both media, with negligible ethanol production (less than 1.5 g/L) (Figure 2c). Final biomass levels were considerably higher for CH+B and the yields were remarkably high: 39.8 g/L on YP+G and 54.5 g/L on CH+B, after 20 and 18 hours, respectively (Table 1). These high yields suggest that this yeast is capable of using compounds present in the nitrogen source (YP or CH) and the BALI sugar, which the other yeast strains cannot use. The protein content of yeast biomass was around 50 % (w/w) on both media.

Thermosacc® Dry consumed the supplied glucose within 8 h on both media, resulting in undesirable high ethanol concentrations of up to 27 g/L (Figure 1d). Thermosacc® Dry is a *Saccharomyces cerevisiae* strain specifically developed for ethanol production. Therefore, this strain was expected to be less suitable for SCP production, as was indeed confirmed by the results shown in Figure 2. Similarly to *C. jadinii* on YP+G, the diauxic nature of the growth curve is visible for both media: after glucose depletion the accumulated ethanol was consumed during the subsequent 12 h-14 h, resulting in CDW concentrations of 27.1 g/L and 22.5 g/L for CH+B and YP+G, respectively (Table 1). Towards the end of the glucose consumption phase, the protein content for yeast growing on YP+G was very high

(62.7 %, w/w) but this level was decreased to 47.7 % (w/w) after 22 h. For yeast grown on CH+B, the protein content was rather stable, reaching 54.1 % (w/w) at 20 h.

Whereas these experiments reveal clear differences between the yeasts, some general trends are visible, which seem to apply to all tested yeasts. While BALI sugars may be considered as a drop-in replacement for other glucose sources, such as starch-derived glucose, the BALI sugar comes with the potential benefit of containing an additional 16 g of sugar per 50 g of glucose (Table S1). Likely as a consequence of this, all yeasts gave higher CDW yields in the CH+B fermentations. Indeed, analysis of the contents of some common monosugars (galactose, arabinose, xylose and mannose) showed that these were largely consumed by the yeasts (Table S2). The protein content of the yeast cells was 50 % or higher in all but one case. The strain, growth medium and growing conditions all may have impact on the content of crude protein. The values obtained here are within protein levels that are considered reasonable in the context of SCP production; protein contents in yeasts normally vary between 45-55 % (2,9,33) .

The maximum yields obtained for yeasts growing on carbohydrates ($Y_{x/glucose}$) under aerobic conditions typically range between 0.4 and 0.5 g biomass per g of sugar (15). Similar trends can be observed in our study for all yeasts with the exception of *B. adeninivorans* which showed $Y_{x/glucose}$ values of 0.76 and 1.15 g/g for YP+G and CH+B, respectively. Correcting the yield on CH+B for the additional sugars in B, still leaves a yield ($Y_{x/sugars}$) of 0.87 g per g of sugar. These high yields confirm the results from the microtiter plate experiments and are likely due to the ability of this yeast to utilize a wide variety of substrates as a carbon and nitrogen source (30). *C. jadinii* and Thermosacc® Dry had the lowest $Y_{x/sugars}$ values when using CH+B as a medium (0.49 g/g and 0.42 g/g, respectively) and these values were very similar to the $Y_{x/glucose}$ values obtained when using YP+G (0.46 g/g and 0.42 g/g, respectively). $Y_{x/sugars}$ and $Y_{x/glucose}$ values for *W. anomalus* grown on CH+B and YP+G were 0.58 g/g and 0.52 g/g respectively, suggesting that this yeast utilized some other compounds next to sugars, albeit not to the same extent as *B. adeninivorans*. Utilization of amino acids both as nitrogen- and carbon sources has been described for a variety of yeast species (34), and is especially efficient in *B. adeninivorans*. This may explain different biomass yields on CH+B.

Protein yields ranged from 0.2 g to 0.29 g per g of glucose (YP+G) or total sugar (CH+B), with the exception of the *B. adeninivorans* fermentations which yielded approximately 0.4 g protein per g of sugar (Table 1). These yields are similar to those described in the literature. Lee et al., (35) achieved a high biomass yield of 0.67 g per g of glucose for batch fermentations with *C.jadinii,* which, assuming a 50 % protein content, corresponds to a respectable protein

yield of some 0.33 g/g. Also using batch fermentation of *C.jadinii,* Nigam et al., (36) produced SCP from pineapple cannery effluent and obtained a maximum cell biomass and total protein yield of 0.30 and 0.17 g per g of sugar. The high protein yields obtained with *B. adeninivorans* can likely be explained in part by the abovementioned ability of this yeast to assimilate a wide range of nitrogen-containing carbon sources (proteins, purines etc.). The enhanced growth of this yeast is also reflected in the consumption of free amino nitrogen that was indeed higher for *B. adeninivorans* compared to the other yeast strains (Figure S3).

In summary, the data shown in Figure 2 and Table 1 demonstrate that CH+B is a promising fermentation medium for production of yeast-based single cell protein production. Compared to YP+G, this medium yielded higher CDW and gave higher protein yields, both in terms of total amounts and the amount of protein per amount of sugar (Table 1). CH has a higher free amino nitrogen content (Figure S3), and presumably a higher purine content than YP. The better performance of the CH+B medium is of course partly due to the additional sugars in B, compared to G, as discussed above. *B. adeninivorans* is especially interesting, due to its very high biomass yields.
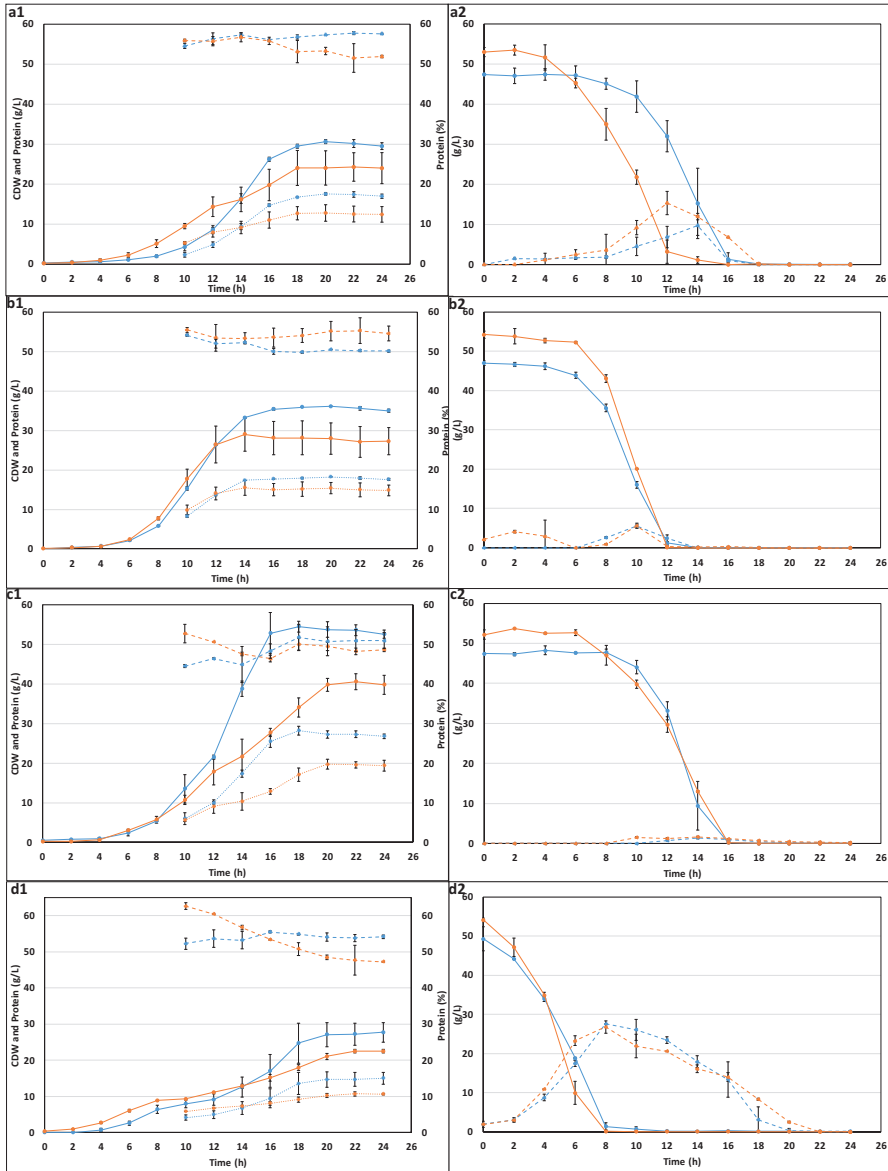
Figure 2. Batch cultivation of four yeast strains on YP+G (orange curves) or CH+B (blue curves) in a 2.5 L benchtop fermenter. The starting volume was 1.5 L and the fermentation lasted 24 h. Panels

labeled 1 show accumulation of cells (solid lines) and protein (dotted lines), as well as the protein content of the cells (dashed lines); panels labeled 2 show glucose (solid lines) and ethanol (striped lines). a, *C. jadinii*; b, *W. anomalus*; c, *B. adeninivorans*; d, Thermosacc® Dry. Growth was monitored by measuring the CDW (g/L, solid lines) every 2 h. For the samples taken from 10 h and onwards, the protein content (striped lines) of the dried cells was measured using the Kjeldahl method (w/w %). The concentration of yeast protein (g/L; dotted lines) was calculated by multiplying CDW (g/L; solid lines) with the protein content (w/w %). Acetic acid and lactic acid production were negligible for all yeasts on both media (results not shown). Values are means ± SD (n = 2).

Table 1. Data for growth of *C. jadinii*, *W. anomalus*, *B. adeninivorans* and Thermosacc® Dry grown in 2.5 L benchtop fermenters. The media used were YP+G or CH+B, and the start volume of the fermentation was 1.5 L. The media contained 5.86 g/L Kjeldahl nitrogen (36.6 g/L protein) and approximately 50 g/L glucose (see t=0 point in Fig2, right panels; note that for B, 50 g/L glucose corresponds to 66.0 g/L total sugars; see Table S1). The data shown are for the time point (indicated in the Table) at which the concentration of yeast protein (g/L) was the highest. Data for 24 h time points appear in Table 2. Y values refer to yields of CDW (X) or protein (P) per gram of consumed glucose, as measured (right panels in Fig. 2) or per gram of total sugar, as calculated from Table S1 (for B only). Values are means ± SD (n = 2).

	C. jadinii		*W. anomalus*		*B. adeninivorans*		Thermosacc® Dry	
Medium	YP+G	CH+B	YP+G	CH+B	YP+G	CH+B	YP+G	CH+B
Time (h)	20	20	20	20	20	18	22	20
CDW (g/L)	24.1 ± 4.3	30.6 ± 0.5	28.0 ± 3.9	36.1 ± 0.1	39.8 ± 1.6	54.5 ± 1.4	22.5 ± 0.5	27.1 ± 3.4
Max. growth rate (h^{-1})	0.4 ± 0.1	0.3 ± 0.0	0.6 ± 0.1	0.4 ± 0.0	0.6 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	0.2 ± 0.1
Protein (%)	53.3 ± 0.9	57.4 ± 0.1	55.2 ± 2.5	50.5 ± 0.0	49.5 ± 0.0	51.8 ± 3.3	47.7 ± 4.1	54.1 ± 1.2
Protein (g/L)	12.8 ± 2.1	17.6 ± 0.3	15.4 ± 1.5	18.2 ± 0.0	19.7 ± 1.2	28.2 ± 1.1	10.7 ± 0.6	14.7 ± 2.1
$Y_{X/sugars}$		0.49 ± 0.01		0.58 ± 0.01		0.87 ± 0.02		0.42 ± 0.08
$Y_{P/sugars}$		0.28 ± 0.00		0.29 ± 0.00		0.45 ± 0.02		0.23 ± 0.05
$Y_{X/glucose}$	0.46 ± 0.09	0.65 ± 0.01	0.52 ± 0.06	0.77 ± 0.01	0.76 ± 0.05	1.15 ± 0.03	0.42 ± 0.01	0.55 ± 0.10
$Y_{P/glucose}$	0.24 ± 0.04	0.37 ± 0.01	0.28 ± 0.02	0.39 ± 0.00	0.38 ± 0.03	0.60 ± 0.02	0.20 ± 0.01	0.30 ± 0.06

3.4. Characterization of yeast biomass

The chemical composition of freeze-dried yeast biomass obtained from the bioreactor cultivations after 24 h of fermentation was determined. Table 2 shows the content of protein, nucleic acids, lipids, carbohydrates and ash. The lipid content of the yeasts (0.4-1.8 %) was lower than what is typically found in literature (2-6 %) (33), and it was somewhat higher for yeasts grown on CH+B. Lipid accumulation is generally known to be induced by nitrogen starvation (37). Figure S3 shows that nitrogen was available during the whole fermentation, in all fermentations, which may explain the low levels of lipids. The contents of nucleic acids (2.5 - 5.8 %; Table 2) were also low compared to previous studies (5 - 12 %) (15,33). However, in the present experiment, nucleic acid analyses were performed on yeast biomasses obtained after 24 h of batch cultivation. At this point, the yeast cells were probably in a stationary phase (i.e., stable CDW for the last 4 to 10 hours before harvesting of cells; Figure 2). It has previously been observed that microbial cells in stationary phase have a low concentration of nucleic acids, primarily due to a reduction of the RNA content (38).

Table 3 provides an overview of selected minerals in the yeast biomass. The total amount of minerals was slightly higher for yeast grown on CH+B, especially for Thermosacc® Dry. *W. anomalus* contained the lowest amount of minerals. The most abundant macro elements were potassium, phosphorus, sulfur, and sodium, while most micro elements were found in very low concentrations.

Table 4 presents the amino acid composition of the yeast cells at the time points (h) where of the protein concentration (g/L) reached its highest levels during the batch fermentation. Generally, the amino acid compositions depicted in Table 4 are similar to previously published amino acid compositions of yeast, including characteristic high contents of threonine and lysine and low contents of S-containing amino acids such as methionine and cysteine (10).

Since yeast potentially may be used as an ingredient in fish feed (39), we compared the amino acid composition of the four yeast strains with the amino acid compositions of fish- and soybean meals. The measured sums of amino acids varied between 393.6 and 475.2 g/kg dry matter for the four yeast strains (Table 4). The total amino acid contents of a standard fish meal and soybean meal were determined to be 526.4 and 497.8 g/kg dry matter, respectively (Table 4). These latter values are slightly higher than those observed for the yeasts, but in some cases, the difference is small: *C. jadinii* on CH+B gave 475.2 g/kg, versus 497.8 g/kg for soybean meal. The total amounts

of amino acids were slightly higher when using CH+B as a medium, except for *W. anomalus,* which is in accordance with the Kjeldahl-based protein concentrations (Table 2).

A PCA analysis (Figure S4) showed that the fermentation medium only slightly affected the amino acid composition of the four yeast strains. The PCA plot also shows that the amino acid compositions of *C. jadinii* and *W. anomalus* are most similar to the composition of fish meal. Table 4 shows the results of ANOVA analysis done to detect differences at the individual amino acid level between the yeasts and the two reference protein sources. Among other things, the Table shows that the differences between fish and soybean meal primarily concern Met, Lys, Gly, Ala and Phe.

