Thesis for the Degree of Doctor of Philosophy

Integration of filamentous fungi in ethanol dry-mill biorefinery

Jorge A. Ferreira

UNIVERSITY
OF BORÅS

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Swedish Centre for Resource Recovery
University of Borås
SE-501 90 Borås, Sweden

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Cover: Dark field microscopy photograph of submerged growth of *Neurospora intermedia*

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ABSTRACT

The industrial production of bioethanol as a replacement to gasoline is well-established worldwide, using starch- or sugar-rich substrates. Additionally, the bioethanol plants produce animal feeds derived from fermentation leftovers. The biorefinery character of bioethanol plants can be enhanced via process diversification. This entails the production of more value-added products, which can be accomplished by including edible filamentous fungi as the second biocatalysts while taking advantage of the available equipment for cost-effective inclusion. The process diversification can be achieved either via valorisation of the process leftovers or via inclusion of other residual substrates.

In dry-mill biorefineries, baker's yeast is unable to consume residual pentose sugars and other more complex substrates in the process leftovers so called whole stillage and thin stillage. Edible ascomycetes and zygomycetes fungi can be used to accomplish yeast and consume those residual substrates in stillage as well as from external substrates of lignocellulosic origin, e.g. spent sulphite liquor and wheat straw. The conversion of these substrates to ethanol, and biomass rich in protein, lipids, respective essential amino acids and fatty acids as well as chitosan was investigated in this thesis.

Among the filamentous fungi studied, *Neurospora intermedia* was the best ethanol producer from thin stillage. Process developments included primary shake-flasks experiments, followed by pilot scale-up using 26 L, 2.3 m^3 and 80 m^3 bioreactors. The 26 L bioreactor, as a bubble column led to similar performance as an airlift bioreactor, and also a continuous mode could be successfully used instead of a batch process. By using a dilution rate of 0.1 h^{-1}, around 5 g/L of ethanol and 4 g/L of biomass rich in protein, lipids, amino acids and fatty acids essential to humans were obtained. The inclusion of the process can potentially lead to a spent medium lower in solids and viscosity which may facilitate the energy-intensive evaporation and drying steps as well as the water recycling back to the process. By applying a two-stage cultivation with whole stillage, up to 7.6 g/L of ethanol could be produced using 1 FPU cellulase/g suspended solids and 5.8 g/L of biomass containing 42% (w/w) crude protein. In the first stage (ethanol production), *N. intermedia* was used, while *Aspergillus oryzae* was the biocatalyst in the second stage for further biomass production. Both strains were able to degrade complex substrates both in liquid and solid fraction of whole stillage. The extrinsic substrates included spent sulphite liquor and pretreated wheat straw slurry. When the former was used, up to around 7 g/L of *Rhizopus* sp. could be obtained in a 26 L airlift bioreactor. The biomass was rich in protein and lipids (30–50% and 2–7% on a dry weight basis, respectively). The monomers of the latter were continuously filtered for production of biomass under simultaneous saccharification, fermentation and filtration. Biomass yields of up to 0.34 g/g of consumed monomeric sugars and acetic acid were obtained.

The inclusion of the process for valorisation of thin stillage can potentially lead to the production of 11,000 m^3 ethanol and 6,300 tonnes of biomass at a typical facility producing 200,000 m^3 ethanol/year.

Keywords: airlift bioreactors, ascomycetes, biomass, bubble column, ethanol, feed, *Neurospora intermedia*, thin stillage, zygomycetes

LIST OF PUBLICATIONS

This thesis is mainly based on the results presented in the following articles:

I. **Ferreira, J. A.**, Lennartsson, P. R., Niklasson, C., Lundin, M., Edebo, L., Taherzadeh, M. J., Spent sulphite liquor for cultivation of an edible *Rhizopus* sp. *Bioresources*. 2012: **7**, pp. 173-188.

II. **Ferreira, J. A.**, Lennartsson, P. R., Edebo, L., Taherzadeh, M. J., Zygomycetes-based biorefinery: Present status and future prospects. *Bioresource Technology*. 2013: **135**, pp. 523-532.

III. **Ferreira, J. A.**, Lennartsson, P. R., Taherzadeh, M. J., Production of ethanol and biomass from thin stillage using food-grade *Zygomycetes* and *Ascomycetes* filamentous fungi. *Energies*. 2014: **7**, pp. 3872-3885.

IV. **Ferreira, J. A.**, Lennartsson, P. R., Taherzadeh, M. J., A pilot study on production of ethanol and biomass from thin stillage by *Neurospora intermedia* for process diversification. *Engineering in Life Sciences*. DOI: 10.1002/elsc.201400213.

V. Bátori, V., **Ferreira, J. A.**, Taherzadeh, M. J., Lennartsson, P. R., Ethanol and protein from ethanol plant by-products using edible fungi *Neurospora intermedia* and *Aspergillus oryzae*. *Submitted*.

VI. FazeliNejad, S., **Ferreira, J. A.**, Brandberg, T., Lennartsson, P. R., Taherzadeh, M. J., Fungal protein and ethanol from lignocelluloses by *Rhizopus* pellets under simultaneous saccharification, filtration and fermentation (SSFF). *Manuscript*.

STATEMENT OF CONTRIBUTION

Jorge A. Ferreira's contributions to each of the above publications are:

Paper I: Responsible for most of the experimental work, data analysis and writing of the manuscript.

Paper II: Responsible for most of the literature survey, data collection and most of the writing of the manuscript.

Paper III: Responsible for part of the idea, most of the experimental work, data analysis and most of the writing of the manuscript.

Paper IV