Table 2. Proximate composition of yeasts grown on YP+G or CH+B after 24 h batch fermentation. The Table shows mean values derived from duplicate fermentations ± standard deviation. Prior to the analysis, yeast cells were washed and freeze-dried. Values are means ± SD (n = 2).

	C. jadinii		*W. anomalus*		*B. adeninivorans*		Thermosacc® Dry	
	YP+G	CH+B	YP+G	CH+B	YP+G	CH+B	YP+G	CH+B
Crude protein[a]	51.9 ± 0.3	57.6 ± 0.2	54.6 ± 1.9	50.2 ± 0.3	48.7 ± 0.5	51.0 ± 2.0	47.3 ± 0.1	54.2 ± 0.5
Nucleic acids	4.4 ± 0.1	4.9 ± 0.2	5.8 ± 0.0	4.1 ± 0.4	2.5 ± 0.2	2.8 ± 0.1	3.1 ± 0.4	2.6 ± 0.0
Crude lipid	0.5 ± 0.2	1.1 ± 0.5	ND	1.2 ± 0.0	0.2 ± 0.1	1.2 ± 0.4	0.4 ± 0.2	1.8 ± 0.1
Est Total Carbohydrates[b]	42.3	35.1	41.2	43.5	45.6	42.3	48.2	37.7
Ash	5.3 ± 0.3	6.2 ± 0.6	4.2 ± 0.4	5.1 ± 0.1	5.5 ± 0.2	5.5 ± 0.5	4.1 ± 0.3	6.3 ± 0.0

[a] The protein content equals N x 6.25, which means that non-protein nitrogen is included

[b] Est Total Carbohydrates = 100 – Crude Protein – Crude lipid – Ash. Nucleic acids are not included here since these are also covered by Kjeldahl nitrogen.

Table 3. Macro and trace minerals determined by ICP – MS in freeze dried yeast biomass. Yeasts were grown on YP+G and CH+B media and harvested after 24 h. Values are means ± SD (n = 2). ND, not detected. No detectable levels of As, Cd, Pb and Br were found in any of the yeast samples.

	C. jadinii		*W. anomalus*		*B. adeninivorans*		Thermosacc® Dry	
Medium	YP + G	CH + B	YP + G	CH + B	YP + G	CH + B	YP + G	CH + B
Macro Minerals (g/kg dry matter)								
Na	1.5 ± 0.1	3.0 ± 0.6	1.1 ± 0.2	1.7 ± 0.8	4.8 ± 0.5	8.8 ± 1.6	1.5 ± 0.4	1.5 ± 0.2
Mg	0.8 ± 0.0	1.0 ± 0.2	0.5 ± 0.0	0.9 ± 0.0	0.6 ± 0.0	1.0 ± 0.0	0.7 ± 0.0	1.1 ± 0.0
P	11.5 ± 0.7	13.0 ± 2.8	7.5 ± 1.0	10.6 ± 0.5	8.2 ± 0.7	8.9 ± 0.6	6.0 ± 0.0	13.0 ± 0.0
S	4.6 ± 0.6	6.4 ± 1.6	3.2 ± 0.4	4.9 ± 1.9	5.4 ± 0.0	8.9 ± 0.4	3.9 ± 0.2	7.6 ± 0.9
K	17.5 ± 0.7	13.5 ± 3.5	11.5 ± 0.7	7.6 ± 3.3	18.0 ± 1.4	11.5 ± 0.7	15.5 ± 0.7	17.0 ± 0.0
Ca	0.1 ± 0.0	0.6 ± 0.1	ND	0.3 ± 0.0	ND	0.7 ± 0.0	ND	3.5 ± 0.4
Cl	0.1 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	2.4 ± 0.0	3.9 ± 0.0	0.1 ± 0.1	0.6 ± 0.0
Trace Minerals (mg/kg dry matter)								
Cr	1.7 ± 0.1	0.8 ± 0.3	2.3 ± 0.5	0.5 ± 0.0	3.1 ± 0.4	0.7 ± 0.2	5.5 ± 0.7	0.9 ± 0.4
Mn	1.8 ± 0.0	14 ± 0.0	1.3 ± 0.0	11.6 ± 0.0	1.4 ± 0.0	15 ± 0.0	2.0 ± 0.0	10.8 ± 0.0
Co	0.5 ± 0.0	0.1 ± 0.0	0.4 ± 0.0	ND	0.5 ± 0.0	ND	0.9 ± 0.0	ND
Cu	3.6 ± 2.9	15.5 ± 3.5	9.8 ± 1.7	9.0 ± 0.3	8.8 ± 1.7	1.7 ± 0.8	14.5 ± 3.5	0.9 ± 0.1
Se	ND	0.6 ± 0.0	ND	0.6 ± 0.0	ND	0.7 ± 0.0	ND	0.6 ± 0.0
Al	3.6 ± 1.3	4.1 ± 2.6	3.0 ± 0.4	3.3 ± 0.6	3.7 ± 0.2	4.1 ± 1.1	2.7 ± 0.7	4.3 ± 0.8
Fe	44.0 ± 1.4	255 ± 7.1	34.5 ± 7.8	55.8 ± 2.6	38.5 ± 2.1	160.0 ± 14.1	53.5 ± 6.4	75.0 ± 5.7
Ni	0.8 ± 0.2	0.5 ± 0.3	1.0 ± 0.5	0.3 ± 0.1	1.6 ± 0.4	0.6 ± 0.3	2.6 ± 0.8	0.5 ± 0.5
Zn	105 ± 7.1	165 ± 21.2	71 ± 8.5	146.7 ± 23.6	70.5 ± 2.1	105.0 ± 7.1	91 ± 1.4	150 ± 14.1
Total Elements (g/kg dry matter)	37.3	38.8	25.5	27.4	41.3	44.8	30.7	45.3

Table 4. Amino acid composition of yeast biomass obtained after fermentation on YP+G or CH+B medium. Values are means ± SD (n = 2). ANOVA analyses were performed for each yeast grown on YP+G or CH+B, including a comparison of means for all pairs using Tukey-Kramer HSD. Amino acids levels that differ significantly ($\alpha = 0.05$) from levels in fish meal or soybean meal are marked by * and °, respectively. EAAs, essential amino acids; NEAAs, non-essential amino acids.

		C.jadinii		*W. anomalus*		*B. adeninivorans*		Thermosacc dry®		Fish meal [c]	Soybean meal [d]
Medium		YP+G	CH+B	YP+G	CH+B	YP+G	CH+B	YP+G	CH+B		
Time (h)[b]		20	20	20	20	20	18	22	20		
EAAs[a]	Met, M	5.1 ± 0.2 *°	6.2 ± 0.1 *°	3.9 ± 0.1 *°	4.6 ± 0.0 *°	5.1 ± 0.0 *°	5.6 ± 0.2 *°	6.0 ± 0.1 *°	7.0 ± 0.3 *	16.1 °	7.7 *
	Thr, T	24.5 ± 0.8	26.3 ± 0.3	21.8 ± 0.8	21.4 ± 0.1	21.3 ± 0.1	23.8 ± 3.0	21.5 ± 0.2	25.1 ± 0.9	25.4	20.2
	Val, V	25.0 ± 0.0	29.0 ± 0.3	22.9 ± 0.6 *	24.7 ± 0.3	20.5 ± 0.2 *°	21.3 ± 0.8 *°	25.2 ± 0.6	25.0 ± 0.4	26.4	24.1
	Ile, I	21.5 ± 0.3	24.6 ± 0.4	20.6 ± 0.9 *	21.4 ± 0.0	16.1 ± 0.1 *°	17.1 ± 0.6 *°	19.3 ± 0.5 *°	23.1 ± 0.2	23.7	23.1
	Leu, L	32.5 ± 0.8 *°	38.2 ± 0.6	31 ± 1.4 *°	32.7 ± 0.2 *°	33.1 ± 0.3 *°	33.9 ± 1.3 *°	29.1 ± 0.4 *°	35.3 ± 0.3 *	42	39
	His, H	9.2 ± 0 *°	11.5 ± 0.4	9.2 ± 0.1 *°	13.6 ± 0.8	8.5 ± 0.3 *°	13.5 ± 0.3	8.8 ± 0.1 *°	10.2 ± 0.2 *°	11.8	13.5
	Lys, K	42.2 ± 1.8 °	39.5 ± 0.5 *°	42.5 ± 0.4	43.1 ± 0	26.8 ± 0.9 *°	31.1 ± 0.7 *	32.4 ± 0.7 *	36.8 ± 0.1 *°	45.5 °	32.3 *
	Arg, R	31.9 ± 1.7	28.5 ± 0.0	34.6 ± 2.6	20.8 ± 0.2 *°	21.0 ± 0.3 *°	23.0 ± 0.9 *°	24.7 ± 0.4 *°	23.6 ± 3.6 *°	35.3	37.4
	Phe, F	18.1 ± 0.5 *°	21.5 ± 0.4 °	17.4 ± 0.8 *°	19.4 ± 0.5 °	14.0 ± 0.2 *°	17.3 ± 0.6 *°	16.6 ± 0.3 *°	18.9 ± 0 *°	22.0 °	26.5 *
	Trp, W	6.2 ± 0.1	7.1 ± 0.0	5.8 ± 0.3 *	5.8 ± 0.1 *	4.9 ± 0.0 *	6.3 ± 0.1 *°	5.2 ± 0.0 *	6.1 ± 0.1 *	6.9	6.8
NEAAs[a]	Asp, D	44.3 ± 1.9 *	46.7 ± 0.6 *°	48.4 ± 0.2	42.2 ± 0.7 *°	36.8 ± 0.5 *°	40 ± 4.9 *°	42.1 ± 0.9 *°	43.8 ± 1.2 *°	54.7	59.5
	Ser, S	23.7 ± 1.1	24.5 ± 0.2	26.7 ± 0.0	23.5 ± 0.0	22.6 ± 0.2 °	21.3 ± 1.1 *°	21.6 ± 0.1 *°	22.7 ± 0.0	25.3	25.8
	Glu, E	72.1 ± 0.8	76.1 ± 1.8	68.6 ± 0.7 °	64.3 ± 0.9 °	69.1 ± 3.7 °	74.6 ± 10.0	73.5 ± 2.4	73.8 ± 3.1	83.9	92.1
	Pro, P	22.0 ± 1.7	18.4 ± 0.6	23 ± 0.4	17.1 ± 0.9	29.2 ± 0.6	22.7 ± 3.7	17.7 ± 0.2	19.8 ± 1.6	23.1	24.1
	Gly, G	30.2 ± 3.2	23.4 ± 0.6 *	29.1 ± 1.0 °	20.3 ± 0.0 *	24.2 ± 0.7	19.1 ± 1.3 *	21.2 ± 0.6 *	21.3 ± 0.2 *	30.8	21.6 *
	Tyr, Y	14.6 ± 0.8	18.2 ± 1.2 °	15.0 ± 0.4	17.7 ± 0.6	10.9 ± 0.2 *°	12.9 ± 0.6	12.7 ± 0.2	15.5 ± 0.1	15.2	14.7
	Cys, C	6.2 ± 1.0	5.4 ± 0.2	3.3 ± 0.1 *°	4.0 ± 0.0 *	3.2 ± 0 *°	3.5 ± 0.2 *°	4.1 ± 0.0 °	4.3 ± 0.4 °	5.7	6.9
	Ala, A	28.5 ± 0.4 *°	29.0 ± 0.3 °	25.3 ± 0.4 *	23.6 ± 0.3 *	25.2 ± 0.4 *	26.2 ± 1.8 *	25.6 ± 0.9 *	27.8 ± 0.2 *°	32.6 °	22.4 *
	SUM AA	458.7	475.2	450	421	393.6	414.1	408	440.9	526.4	497.8

[a] All values are in g/kg of dry matter.

[b] Time point (h) during batch fermentations where the yeast protein concentration (g/L) was at its maximum.

FTIR spectra of freeze-dried yeast cells showed expected features and were similar for all four yeasts, independent of the medium used (Fig. 3 & S5). Fig. 4 shows spectra for *C. jadinii*, grown on YP+G or CH+B, and sampled at 6 h and 24 h; spectra for the other yeasts are provided in Fig. S5. The spectra are dominated by N-H, C=O, C-N, C-C stretching and N-H bending vibrations from the amide groups of proteins (3280-3225, 1640, 1580-1510, 1350-1200 cm^{-1}) and by C-O, C-C, C-O-H and C-O-C stretching and deformation vibrations from carbohydrates (900-1200 cm^{-1}). Minor contributions of C-H and C=O stretching vibrations from lipids (3010-2850, 1740 cm^{-1}) and PO2- stretching vibrations from nucleic acids and phospholipids (1240 cm^{-1}) are also visible. There are no major differences between the two sampling time points (Fig. 4 & S5) except that the early samples (6 h) show a stronger lipid signal for fermentations on CH+B medium, which is probably due to the soluble lipids present in CH.
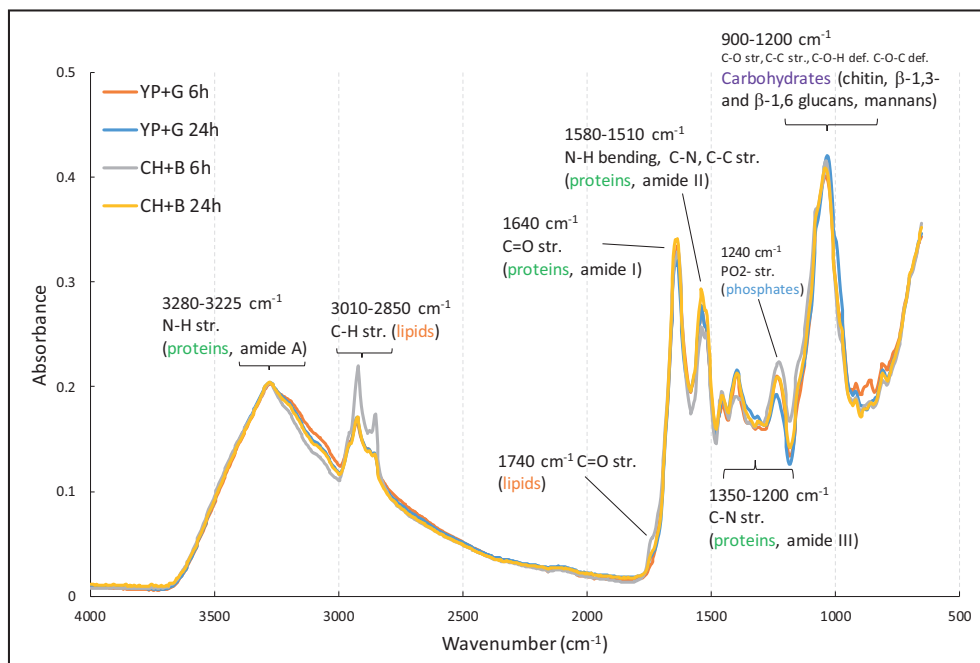
Figure 3. FTIR spectra of *C. jadinii* biomass obtained after 6 h or 24 h growth on YP+G or CH+B. The main vibration bands (and modes), with corresponding bio-macromolecular groups, are indicated.

Conclusions

In conclusion, this proof-of-concept study demonstrates a possible way of upgrading low value industrial side streams to yeast biomass that can be used as a high quality feed ingredient. Bioreactor experiments showed that yeast biomass and protein production values on BALI spruce hydrolysate + chicken by-products hydrolysate were similar or better than when using a traditional glucose + yeast extract medium. The protein content of the yeast biomass was high (around 50 w/w %), while the nucleic acid content was very low; these are both favorable features of SCP. Importantly, the amino acid profile of the yeasts were similar to those of fishmeal. *B. adeninivorans* is a promising candidate for single cell protein production due to its exceptional ability to utilize a wide range of compounds for growth without producing ethanol. While *C. jadinii* is well established as high-potential SCP with beneficial functional properties (43), less is known for *B. adeninivorans*. Further studies on the performance of *B. adeninivorans* as SCP in diets for animals and fish are needed, and considering the results presented here, of major interest.

Further research is needed to develop an economically viable yeast production process based on industrial side streams as substrates. The cost of the chicken by-product hydrolysates could be decreased by applying only endogenous enzymes for hydrolysis. It may also be possible to replace part of the presumably "rich" chicken by-product hydrolysates by cheap inorganic nitrogen sources. Repeated batch or continuous fermentation modes are known to be most suitable for microbial biomass production processes (15) and should be explored in future work. Downstream processing of the yeast biomass (drying steps, possible mechanical or autolytic lysis of the cells, formulation) also needs to be addressed since such processing will affect nutritional value. For example, the yeasts may be used as whole cells, or they may be subjected to a cell-wall destructing treatment that may increase digestibility. Other processing steps, such as removal of nucleic acids, could also be envisaged. However, for use in diets for salmon, this will probably not be necessary as the urolytic pathway in salmon can handle very high dietary levels of nucleic acids (44).

Importantly, besides providing protein, minerals and vitamins, yeasts in feed may have positive health effects as a result of the presence of bioactive and immunostimulating compounds such as ß-glucans and α-mannans (13). Therefore, further detailed compositional analysis of the yeast cell walls is of interest. Finally, fish feeding experiments should be carried out in order to assess the true potential of these yeast as a feed ingredient (39).

Abbreviations

SCP: Single Cell Protein, YNBAS: Yeast nitrogen base without amino acids with ammonium sulfate, YNBU: Yeast nitrogen base without amino acids with urea, YP: Yeast extract and meat peptone, CH: Chicken by-products hydrolysate, G: Glucose, B: BALI^{TM} spruce hydrolysate, CDW: Cell dry weight, HPLC: High-performance liquid chromatography, ICS: Ion chromatography system, ICP-MS: Inductively coupled plasma mass spectrometry, FTIR: Fourier-transform infrared spectroscopy, PCA: Principal component analysis, AA: Amino acid, EAAs: Essential amino acid, NEAA: Non-essential amino acid, ANOVA: Analysis of variance, MTPS: Microtiter plate system, $Y_{X/sugars}$: Yield, g dry yeast per g sugar fed (g/g), $Y_{P/sugars}$: Yield, g dry yeast protein per g sugar fed (g/g), $Y_{X/glucose}$: Yield, g dry yeast per g consumed glucose(g/g), $Y_{P/glucose}$: Yield, g dry yeast protein per g consumed glucose (g/g).

Declarations

Ethics approval and consent to participate: not applicable

Consent for publication: not applicable

Availability of data and material: All data generated or analyzed during this study are included in this published article (and its additional files).

Competing interests: The authors declare that they have no competing interests.

Author contributions:

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Conceived the research idea: DL, GK, SH, VE. Design of the work: DL, GK, VP, SH, VE. Methodology: DL, GK, SH, VE. Performed the experiments: DL, GK, LH, LM. Analyzed the data: DL, GK, LT, SH, VE. Discussed the results: all authors. Wrote the manuscript: DL, GK, VP, SH, VE. Discussed and revised the manuscript: all authors.

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128

129   **List of additional files:**

130   Supplementary Material (Figure S1-S5, Table S1-S2)

131

Production and characterization of yeasts grown on media composed of spruce-derived sugars and protein hydrolysates from chicken by-products

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Contents:

Figure S1. Cell dry weight (CDW) in microtiter plate experiments. Microtiter plate experiments were carried out to assess the growth of four yeast strains on 10 different media. Conditions: 24h cultivation in the Duetz-system with deepwell microtiter plates (2.5 mL/11mL) for 24h, initial pH: 5.0, shaking speed: 450 rpm. The CDW values are means ± SD (n = 3). The media contained 5.86 g/L Kjeldahl nitrogen (36.63 g/L protein) and 50 g/L glucose (note that for B, 50 g/L glucose corresponds to 66 g/L total sugars; see Table S1). Abbreviations: YNBAS, yeast nitrogen base without amino acids and with ammonium sulfate; YNBU, yeast nitrogen base without amino acids and with urea; YP, yeast extract and meat peptone; CH, chicken by-products hydrolysate; B, BALI™ hydrolysate; G, glucose.
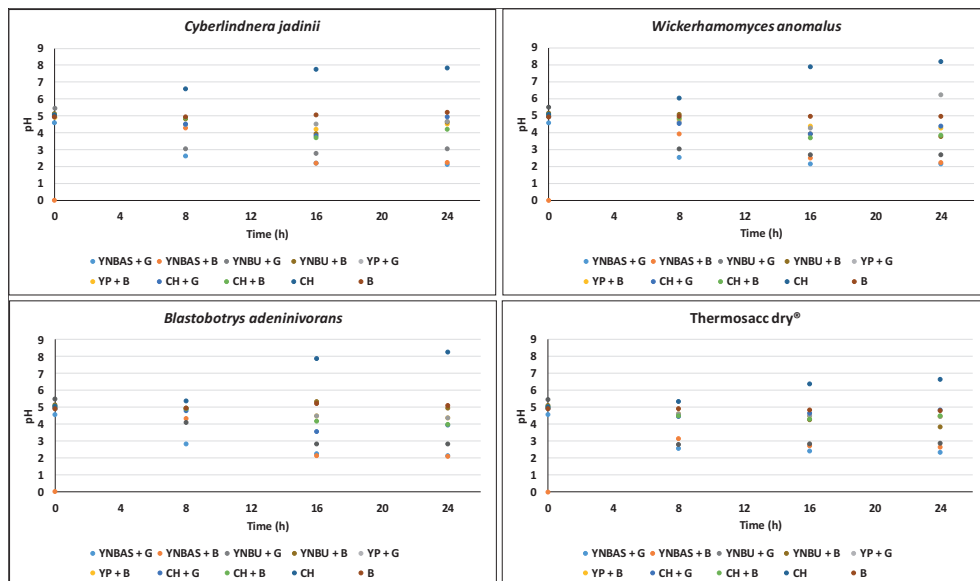
Figure S2. pH in microtiter plate growth experiments. The graphs show the pH during the growth experiments depicted in Figure S1. Values are means ± SD (n = 3). Abbreviations: YNBAS, yeast nitrogen base without amino acids and with ammonium sulfate; YNBU, yeast nitrogen base without amino acids and with urea; YP, yeast extract and meat peptone; CH, chicken by-products hydrolysate; B, BALI™ hydrolysate; G, glucose.
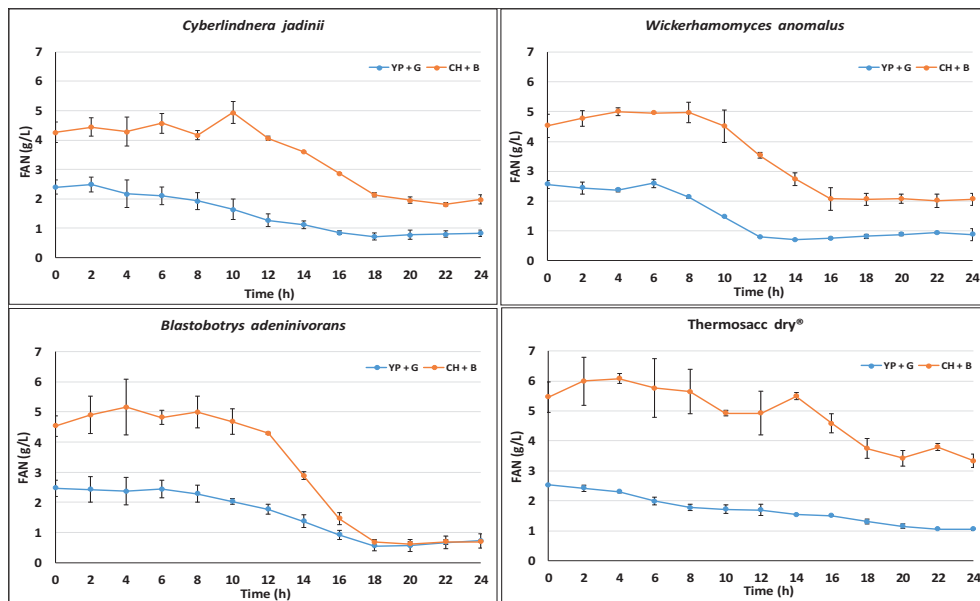
Figure S3. Free amino nitrogen (FAN) in 1.5 L batch fermentations. The graphs show the level of free amino nitrogen (g/L) during growth of four yeast strains in 2.5 L total volume benchtop bioreactors. Values are mean ± SD (n = 2). Abbreviations: YP, yeast extract and meat peptone; CH, chicken by-products hydrolysate; B, BALI™ hydrolysate; G, glucose.

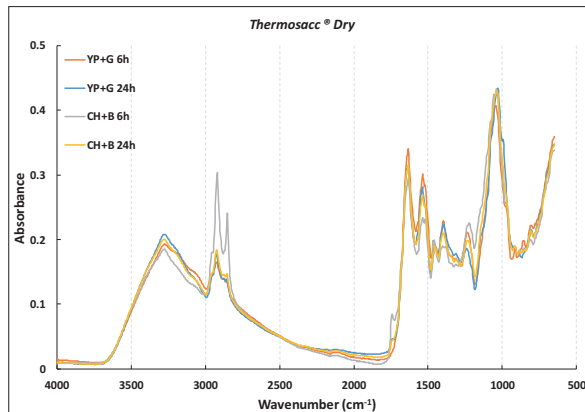**Figure S4.** Principal component analysis (PCA) of amino acid compositions. a) score plot and b) loading plot.

Wickerhamomyces anomalus

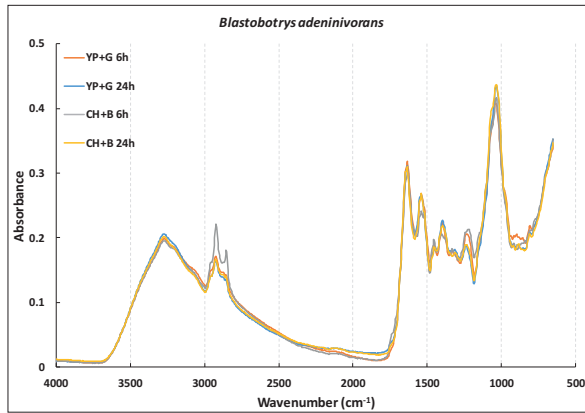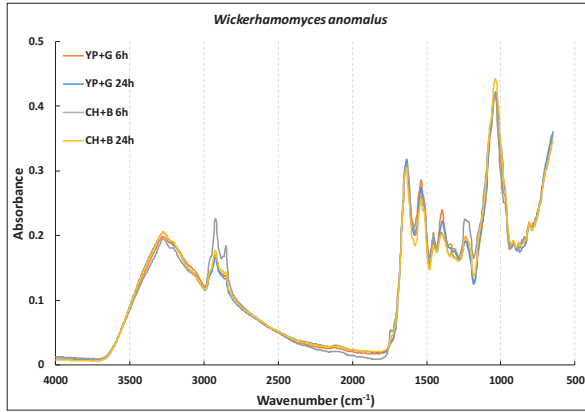*Blastobotrys adeninivorans*
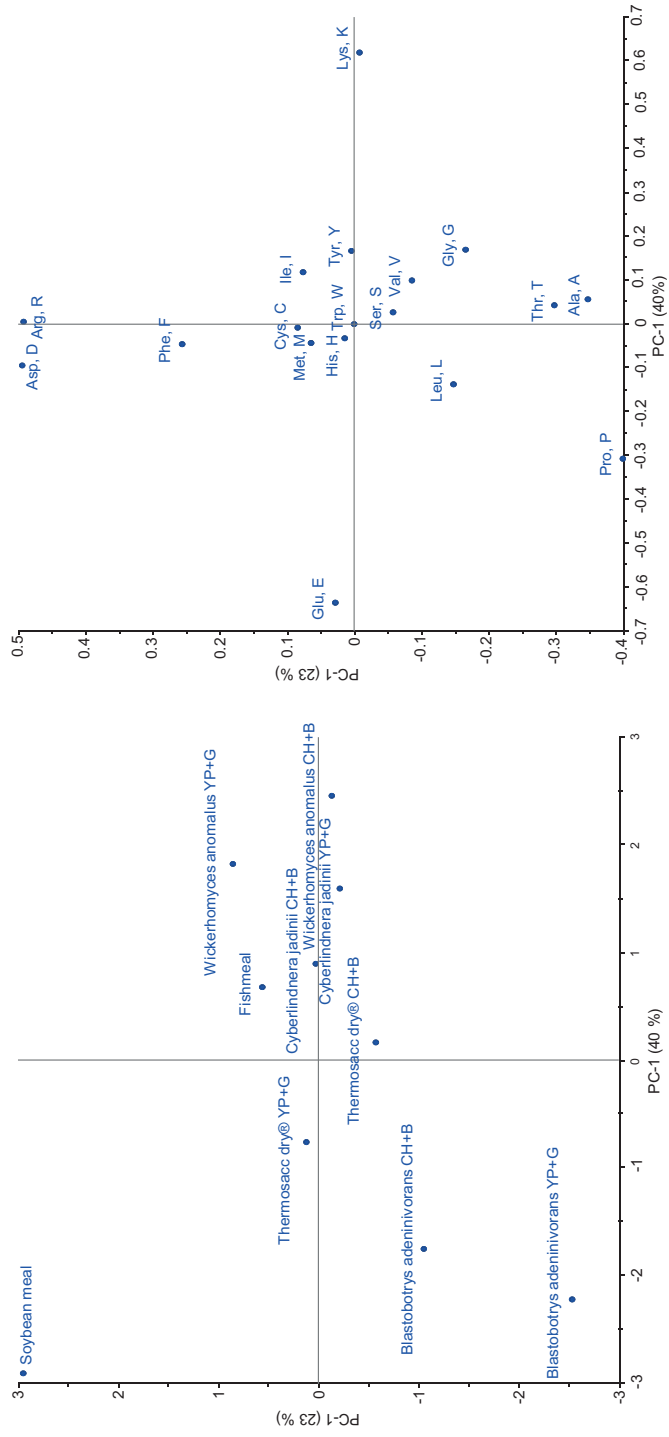
Thermosacc ® Dry

Figure S5. FTIR spectra of freeze-dried yeasts. The graphs show FTIR spectra of freeze-dried *W. anomalus*, *B. adeninivorans* and Thermosacc® Dry cells harvested after 6h and 24h cultivation in 2.5 L total volume benchtop bioreactors, using the indicated media. Spectra for C. *jadinii* are provided in the main manuscript. The obtained raw spectra were subjected to EMSC (Extended Multiplicative Signal Correction). Abbreviations: YP, yeast extract and meat peptone; CH, chicken by-products hydrolysate; B, BALI™ hydrolysate; G, glucose.

Table S1. Composition of the BALI™ spruce hydrolysate.

Content	BALI™
Dry matter (%, w/w)	62.3
Density (kg/L)	1.29
Total sugars (% DM)	90.0
Glucose	68.2
Xylose	5.4
Mannose	6.5
Other sugars[a]	9.9
Acids[b] (% DM)	1.2
Glycerol (% DM)	0.2
Lignin (% DM)	5.3

[a]Sum of fructose, arabinose, galactose, gentobiose and cellobiose. [b]Sum of lactic, formic and acetic acid.

Table S2. Monosaccharide analysis for 1.5L batch fermentations. The table shows the content of selected monosaccharides (g/L) in the fermentation medium (CH + B, which is chicken hydrolysate + BALI sugar) before (0 h) and after (24 h) fermentation. Values are means ± SD (n = 3). ND, Not detectable; -, not determined.

Time points (h)	Yeast strain	Galactose	Arabinose	Xylose	Mannose
0		1.0[1]	0.6[1]	4.0[1]	4.8[1]
24	*C. jadinii*	0.06 ± 0.01	0.02 ± 0	0.01 ± 0.01	ND
	W. anomalus	ND	ND	ND	ND
	B. adeninivorans	ND	ND	ND	ND
	Thermosacc Dry®	0.02 ± 0	ND	0.29 ± 0.12	ND

[1] Calculated based on Table S1.

Paper III

Spruce sugars and poultry hydrolysate as growth medium in repeated fed-batch fermentation processes for production of yeast biomass

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Abstract

The production of microbial protein in the form of yeast grown on lignocellulosic sugars and nitrogen-rich industrial residues is an attractive approach for reducing dependency on animal and plant protein. Growth media composed of enzymatically saccharified sulfite-pulped spruce wood, enzymatic hydrolysates of poultry by-products and urea was used for the production of single cell protein. Strains of three different yeast species, *Cyberlindnera jadinii*, *Wickerhamomyces anomalus* and *Blastobotrys adeninivorans, were* cultivated aerobically using repeated fed-batch fermentation up to 25 L scale. *Wickerhamomyces anomalus* was the most effective candidate in terms of substrate consumption with yields of 0.6 g of cell dry weight and 0.3 g of protein per gram of glucose, with cell and protein productivities of 3.92 g/L/h and 1.87 g/L/h, respectively. The protein content of the yeast biomass was 48 %, and the amino acid profiles were similar to those of fish- and soybean meal. *W. anomalus* was susceptible to disruptive methods commonly used in downstream processing of yeasts, such as homogenization and partial enzymatic hydrolysis, as visualized by scanning electron microscopy. While further optimization of both the fermentation process and downstream processing is needed, we conclude that *W. anomalus* can be potentially more efficient than *C. jadinii* and *B. adeninivorans* for the semi continuous and continuous production of microbial protein. Using the conditions developed here for producing *W. anomalus*, it would take 25 industrial (300 m^3) continuously operated fermenters to replace 10 % of the fish feed protein used in Norway.

Key words: microbial protein, yeast, spruce, protein hydrolysate, repeated fed-batch fermentation, aquaculture, optimization, downstream processing, repeated batch

1. Introduction

A possible future shortage of feed-protein will force mankind to explore alternative protein sources that could replace conventional soymeal- or fish meal [1]. Several large industrial organic side-streams could potentially be upgraded to feed-protein using fermentation processes [2]·[3]. Single cell protein (SCP) refers to cells of microorganisms such as algae, fungi or bacteria which are produced in bioreactors and then used as a protein source in human food or animal feed. Yeast is the most widely accepted microorganism for SCP production [4], because of its superior nutritional quality and acceptability among consumers [5]. Generally, yeast consists of 45-55 % (w/w) protein [6], has a beneficial amino acid profile according to FAO guidelines [7], and is a good source of vitamins [8]. Furthermore, yeast cell walls contain different proportions of mannan-oligosaccharides, ß-glucan, and chitin [9] with potential health-promoting effects [10], such as stimulation of the immune and antioxidant systems in fish [11]. Yeast can be used as whole cell preparations, or the cell wall might be partly broken down to make the protein and the cell wall components more accessible, using mechanical force, hydrolytic enzymes or detergents [7].

A wide variety of substrates have been utilized to cultivate different microorganisms, but to achieve large-scale production and to reduce the cost of SCP, relatively cheap agroindustrial by-products need to be considered as growth medium ingredients [3]. Yeasts can utilize by-products from agriculture, forestry (lignocellulosic residues) and food industries (hydrolysates from meat and fish by-products) as carbon and nitrogen sources for single cell protein production [12]·[13]. Nitrogen might also be sourced from a combination of protein hydrolysates and inorganic nitrogen like ammonium salts, nitrates and urea, which are relatively cheap nitrogen sources [14]. Yeasts can utilize various inorganic nitrogen compounds as a sole source of nitrogen [15].

In general, fermentation processes can be classified into batch, fed-batch and continuous fermentations. The chosen mode of operation is to a large extent dictated by the kind of products one is aiming for and process economy. A batch fermentation is a closed culture system that contains a finite amount of nutrients that will be consumed after a relatively short period of time, and is thus not ideal for the purpose of SCP production [16]. Fed-batch fermentations are initially established in batch mode and then continuously, or sequentially, fed with fresh medium without removal of culture [17], but have not been established for the production of SCP at a large industrial scale [18]. During continuous fermentations, fresh medium is constantly fed into the fermenters at a constant rate while used media containing microbes is continuously harvested. Therefore, medium conditions do not change over time, and exponential growth can be maintained throughout the whole cultivation [19]. Continuous fermentation has been the

preferred strategy to produce SCP industrially [20],[21]. Another good strategy is called repeated fed-batch fermentation, which it is a semi-continuous system of operation where a portion of culture is harvested at regular intervals and replaced by an equal volume of fresh medium [22]. It is considered as one of the best fermentation set-ups for economical SCP production [23]. In contrast to batch fermentation, production of biomass can in this case be prolonged over time, while low dissolved oxygen levels due to the increase of microbial biomass are avoided since cells are withdrawn and replaced with fresh medium. Compared to continuous fermentation, the harvested culture can potentially have a higher concentration of microbial biomass, which can facilitate the efficiency of the downstream processing.

In this study, media composed of sugars from enzymatically hydrolysed lignocellulosic biomass [24], enzymatically hydrolyzed poultry by-products [13] and urea were used to produce SCP in a semi-continuous mode (repeated fed-batch fermentation). Building on a previous comparative assessment of various yeasts [25], three different yeast strains belonging to the species *Cyberlindnera jadinii* (anamorph name *Candida utilis*), *Wickerhamomyces anomalus* and *Blastobotrys adeninivorans* (synonym *Arxula adeninivorans*) were tested. Repeated fed-batch fermentations at 1.5 L scale were carried out using benchtop fermenters, where concentrations of cells, substrates, side-products and yeast protein were monitored. Production of the best performing yeast strain, *W. anomalus* J121, was scaled up to 25 L, and the resulting yeast biomass was analyzed for protein and amino acid content. Finally, the effect of several cell disruptive methods on the composition of the yeast were investigated using autolysis, exogenous enzymes and mechanical force.

2. Material and methods

2.1 Materials

Protein-rich enzymatic hydrolysates from chicken and turkey cut-offs were provided by BIOCO AS (Hærland, Norway) and were kept at 4 °C until further use. The poultry hydrolysates contained 50.37 ± 0.03 % dry matter and 44.40 ± 0.10 protein %, according to product specifications. Glucose was purchased from VWR chemicals (Radnor, United States), and xylose, mannose, lactic acid, acetic acid, ethanol, sulfuric acid, sodium hydroxide, sodium acetate, potassium phosphate, 37 % formaldehyde, biotin, glucosamine and Glucanex were purchased from Sigma-Aldrich (Missouri, USA). Urea was kindly provided by Yara International ASA (Oslo, Norway). Kjeltabs for Kjeldahl analysis were purchased from Thomson and Capper Ltd. (Cheshire, UK). Enzymatic hydrolysates of BALI™ pretreated spruce were kindly provided by Borregaard AS (Sarpsborg, Norway). The BALI™ pretreatment[26] involves sulfite-pulping of chipped spruce wood (*Picea abies*), with chip size up to 4.5 x 4.5 x 0.8 cm. The carbohydrate composition of the spruce hydrolysate is shown in Table S1.

2.2. Microorganisms

Cyberlindnera jadinii LYCC 7549*, Wickerhamomyces anomalus* J121 (CBS 100487, Swedish University of Agricultural Sciences, Uppsala, Sweden) and *Blastobotrys adeninivorans* LS3 (Swedish University of Agricultural Sciences, Uppsala, Sweden), were stored at -80 °C in cryovials containing 20 % (v/v) glycerol and 80 % (v/v) YPD medium.

2.3. Shake flask experiments and repeated fed-batch fermentations

2.3.1. Shake flask experiments

Shake flask batch fermentations were conducted using media composed of spruce sugar hydrolysate (abbreviated as BALI™, in this study) and different mixtures of poultry hydrolysates and urea. Pre-cultures were prepared by adding 200 µL of a thawed seed culture stored at – 80 °C to 50 mL of the to-be-tested medium in a 250 mL baffled shake flask, followed by incubation at 30 °C, 220 rpm, for 16 h. The initial pH was adjusted to 5.0 using 5 M NaOH or 5 M

H_2SO_4. Shake flasks containing 50 mL fresh medium were then inoculated with overnight pre-cultures to obtain an initial OD of 0.5, as measured at 595 nm with an UV/VIS spectrophotometer (Hitachi U1900, Tokyo, Japan). The shake flasks were incubated at 30 °C and 220 rpm, and samples were taken at 24 h, for the measurement of pH, cell dry weight (CDW), protein content, and soluble sugars. These experiments were performed in duplicates. All media contained 50 g/L BALI™ glucose and 5.86 g/L nitrogen. The nitrogen was supplied by using 6 different blends of poultry protein hydrolysates and urea. More specifically, 0, 20, 40, 60, 80 or 100 % of the nitrogen was supplied by urea.

2.3.2. Repeated fed-batch fermentations at 1.5 L and 25 L scale

The bioreactor cultivations were performed in 2.5 L volume glass fermenters (Minifors, Infors, Bottmingen, Switzerland) with working volumes of 1.5 L, and a 42 L Techfors S stainless steel bioreactor (Infors, Bottmingen, Switzerland) with 25 L working volume, both equipped with two 6-bladed Rushton impellers. Blends of urea and poultry hydrolysates were autoclaved at 121 °C for 15 min in the bioreactors. BALI™ sugar hydrolysate was autoclaved separately, and aseptically added into the bioreactors.

For repeated fed-batch mode, fresh medium containing poultry hydrolysate and urea was prepared for the 2.5 L bioreactors by autoclaving at 121 °C for 15 min. For the 42 L bioreactor, new nitrogen medium was prepared using 80 °C water in 30 L Einar hydrolysis reactors (Belach Bioteknik, Skogås, Stockholm, Sweden) and stored at 4 °C for up to 12-16 hours, until use. Also for the repeated fed-batch experiments BALI™ hydrolysate was autoclaved separately.

Overnight pre-cultures were prepared by adding 0.2 mL or 1.6 mL of seed culture to 50 mL or 400 mL of the selected medium in 250 mL or 2 L baffled shake flasks for the 2.5 L and 42 L bioreactors, respectively. The pre-cultures were incubated at 30 °C, 220 rpm for approx. 16 h, prior to inoculation of the bioreactors, which were inoculated with 3% (v/v) pre-culture. The temperature for all cultivations was 30 °C. The pH was monitored with a pH probe (Mettler Toledo, Greifensee, Switzerland) and automatically maintained at 5.0 by controlled addition of 5 M NaOH or 5 M H_2SO_4. Dissolved oxygen (DO) was set at 30 % saturation and regulated by automatic adjustment of the stirrer speed (300 – 1250 rpm). Cultures were aerated through a sparger at an initial rate of 1.5 L/min or 25 L/min (1 VVM) and a maximum rate of 3 L/min or 50 L/min (2 VVM), for the 2.5 L and 42 L bioreactors, respectively. CO_2 and O_2 analysis was performed with a FerMac 368 off-gas analyzer (Electrolab Biotech, Tewkesbury, UK) for the 2.5

L bioreactors and an Infors HT Gas Analyzer (Infors, Bottmingen, Switzerland) for the 42 L bioreactor. Foam was controlled via a foam sensor with two times diluted Glanapon DB 870 antifoam (Busetti, Vienna, Austria). Fermentation data were recorded using IRIS process control software (Infors). The repeated fed-batch fermentation was carried out using a V_{out}/V_f value of 0.75 (i.e., 75 % of the total volume was harvested and replaced by fresh medium in each cycle). The total cultivation time was kept constant at 72 h or 76 h using cultivation cycles of 8 h or 12 h, respectively. The first harvest after the initial batch-phase always occurred after 16 h. The cultivation broth was aseptically collected, and a sterilized or pasteurized (the nitrogen medium fraction for the 42 L bioreactor) fresh medium was added into the fermenters with the use of a peristaltic pump connected to the inoculation port. During the fermentation experiments, samples were taken every four hours for analysis of soluble medium components and yeast biomass.

2.3.3. Downstream processing

Yeast produced during the repeated fed bath fermentation in the 42 L bioreactor was collected and kept at 4 °C. The broth, containing the medium and the yeast cells, was centrifuged by using a GEA Westfalia Separator Easyscale 10.S (GEA, Bönen, Germany) with a flowrate of 70 L/h and discharge every 120 seconds. The phase containing the yeast was resuspended in water (1:1, v/v) and washed one time with tap water using a flowrate of 50 L/h and 90 seconds discharge time. Different aliquots were stored as a yeast paste (i.e., a dry matter content of approximately 15 %) at -20 °C until further use.

For disruption experiments, 25 mL thawed cell paste was transferred to a 50 mL centrifuge tube with blue cap and water was added up to 50 mL to wash the cells. After collecting the cells using a centrifuge at 4 °C and 4700 g for 5 min, the washing step was repeated once. An autolysis treatment was carried out by incubating washed yeast cells in water (7.5 % DM) at 55 °C for 20 h at 220 rpm without pH adjustment. A hydrolytic treatment with Glucanex was done using an identical cell suspension, which was supplemented with 1:200 (w/w) Glucanex with 200 mM NaOAc pH 6 (hydrolytic treatment with Glucanex), followed by incubation at 37 °C for 24 h at 220 rpm. Identical 7.5 % DM cell suspensions were also subjected to a mechanical treatment (cell disruption by high-pressure homogenization) using a microfluidizer (Microfluidizer™ SIMATIC HMI LM20) at 30.000 psi, for three consecutive times. The cell suspensions subjected to autolysis, hydrolytic treatment with Glucanex, homogenization with a microfluidizer and to no treatment were frozen at -80 °C, and then freeze-dried using an Alpha 2-4 LD plus freeze

drier (Martin Christ, Osterode am Harz. Germany) set at -60 °C and 0.01 mbar for a minimum of 24 hours until the samples were dry. All these experiments were performed in triplicates.

2.4 Analytical Methods

2.4.1. Cell dry weight (CDW)

Fermentation broth samples (50 mL for shake flasks, 25 mL for bioreactors) were centrifuged at 4700 g for 5 min at 4 °C and the supernatant was collected for further analyses (sugars, organic acids, ethanol, protein). Then, the yeast biomass was washed twice with cold distilled water, frozen at -80 °C and then freeze-dried using an Alpha 2-4 LDplus freeze drier (Martin Christ, Osterode am Harz, Germany) at -60 °C and 0.01 mbar vacuum for a minimum of 24 hours until samples were dry. The dried cells were weighed to determine CDW, and were used for analysis of protein content and amino acids.

2.4.2. Monosaccharides, organic acids and ethanol

Monosaccharides (D-glucose, D-xylose), organic acids (lactic acid, acetic acid) and ethanol present in the fermentation broth were analyzed by high performance liquid chromatography (HPLC) with refractive index detection. The samples were diluted 10 times with distilled water and 200 µL of the diluted sample was vacuum-filtered using 96 well filter plates (0.45 µm). Samples were separated on a Rezex ROA-organic acid H+, 300 x 7.8 mm (Phenomenex, Torrance, CA, USA) analytical column fitted with a cation-H cartridge guard column, using a column temperature of 65 °C, 5 mM H_2SO_4 as eluent and a flow rate of 0.6 mL/min.

2.4.3. Protein content

The total nitrogen content of the poultry hydrolysates and the freeze-dried yeast biomass was measured according to the Kjeldahl method (European Commission [EC] regulation No: 152/2009, pp 15 – 19) using a Kjeltec TM 8400 (FOSS, Tecator, Hoganas, Sweden) after acid digestion in an autodigestor (FOSS, Tecator, Hoganas, Sweden). The protein content of samples was estimated by multiplying total nitrogen by a factor of 6.25.

2.4.4. Amino acids

Analysis of the content of amino acids (except tryptophan) in freeze-dried yeast was performed according to EC regulation No: 152/2009 (pp. 23 – 32) using a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, UK). Tryptophan was analyzed according to EC regulation No: 152/2009 (pp. 32 – 37) using a Dionex Ultimate 3000 HPLC system (Dionex Softron GmbH, Germering, Germany) connected to a RF-535 fluorescence detector (Shimadzu., Kyoto, Japan). All amino acids were quantified by using external standards (Dionex Ltd., Surrey, UK).

2.4.5. Scanning electron microscope (SEM)

For SEM images the yeast cell suspensions were mixed with 37 % (v/v) formaldehyde reaching a final concentration of 3.7%, and incubated at room temperature for 20 min. The yeast suspensions were then centrifuged for 5 min at 8000 g, resuspended in 0.1 M potassium phosphate and kept at 4 °C until imaging. Prior to scanning electron microscopy, the cells were washed several times in 0.05 M Pipes buffer, pH 7.0, and dehydrated with 10 min stages in ascending ethanol series (30 – 100 %). The samples were processed in a BAL-TEC Critical Point Dryer (CPD 030, Witten, Germany) and a thin conductive coating of gold/palladium was applied to the samples using a Polaron Sputter Coater (SC 7640, Kent, UK). The coated samples were mounted on brass stubs and examined and photographed with a Zeiss EVO-50-EP scanning electron microscope at an accelerating voltage of 15 kV in the secondary emission mode.

2.4.6. Statistical Analysis

Data handling and statistics were performed using the Excel software package (Microsoft Excel 2013, Microsoft Corp., Redmond, WA).

3. Results and Discussion

3.1. Characterization of BALI™ and poultry hydrolysates

Table S1 shows that glucose is the main carbon source in the spruce BALI™ hydrolysates constituting 76 % of the total sugar. The protein content of the protein-rich hydrolysates (named poultry hydrolysates in this study), based on

the Kjeldahl method, was 444 ± 1 g/L. In the growth experiments described below carbohydrates were dosed based on glucose, whereas the nitrogen source was dosed based on nitrogen.

3.1. Preliminary 1.5 L batch fermentations using poultry hydrolysates

Initially, several batch fermentations were carried out at 1.5 L scale in bioreactors to compare the growth performance of the three yeast strains growing on a blend of BALI[TM] sugar and poultry protein hydrolysate. Table S2 shows the CDW (g/L) and protein content (%) after 12h and 24h. In general, all the yeast strains had a protein content in the range 47-51% after 12 h of fermentation, but prolonged incubation somewhat reduced this. *C. jadinii* showed both the slowest growth rate and the lowest final production of microbial biomass. Both *W. anomalus* and *B. adeninivorans* grew faster and achieved a much higher biomass concentration after 24 h, in particular *B. adeninivorans* which reached a CDW of 44.0 g/L (as compared to 29.7 g/L and 18.0 g/L for *W. anomalus* and *C. jadinii* showed, respectively. A similar trend has been observed previously for these three yeast strains when grown on a medium composed of BALI[TM] sugars and an in-house prepared chicken hydrolysate [25]. Thus, in these batch fermentations *B. adeninivorans* showed superior growth performance, probably due the good ability of this yeast to utilize peptides as a carbon source [27]. Of note, however, *B. adeninivorans* showed the lowest protein content after 24 h (41.9 %).

3.2. Assessing the ratio of organic and inorganic nitrogen

To test the importance of the protein hydrolysate for growth, a screening of growth was performed where different amounts of protein hydrolysate were substituted with inorganic nitrogen (urea). The experiments were conducted in shake flasks and the results in terms of CDW (g/L) and protein content (%) are shown in Figures 1 and 2, respectively. After 24h, the growth based on cell dry weight was between 9.8 – 13.2 g/L for all the yeasts and all fermentations containing poultry hydrolysates (100 - 20 %). When only urea was used as a nitrogen source (condition 6) there was hardly any growth. The observed growth yields were much lower than 50 % (in this case, 25 g/L) which is typically achieved for yeast under aerobic conditions [28]. Analysis of the supernatants after 24h showed that glucose was completely consumed for *C. jadinii* and *W.anomalus*, while *B.adeninivorans* did not consume all glucose. Without protein hydrolysate in the medium, less than 20 % of the glucose was consumed for any yeast. Ethanol concentrations between 17.0 and 24.7 g/L were observed for all the *C. jadinii* and *W. anomalus* cultivations containing poultry hydrolysates. For *B.adeninivorans* ethanol concentrations were in the range of 4.8 and 11.7 g/L (data not shown). The

pH was measured after 24 h (data not shown), and it was observed that the buffer capacity decreased when less poultry hydrolysate was included in the medium, with pH values after 24 h being 4.77, 4.36 and 4.50 in the cultures with 80 % poultry hydrolysate and 20 % urea, and 4.30, 3.89 and 3.32 in the cultures with 20 % poultry hydrolysate and 80 % urea, for *C. jadinii, W.anomalus*, and *B.adeninivorans*, respectively. In the cultures with only urea, the final pH values varied between 5.53 and 5.81. Acetic acid was also measured and concentrations ranged between 0 and 3 g/L (data not shown). All in all, these results show that the shake flask cultures were clearly limited regarding oxygen supply, causing high ethanol concentrations, and by medium acidification.
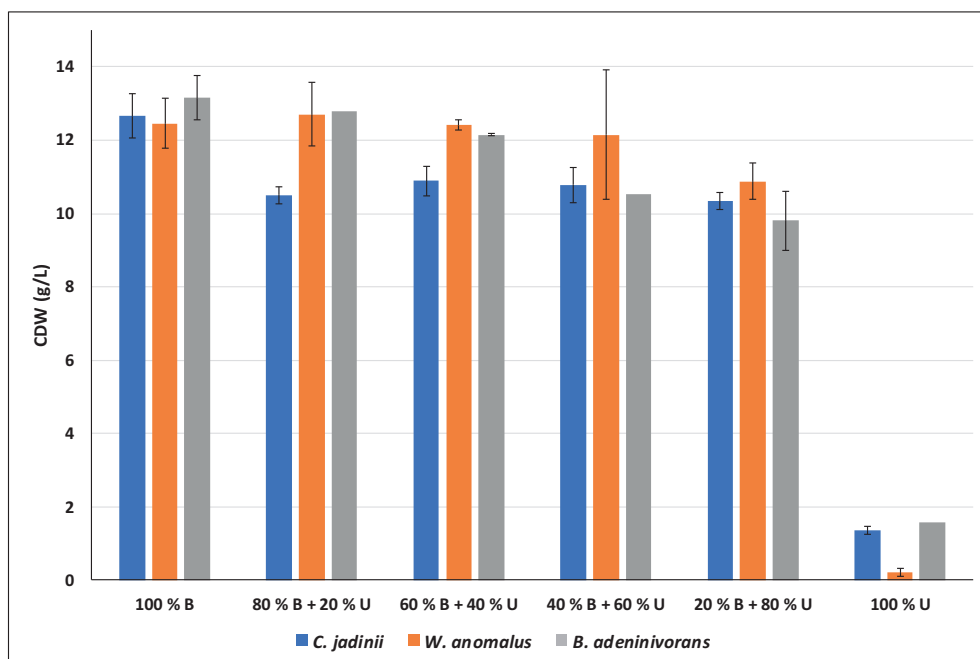
Figure 1. Growth of three yeast strains in shake flasks using 6 different media using different combinations of poultry hydrolysates and urea. The graph shows CDW (g/L) after 24h cultivation (values are means ± SD; n = 2). B: BIOCO poultry hydrolysates, U: Urea. Conditions: Glucose, 50 g/L; Kjeldahl nitrogen, 5.86 g/L; $OD_{initial}$ = 0.5; volume: 50 mL; $pH_{initial}$ = 5.0; incubation at 30°C with 220 rpm shaking. The pH and pO_2 were not controlled.
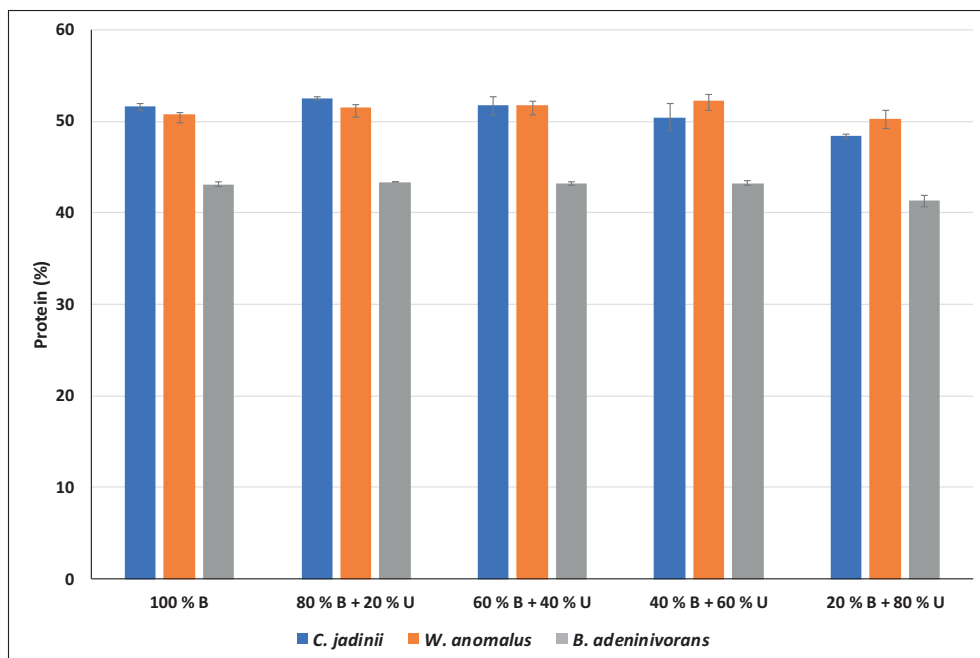
Figure 2. Protein content of three yeast strains grown in shake flasks using 5 different media using different combinations of poultry hydrolysates and urea. The graph shows protein content (%) after 24h cultivation (values are means ± SD; n = 2). B: BIOCO poultry hydrolysates, U: Urea. Conditions: Glucose, 50 g/L; Kjeldahl nitrogen, 5.86 g/L; $OD_{initial}$ = 0.5; volume: 50 mL; $pH_{initial}$ = 5.0; incubation at 30°C with 220 rpm shaking. The pH and pO_2 were not controlled. The protein content in cells grown on urea only (see Fig. 1) was not determined.

The general trends visible in Figures 1 and 2 are that biomass production went down as the amount of poultry hydrolysates hydrolysate was reduced whereas the protein content of the cells, around 50 % for *C. jadinii* and *W.anomalus* and 43 % for *B.adeninivorans* was hardly affected. Although the growth effects are likely primarily due to pH effects, the data in Figures 1 and 2 do make clear that the cells can convert urea to protein, but also that they need some of the components provided by the poultry hydrolysate (since on urea only growth was strongly restricted. It is well known that inorganic nitrogen can provide a considerable amount of nitrogen when combined with an organic nitrogen source that supplies additional benefits such as various trace elements [28] [29].

3.3. 2.5L Bioreactor runs

Shake flasks experiments suffer from a lack of pH control, as well as sub-optimal aeration (pO$_2$) and stirring. Therefore, *C. jadinii*, *W. anomalus* and *B. adeninivorans* were cultivated in 2.5 L fermenters in repeated fed-batch mode. The main objectives were to identify the best medium ratios of poultry hydrolysate and urea to support high productivity (Q_x), while producing yeast biomass containing around 50 % protein [6][18].

3.3.1. Using 100 % poultry hydrolysates

We then carried out repeated fed-batch fermentations in 1.5 L fermenters with all three yeast, using 100 % poultry hydrolysate as nitrogen source. Raw data from these fermentations are provided in Figure S1, while Table 1 summarizes the key features. This procedure worked very well for *C. jadinii* and *W. anomalus* but not for *B. adeninivorans*, which left much of the glucose unused and, thus, showed low productivity. *C. jadinii* and *W. anomalus* used most of the glucose, showed good biomass productivity, amounting to 2.51 and 2.61 g/L/h, respectively. The protein content of the produced cells was 40.9 % and 48.4 %, respectively. While *C. jadinii* reached high cell densities, this came at the cost of protein content, which was lower than previously observed [25]. A similar trade-off between cell mass and protein content was observed by Sharma et al., 2018 [30]. Overall, *W. anomalus* was clearly the best protein producer when grown in medium containing the poultry hydrolysate as the only nitrogen source.

Table 1. Growth characteristics for 1.5 L repeated fed-batch fermentations of *C. jadinii*, *W. anomalus* and *B. adeninivorans* grown on 100 % poultry hydrolysates and BALI™ hydrolysates. The V$_{out}$/V$_f$ was 0.75, every 8 h, and starting at 16 h. The data shown are average values for the samples taken at 24 h and at the end of the subsequent six repeated batches (32 - 72 h). The nitrogen concentration in the medium was 5.86 g/L, whereas the glucose concentration was approximately 50 g/L (The actual glucose concentrations were measured by HPLC at each sampling point and right after each harvest/refill procedure, and these measurements were used for the calculations). Figure S1 provides the actual values of the parameters during the course of the fermentation.

	C. jadinii	*W. anomalus*	*B. adeninivorans*
Increase in CDW (g/L)[1]	20.05 ± 2.01	20.87 ± 2.44	14.69 ± 8.62
Protein (%)	40.89 ± 1.54	48.38 ± 0.91	47.44 ± 2.77
Increase in protein (g/L)[1]	8.18 ± 0.65	10.09 ± 1.09	6.99 ± 4.17
Unconsumed glucose (g/L)	2.90 ± 1.55	0.99 ± 0.77	25.06 ± 8.72
$Y_{X/glucose}$[2]	0.48	0.54	0.50
$Y_{X/sugars}$[3]	0.37	0.41	0.38
$Y_{p/glucose}$[2]	0.20	0.26	0.24
$Y_{p/sugars}$[3]	0.15	0.20	0.18
Q_x [4]	2.51	2.61	1.51
Q_p [5]	1.02	1.26	0.71

[1] Increase over the eight hours growth period following each harvest/refill procedure
[2] Yield of cell biomass (X) or protein (P) per consumed glucose
[3] Yield of cell biomass (X) or protein (P) per total added sugar
[4] Cell biomass productivity in g/L/h
[5] Protein productivity in g/L/h

3.3.2. Using 40 % poultry hydrolysate and 60 % urea

Inorganic nitrogen is cheaper than protein hydrolysates and may be easier to take up and metabolize. Thus, substituting part of the protein hydrolysate with inorganic nitrogen may be beneficial. The same set of fermentation experiments were repeated, but this time 60 % of the nitrogen in the protein hydrolysate was substituted with urea nitrogen. Compared to the fermentations using 100 % poultry hydrolysates as N source, the cell biomass production substantially changed for *W.anomalus* (increased from 20.9 to 28.9 g/L) but the protein content decreased to 37.7 % (Table 2; raw data in Fig. S2). For *C. jadinii* the protein content increased to 44.9%, while biomass production was similar to the fermentation with 100 % poultry. The *B. adeninivorans* fermentations showed better performance than in the fermentations with 100 % poultry hydrolysate, but did still not consume glucose very well, indicating that the fermentation strategy still was not optimal for this yeast. In accordance with previous observations *B. adeninivorans* performed better than expected on the basis of the low glucose consumption, which is likely due to the ability of this yeast to effectively use compounds in the poultry hydrolysate as carbon source [25]·[27].

All in all, while comparing the results of Table 1 (100 % poultry hydrolysate) and Table 2 (40 % poultry hydrolysate) does not provide immediate clues as to how to further optimize the process, this comparison clearly shows that varying the ratio of the organic and the inorganic nitrogen source has considerable effects.

Table 2. Growth characteristics for 1.5 L repeated fed-batch fermentations of *C. jadinii*, *W. anomalus* and *B. adeninivorans* grown on BALI™ hydrolysate with a mixture of 40 % poultry hydrolysate and 60 % urea as nitrogen source. The V_{out}/V_f was 0.75, every 8 h, and starting at 16 h. The data shown are average values for the samples taken at 24 h and at the end of the subsequent six repeated batches (32 - 72 h). The nitrogen concentration in the medium was 5.86 g/L, whereas the glucose concentration was approximately 50 g/L (The actual glucose concentrations were measured by HPLC at each sampling point and right after each harvest/refill procedure, and these measurements were used for the calculations). Figure S2 provides the actual values of the parameters during the course of the fermentation.

	C. jadinii	*W.anomalus*	*B.adeninivorans*
Increase in CDW (g/L) [1]	21.08 ± 1.45	28.88 ± 1.11	18.51 ± 3.18
Protein (%)	44.87 ± 4.03	37.66 ± 2.19	44.45 ± 3.16
Increase in protein (g/L) [1]	9.43 ± 1.69	10.88 ± 0.89	8.19 ± 1.31
Unconsumed glucose (g/L)	3.48 ± 2.50	0.55 ± 0.13	25.53 ± 6.83
$Y_{X/glucose}$ [2]	0.55	0.69	0.89
$Y_{X/sugars}$ [3]	0.41	0.53	0.68
$Y_{p/glucose}$ [2]	0.24	0.26	0.39
$Y_{p/sugars}$ [3]	0.19	0.20	0.30
Q_x [4]	2.63	3.61	2.31
Q_P [5]	1.18	1.36	1.02

[1] Increase over the eight hours growth period following each harvest/refill procedure
[2] Yield of cell biomass (X) or protein (P) per consumed glucose
[3] Yield of cell biomass (X) or protein (P) per total added sugar
[4] Cell biomass productivity in g/L/h
[5] Protein productivity in g/L/h

3.3.3. Using 60 % poultry hydrolysates with 40 % urea

The experiments described above showed that *W. anomalus* had the highest productivity regarding both biomass (Q_x in the range 2.61-3.61) and protein (Q_P in the range 1.26-1.36) production, although it must be noted that the higher productivities depicted in Table 2 come at the cost of a low protein content of the cells. In a previous study [25], we had found that *B. adeninivorans* outperformed *C. jadinii and W. anomalus* during batch fermentations using BALI™ hydrolysates and in-house generated chicken hydrolysates, due to its ability to not only grow on sugars but also on different nitrogen sources [31]·[27]. In the repeated fed-batch setup used in this study *B. adeninivorans* had not been able to consume the glucose during the 8 h cycles. Thus, in a next series of experiments, this time using 60 % poultry and 40 % urea as nitrogen source, the cycle time for *B. adeninivorans* was increased to 12 h in an attempt to obtain

better glucose utilization. The results, summarized in Table 3 (raw data in Fig. S3) show that the longer cycle time indeed resulted in increased consumption of glucose and that glucose consumption was more stable. However, sugar consumption was still not complete and, moreover, performance wise (yields, productivities, protein content; see table 3), the longer cycle time did not improve the overall process. For *W anomalus*, the change from 40 % to 60 % poultry hydrolysate resulted in improved performance such as a higher protein content (41.2 % versus 37.7 %) and higher productivity of both cell biomass (3.72 versus 3.61 g/L/h) and protein (1.53 versus 1.36 g/L/h).

Table 3. Growth characteristics for 1.5 L repeated fed-batch fermentations of *W. anomalus* and *B. adeninivorans* grown on BALI[TM] hydrolysate with a mixture of 60 % poultry hydrolysate and 40 % urea as nitrogen source. The V_{out}/V_f was 0.75, every 8 h (*W. anomalus*) or every 12 h (*B. adeninivorans*), and starting at 16 h. The data shown are average values for the samples taken at 24 h and 28 h, respectively and at the end of the subsequent six (*W. anomalus*) or four (*B. adeninivorans*) repeated batches. The nitrogen concentration in the medium was 5.86 g/L, whereas the glucose concentration was approximately 50 g/L (The actual glucose concentrations were measured by HPLC at each sampling point and right after each harvest/refill procedure, and these measurements were used for the calculations). Figure S3 provides the actual values of the parameters during the course of the fermentation.

	W.anomalus	*B.adeninivorans*
Increase in CDW (g/L) [1]	29.78 ± 3.51	27.62 ± 1.30
Protein (%)	41.22 ± 1.19	42.45 ± 1.12
Increase in protein (g/L) [1]	12.24 ± 1.14	11.73 ± 0.67
Unconsumed glucose (g/L)	0.15 ± 0.03	11.37 ± 2.83
$Y_{X/glucose}$ [2]	0.71	0.81
$Y_{X/sugars}$ [3]	0.54	0.61
$Y_{p/glucose}$ [2]	0.29	0.34
$Y_{p/sugars}$ [3]	0.22	0.26
Q_x [4]	3.72	2.30
Q_p [5]	1.53	0.98

[1] Increase over the eight hours growth period following each harvest/refill procedure
[2] Yield of cell biomass (X) or protein (P) per consumed glucose
[3] Yield of cell biomass (X) or protein (P) per total added sugar
[4] Cell biomass productivity in g/L/h
[5] Protein productivity in g/L/h

3.3.4. Using 60 % poultry hydrolysate and 40 % urea with biotin, and 80 % poultry hydrolysate with 20 % urea

In the experiments described above *W. anomalus* was superior in terms of productivity, but in several of the fermentations the protein content of the cells was rather low. In particular, while *W. anomalus* grew well on urea, replacement of poultry hydrolysate with urea led to lowered protein contents (e.g. 48.4 % with 100 % poultry hydrolysate, versus 37.7% with 40 % poultry hydrolysate & 60 % urea). Yeasts can assimilate urea in two different ways, either by via the action of an extracellular urease leading to ammonia production, or via import of urea and subsequent assimilation through the urea and amydolyase pathway [32]. In this latter case, addition of biotin is necessary since it works as a cofactor of the urea amidolyase [32]. Therefore, we carried out an additional experiment with the medium composed of 60 % poultry hydrolysate and 40 % urea and added 0.4 mg of biotin per gram of urea. Additionally, a repeated fed-batch fermentation was run using a medium composed of 80 % poultry hydrolysate and 20 % urea, without addition of biotin. Figure S4 and Table 4 show that the addition of biotin had no significant effect on the production of cell biomass and the protein content, which were 30.00 g/L and 40.6 %, respectively, compared to 39.8 g/L and 41.2 % for the similar experiment without added biotin (Table 3).

Figure S4 and Table 4 also show that the protein content increased to 45.0 % after increasing the amount of poultry hydrolysates from 60 % to 80 %, while the production of cell biomass stayed at as high 27.0 g/L. The latter value is slightly lower than the value obtained wit 60 % poultry, but still much higher than the value of 20.9 g/L obtained with 100 % poultry. Apparently, having some urea in the medium is highly favorable for cell biomass production. All in all, the run with 80 % poultry hydrolysate seemed close to optimal, yielding productivity values of 3.38 1.52 g/L/h and 1.52 g/L/h for cell biomass and protein, respectively.

Table 4. Growth characteristics for 1.5 L repeated fed-batch fermentations of *W. anomalus* grown on BALI^{TM} hydrolysate with two different mixture of poultry hydrolysate and urea as nitrogen source (60-40, with added biotin or 80-20). The V_{out}/V_f was 0.75, every 8 h, starting at 16 h. The data shown are average values for the samples taken at 24 h and at the end of the subsequent six repeated batches (32 - 72 h). The nitrogen concentration in the medium was 5.86 g/L, whereas the glucose concentration was approximately 50 g/L (The actual glucose concentrations were measured by HPLC at each sampling point and right after each harvest/refill procedure, and these measurements were used for the calculations). Figure S4 provides the actual values of the parameters during the course of the fermentation.

	W.anomalus	
	Poultry hydrolysate 60 % + UREA 40 % + Biotin	Poultry hydrolysate 80% + UREA 20%
Increase in CDW (g/L) [1]	30.00 ± 2.07	27.04 ± 1.55
Protein (%)	40.61 ± 0.48	45.03 ± 0.81
Increase in protein (g/L) [1]	12.18 ± 0.75	12.17 ± 0.53
Unconsumed glucose (g/L)	0.19 ± 0.01	0.19 ± 0.03
$Y_{X/glucose}$ [2]	0.65	0.62
$Y_{X/sugars}$ [3]	0.49	0.47
$Y_{p/glucose}$ [2]	0.26	0.28
$Y_{p/sugars}$ [3]	0.20	0.21
Q_x [4]	3.75	3.38
Q_p [5]	1.52	1.52

[1] Increase over the eight hours growth period following each harvest/refill procedure
[2] Yield of cell biomass (X) or protein (P) per consumed glucose
[3] Yield of cell biomass (X) or protein (P) per total added sugar
[4] Cell biomass productivity in g/L/h
[5] Protein productivity in g/L/h

3.4. 42 L Bioreactor run

Based on the observations and considerations described above, the combination of 80 % poultry hydrolysate with 20 % urea was selected as medium for upscaling the repeated fed-batch fermentation with *W. anomalus* from 1.5 to 25 L. Figure 3 shows that the 1.5 and 25 L fermentations behaved rather similar, but with somewhat higher Q_x (3.92 g/L/h) and Q_P (1.87 g/L/h), as well as a higher protein content (47.8 %) in the large scale fermentation (see Tables 4 and 5). The biomass yield was 0.66 g biomass per g of glucose, which is in the higher range of yields report for aerobic growth of yeast, typically ranging from 0.4 and 0.5 g biomass per g of sugar [18].

It is important to mention that it is difficult to compare biomass yields and productivity values for SCP production since they are strongly dependent on culture medium composition, the type of yeast and environmental conditions, such as incubation temperature, medium pH, dissolved oxygen, aeration rate and fermentation mode[4]. Moreover, to our knowledge, this is the first study on upscaling of *W.anomalus* production using repeated fed-batch as a bioprocess strategy for SCP production. However, it is still possible and compare the efficiency of the 25 L experiment with *W. anomalus* with results obtained for well-known SCP yeasts such as *Cyberlindnera jadinii* (anamorph name *Candida utilis*). Bajpai et al. [23] reached a Q_X 0.76 g/L/h, i.e. five times lower than in our study (3.92 g/L/h), for repeated fed-batch fermentations of *C. utilis* using the same ratio for the withdrawal and addition of medium (V_{out}/V_f = 0.75) but

with a longer cycle time of 24 h. Lee et al. [33] achieved biomass yields and productivities of 0.67 g/g and 0.24 g/L*h for batch fermentations, 0.51 g/g and 1.95 g/L*h for fed-batch fermentations, and 0.36 g/g and 2.15 g/L*h for continuous fermentations, using *C.utilis*. Gao et al. [34] produced single cell protein (SCP) from soy molasses using *C. tropicalis* and obtained maximum cell densities and protein concentrations of 10.83 g/L and 6.11 g/L in a 10 L bioreactor, using batch fermentation. Overall, comparing the yields and productivity values from literature with the values presented in Table 5, it can be concluded that it was possible to effectively produce a reasonable amount of protein-rich yeast in the form of *W. anomalus*, using a medium composed of spruce derived components (BALI[TM] hydrolysates), poultry by-products and urea.

Table 5. Growth characteristics for a 25 L repeated fed-batch fermentation of *W. anomalus* grown on BALI[TM] hydrolysate and a 80:20 mixture of poultry hydrolysate and urea. The V_{out}/V_f was 0.75, every 8 h, starting at 16 h. The data shown are average values for the samples taken at 24 h and at the end of the subsequent six repeated batches (32 - 72 h). The nitrogen concentration in the medium was 5.86 g/L, whereas the glucose concentration was approximately 50 g/L (The actual glucose concentrations were measured by HPLC at each sampling point and right after each harvest/refill procedure, and these measurements were used for the calculations). Figures 3 and S5 provide the actual values of the parameters during the course of the fermentation.

W.anomalus	
Poultry hydrolysate 80% + UREA 20%	
Increase in CDW (g/L) [1]	31.39 ± 2.77
Protein (%)	47.76 ± 1.13
Increase in protein (g/L) [1]	14.97 ± 1.07
Unconsumed glucose (g/L)	0.08 ± 0.01
$Y_{X/glucose}$ [2]	0.66
$Y_{X/sugars}$ [3]	0.50
$Y_{p/glucose}$ [2]	0.31
$Y_{p/sugars}$ [3]	0.24
Q_x [4]	3.92
Q_p [5]	1.87

[1] Increase over the eight hours growth period following each harvest/refill procedure
[2] Yield of cell biomass (X) or protein (P) per consumed glucose
[3] Yield of cell biomass (X) or protein (P) per total added sugar
[4] Cell biomass productivity in g/L/h
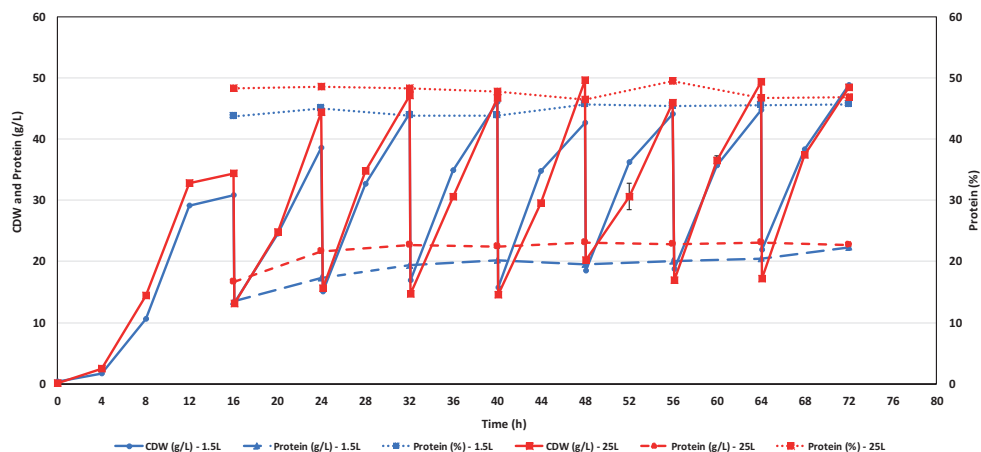[5] Protein productivity in g/L/h

Figure 3. Data for the 1.5 L and 25 L repeated fed-batch fermentations of *W. anomalus* grown on a medium containing an 80:20 mixture of poultry hydrolysate and urea as nitrogen source and BALI™ hydrolysate as sugar source. The V_{out}/V_f was 0.75, every 8 h, and starting at 16 h.

3.5. Amino acid composition

The amino acid compositions of *W.anomalus* harvested during the 25 L fermentation, and of fish meal and soybean meal are presented in Table 6. Microorganisms to be used as feed ingredients would need a beneficial amino-acid profile, with particular attention to the nutritionally important amino acids methionine (Met), cysteine (Cys), lysine (Lys) and arginine (Arg) [35]. The data show that the amino acid composition of *W. anomalus* produced using 80 % poultry hydrolysate and 20 % urea with BALI™ sugar is similar to that of fishmeal and soybean meal, except for sulphur-containing amino acids such as methionine (Met) and cysteine (Cys). Low levels of sulfur-containing amino acids is common for yeast and bacterial biomasses [12] and normally restricts their use as the *sole* protein source in feed [36]. The data also shows that the amino acid composition for *W. anomalus* is similar to the amino acid composition of the well-known feed ingredient *C. jadinii* grown on lignocellulosic substrates [30]·[37]·[38].

Table 6. Amino acid composition of *W. anomalus* obtained after repeatd fed-batch fermentation on a medium containing an 80:20 mixture of poultry hydrolysate and urea as nitrogen source, and BALI[TM] sugar. Values are means ± SD (n = 2). EAAs, essential amino acids; NEAAs, non-essential amino acids.

	Amino acids	*W. anomalus*	Fish meal [b]	Soybean meal [c]
EAAs [a]	Met, M	3.27 ± 0.07	16.1	7.7
	Thr, T	18.91 ± 0.05	25.4	20.2
	Val, V	19.52 ± 0.13	26.4	24.1
	Ile, I	18.41 ± 0.13	23.7	23.1
	Leu, L	28.96 ± 0.05	42.0	39.0
	His, H	11.19 ± 0.18	11.8	13.5
	Lys, K	30.61 ± 0.23	45.5	32.3
	Ala, A	24.11 ± 0.18	32.6	22.4
	Phe, F	16.33 ± 0.06	22.0	26.5
	Trp, W	5.20 ± 0.22	6.9	6.8
NEAAs [a]	Asp, D	40.51 ± 0.15	54.7	59.5
	Ser, S	20.19 ± 0.04	25.3	25.8
	Glu, E	76.50 ± 0.48	83.9	92.1
	Pro, P	17.67 ± 0.58	23.1	24.1
	Gly, G	22.18 ± 0.08	30.8	21.6
	Tyr, Y	11.20 ± 0.01	15.2	14.7
	Arg, R	25.71 ± 0.04	35.3	37.4
	Cys, C	3.27 ± 0.07	5.7	6.9
	SUM AA	395.80	526.4	497.8

[a] All values are in g/kg of dry matter.

[b] The content of amino acids in fish meal (except tryptophan) was taken from Hansen et al., 2010 [39]; the value for tryptophan comes from Skrede et al., 1998 [40].

[c] The content of amino acids in soybean meal was taken from Sriperm et al., 2011 [41].

3.6. Effects of different disruptive methods on *W. anomalus*

W. anomalus has not been used in SCP production and little is known about how this yeast responds to downstream processing processes that are commonly in refining of SCP. To obtain some first insight into this issue, *W. anomalus* cells from the 25 L fermentation were subjected to varying potential processing steps. Cells from different harvesting points during the 25 L fermentation (16, 24, 32, 40, 48, 56, 64 and 72 h) were pooled and subjected to separation using a continuous 2-phase separator, resulting in a yeast cell paste with a dry weight of 15 % (w/v). After washing the cells, as described in the Materials and Methods section, they were subjected to varying disruptive methods followed by analysis of effects of on cell morphology using SEM. The SEM images of the autolyzed cells (Figure 4B) did not differ much from the images of the untreated cells (Figure 4A), while loss of cell integrity and liberation of internal contents were clearly visible in the pictures of homogenized cells (Figure 4C). The SEM images also show a clear disruptive effect provoked by the use of the enzyme preparation Glucanex (Figure 4D). These results indicate that several methods can be applied to disrupt the *W. anomalus* cells, which presumably would affect yeast digestibility. Such effects will be investigated in a follow-up study.
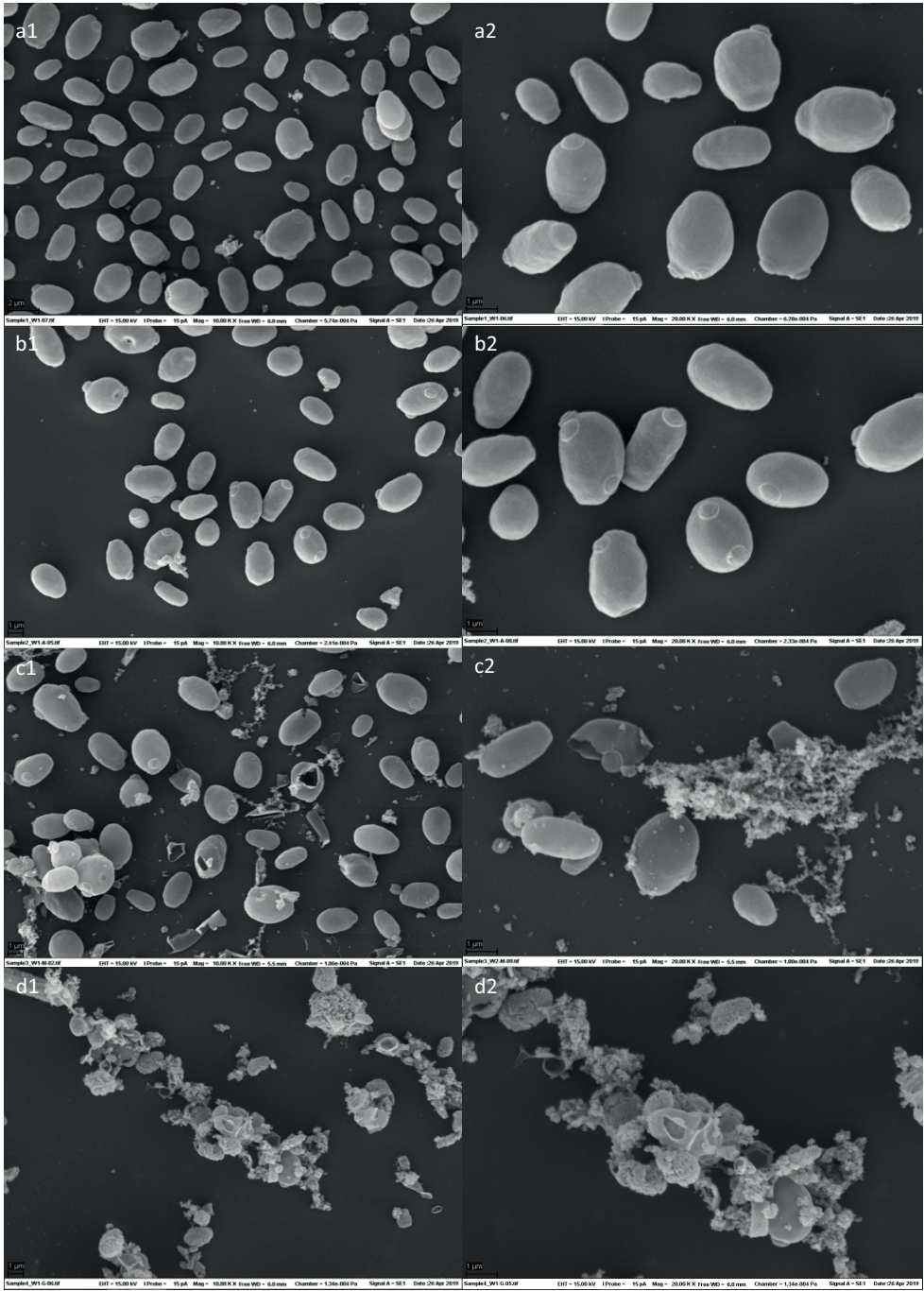
Figure 4. SEM images of *W. anomalus* cells after different potentially disruptive treatments. a, Untreated yeast; b, autolysis; c, homogenization using a microfluidizer; d, enzymatic hydrolysis using Glucanex. For each treatment, two magnifications are shown, 10000 (panels labeled 1) and 20000 (panels labeled 2).

3.7 Possible industrial applications as fish feed

The three major feed companies in Norway jointly used 1.63 million tonnes of ingredients for production of fish feed in 2012 [42]. These ingredients are mainly of plant and marine origin, which combined yield a feed protein content of approximately 50 % (815 000 tonnes) [43]. If the SCP process developed in this study were to replace 10 % of fish feed protein (81 500 tonnes), a total fermentation volume of approximately 5 000 m3 would be needed, based on the results from the large-scale fermentation (protein productivity of 1.87 g/L/h or 0.016 tonnes/L/year). The commercial yeast producer Lallemand Inc. uses bioreactors with volumes of $100 - 300$ m^3 for production of baker's yeast [44] . If bioreactors of 300 m^3 (assuming a working volume of 200 m^3) were used for production of SCP, a yearly production of 81 500 tonnes of fish protein would require 25 of such vessels (see Table S3 for the numbers on which this and the subsequent calculations are based).

Regarding the raw materials, production of 81 500 tonnes of protein would annually require approximately 0.166 million tonnes DM of protein from poultry hydrolysate, 12 570 tonnes of urea and 220 000 tonnes of cellulose, which translates to 0.78 million tonnes of wet spruce [45]·[46], which would amount to 9.6 % of the total annual spruce harvest in Norway [47] . Of note, while the poultry hydrolysate:urea ratio was somewhat optimized in this study, we did not look into how to minimize the amount of the poultry hydrolysate-urea mixtures used. Thus, some further improvements in process economy should be feasible.

In conclusion, this study demonstrates that *W. anomalus* is better suited than and *B. adeninivorans* and the well establish *C. jadinni* to produce microbial protein in a medium composed of a mixture of organic and inorganic nitrogen sources and spruce derived-sugars in a semi-continues mode. The protein content of the yeast biomass produced in the 42 L fermenter was high (around 50 w/w %), and the amino acid profile of *W. anomalus* was reasonable, albeit with the deficit in sulphur-containing amino acids, which is common for yeast and bacterial biomasses. The inclusion of *W.anomalus* has shown promising results in feeding experiments with rainbow trout [48].

However, further feeding experiments with *W. anomalus* as SCP in diets for animals and fish are needed to establish the full potential of the protein-rich *W. anomalus* cells produced by the protocols described in this study. It will also be important to investigate how different downstream processing routes, including cell disruptive methods, affect protein digestibility and nutritional value in animals and fish.

Additional research efforts may also needed to further optimize and develop a more economically viable yeast production process based on industrial side streams as substrates, in combination with cheap inorganic nitrogen sources. The poultry by-product hydrolysates used in this study are probably not best suited for SCP production, because they may find higher value applications in other markets, such as in food. Continuous fermentation modes may be worth further exploration, since these are also considered to be good strategies for microbial biomass production [18].

Abbreviations

SCP: Single Cell Protein

YP: Yeast extract and meat peptone

CDW: Cell dry weight

HPLC: High-performance liquid chromatography

ICS: Ion chromatography system

ICP-MS: Inductively coupled plasma mass spectrometry

AA: Amino acid

EAAs: Essential amino acid

NEAA: Non-essential amino acid

$Y_{X/sugars}$: Yield, g dry yeast per g sugar fed (g/g)

$Y_{P/sugars}$: Yield, g dry yeast protein per g sugar fed (g/g)

$Y_{X/glucose}$: Yield, g dry yeast per g consumed glucose (g/g)

$Y_{P/glucose}$: Yield, g dry yeast protein per g consumed glucose (g/g)

Q_x : Productivity, g dry yeast per liter and hour (g/L/h)

Q_p : : Productivity, g yeast protein per liter and hour (g/L/h)

Conflicts of interest: The authors declare that they have no conflict of interest.

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Spruce sugars and poultry hydrolysate as growth medium in repeated fed-batch fermentation processes for production of yeast biomass

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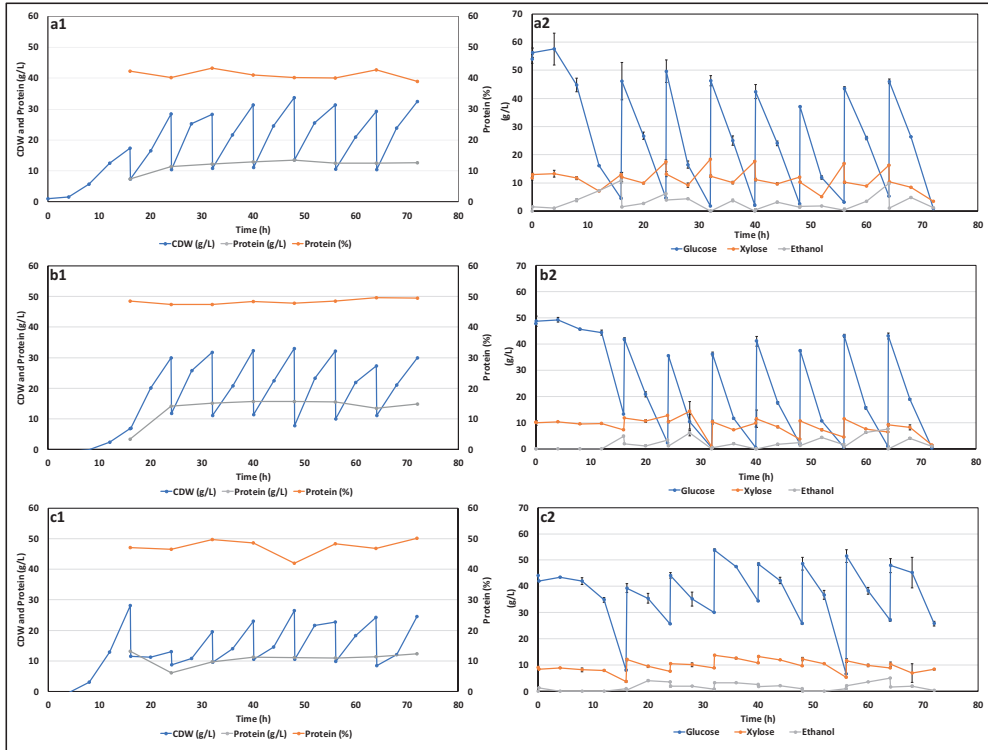
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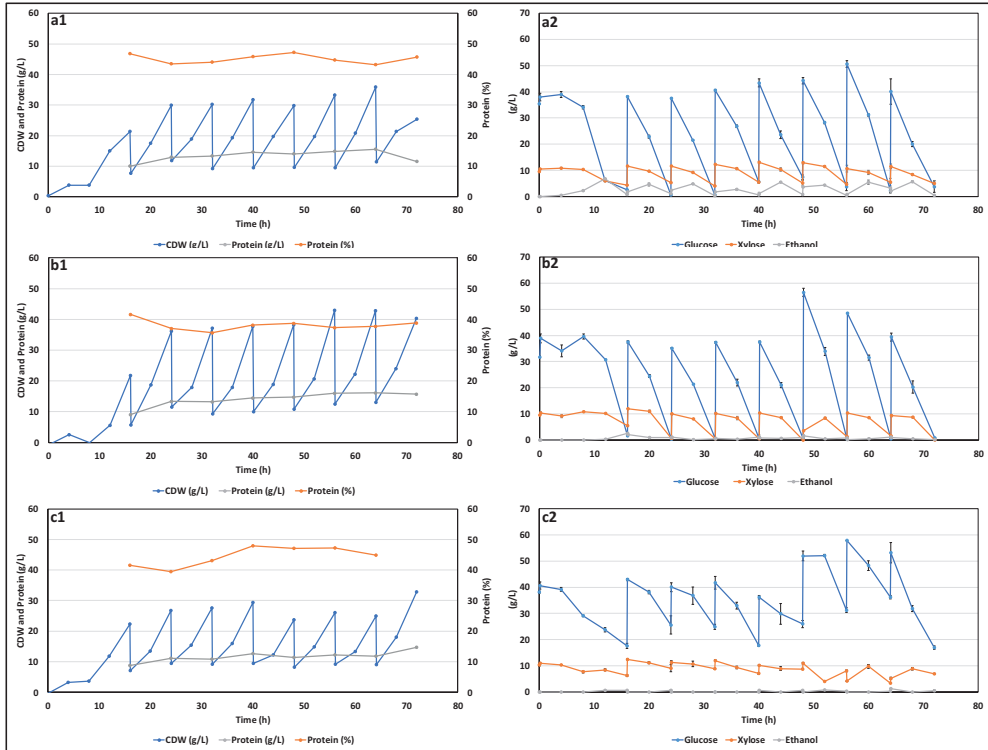
Figure S2. Repeated fed-batch cultivation of three yeast strains grown on 40% poultry hydrolysates and 60% urea with BALI^{TM} hydrolysates in a 2.5 L fermenter. The starting volume was 1.5 L and the fermentation lasted 72 h. Panels labeled 1 show accumulation of cells (blue lines) and protein (grey lines), as well as the protein content of the cells (orange lines); panels labeled 2 show glucose (blue lines), xylose (orange lines) and ethanol (grey lines). a, *C. jadinii*; b, *W. anomalus*; c, *B. adeninivorans*. Growth was monitored by measuring the CDW (g/L, blue lines) every 4 h. For the samples taken from 16 h and onwards, the protein content (orange lines) of the dried cells was measured using the Kjeldahl method (w/w %). The concentration of yeast protein (g/L; grey lines) was calculated by multiplying CDW (g/L; blue lines) with the protein content (w/w %). Acetic acid and lactic acid production were negligible for all yeasts (results not shown).
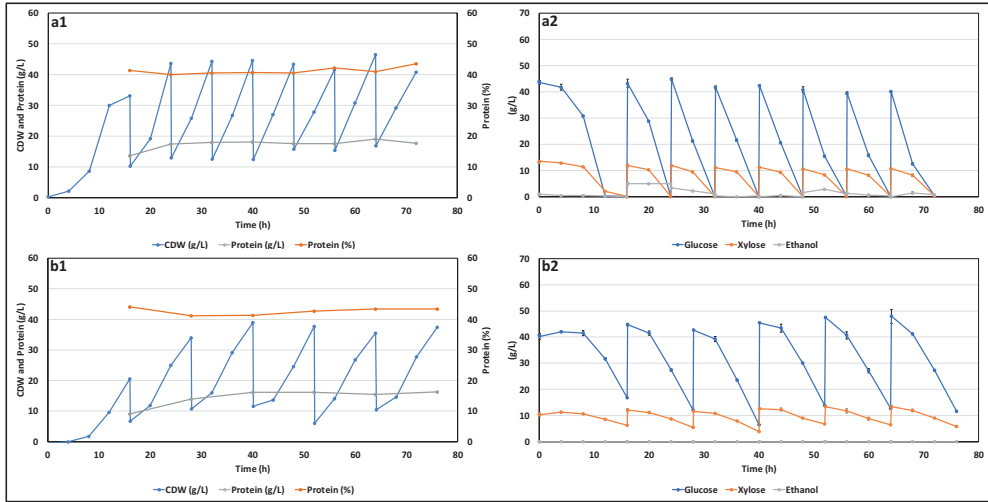
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Figure S3. Repeated fed-batch cultivation of two yeast strains grown on 60% poultry hydrolysates with 40% urea and BALI™ hydrolysates in a 2.5 L fermenter. The starting volume was 1.5 L and the fermentation lasted 72 h and 76 h, respectively. Panels labeled 1 show accumulation of cells (blue lines) and protein (grey lines), as well as the protein content of the cells (orange lines); panels labeled 2 show glucose (blue lines), xylose (orange lines) and ethanol (grey lines). a, *W. anomalus*; b, *B. adeninivorans*. Growth was monitored by measuring the CDW (g/L, blue lines) every 4 h. For the samples taken from 16 h and onwards, the protein content (orange lines) of the dried cells was measured using the Kjeldahl method (w/w %). The concentration of yeast protein (g/L; grey lines) was calculated by multiplying CDW (g/L; blue lines) with the protein content (w/w %). Acetic acid and lactic acid production were negligible for both yeasts (results not shown).
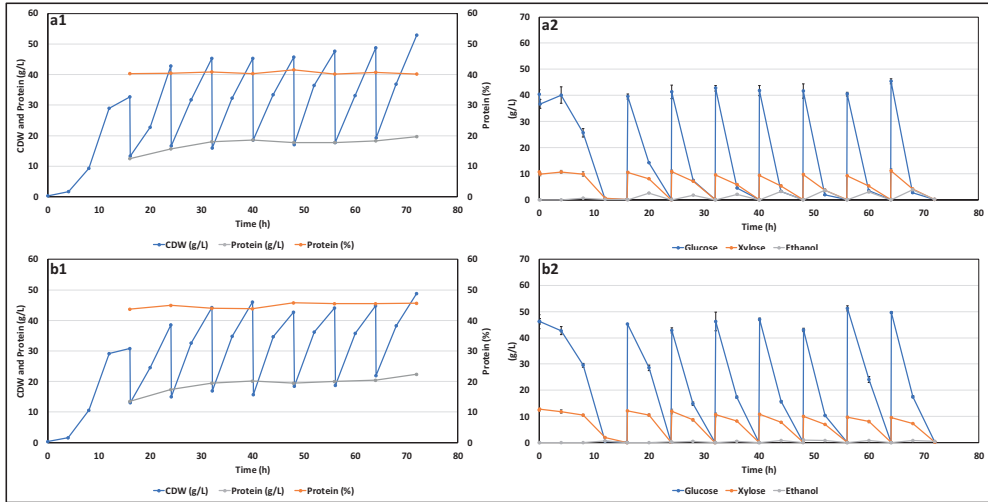
Figure S4. Repeated fed-batch cultivation of *W. anomalus* grown on 60% poultry hydrolysates, 40% urea and biotin or 80% poultry hydrolysates, 20% urea with BALI™ hydrolysates in a 2.5 L fermenter. The starting volume was 1.5 L and the fermentation lasted 72 h. a, 60% poultry hydrolysates, 40% urea and biotin b, 80% poultry hydrolysates, 20% urea. Panels labeled 1 show accumulation of cells (blue lines) and protein (grey lines), as well as the protein content of the cells (orange lines); panels labeled 2 show glucose (blue lines), xylose (orange lines) and ethanol (grey lines). Growth was monitored by measuring the CDW (g/L, blue lines) every 8 h. For the samples taken from 16 h and onwards, the protein content (orange lines) of the dried cells was measured using the Kjeldahl method (w/w %). The concentration of yeast protein (g/L; grey lines) was calculated by multiplying CDW (g/L; blue lines) with the protein content (w/w %). Acetic acid and lactic acid production were negligible on both media (results not shown).
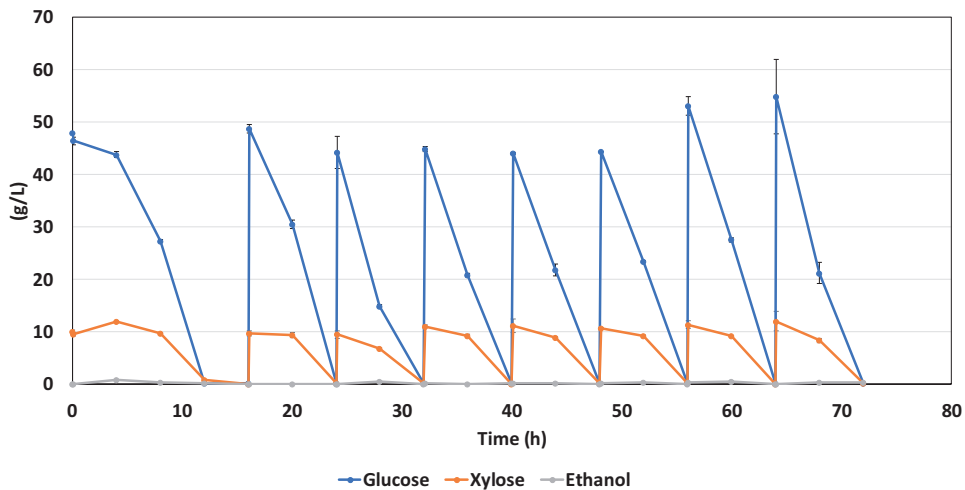
Figure S5. Repeated fed-batch cultivation of *W. anomalus* grown on 80% poultry hydrolysates, 20% urea with BALI™ hydrolysates in a 42 L fermenter. The starting volume was 25 L and the fermentation lasted 72 h. The figure shows glucose (blue lines), xylose (orange lines) and ethanol (grey lines). Acetic acid and lactic acid production were negligible (results not shown). Values are means ± SD (n = 2).

Table S1 Composition of BALI^{TM} spruce hydrolysate

Content	BALI^{TM}
Dry matter (w/w %)	62.3
Density (kg/L)	1.286
Total sugars (% DM)	90.0
Glucose	68.2
Xylose	5.4
Mannose	6.5
Other sugars[a]	9.9
Acids[b]	1.2
Glycerol	0.2
Lignin	5.3

[a] Sum of fructose, arabinose, galactose, gentobiose and cellobiose. [b] Sum of lactic, formic and acetic acid.

Table S2. CDW and protein content in 1.5 L batch fermentations (24h).

		Poultry hydrolysates + BALI^{TM}	
	Sampling time (h)	CDW (g/L)	Protein content (%)
C. jadinii	12	13.4 ± 1.2	47.1 ± 0.7
	24	17.4 ± 1.7	45.1 ± 0.9
W. anomalus	12	17.7 ± 0.4	50.8 ± 0.4
	24	29.7 ± 0.2	45.9 ± 0.3
B. adeninivorans	12	16.4 ± 1.6	47.2 ± 0.4
	24	44.0 ± 0.0	41.9 ± 2.6

Table S3. The numbers and assumptions related to the industrial evaluation of replacing 10 % of the annual Norwegian fish feed protein by yeast produced using the media described in this study.

	Quantity	Unit
Numbers related to replacing 10 % of fish feed by yeast: *		
Produced fish feed per year [42]	1 630 000	tonnes
Replace 10 % of the feed with SCP	163 000	tonnes
Biomass yield on glucose	0.66	g/g
Amount glucose needed for industrial production	250 000	tonnes
Amount of cellulose **	220 000	tonnes
Amount of dry spruce [46]	510 000	tonnes
Amount of wet spruce [47]	780 710	tonnes
% of the annual spruce harvest in Norway [48]	9.6	%
Amount BIOCO hydrolysate (DM) needed (80 %, 4.69 g N/L)	166 200	tonnes
Amount urea needed (20 %, 1.17 g N/L)	12 570	tonnes
Q_X*	3.92	g/L/h
Q_X (converted to tonnes per liter per year) *	0.03434	tonnes/L/year
Total fermentation volume required	4747	m^3
No. of bioreactors of 300 m3 with a working volume of 200 m^3 required to produce the yeast	24	reactors
Numbers related to replacing 10 % of fish feed protein by yeast protein: *		
Amount feed protein per year	815 000	tonnes
Replace 10 % of the protein with SCP	81 500	tonnes
Q_P*	1.87	g/L/h
Q_P (converted to tonnes per liter per year) *	0.01638	tonnes/L/year
Total fermentation volume required	4 975	m^3
No. of bioreactors of 300 m3 with a working volume of 200 m^3 required to produce the SCP	25	reactors

* Based data from the pilot-scale fermentation of *W. anomalus*. Note that the protein content of the yeast was 47.8 %, whereas the protein content of the feed is 50 %.
** Conversion of cellulose to glucose increases the mass by a factor 1.11. Note that the calculation assumes that saccharificaton of cellulose happens with 100 % efficiency.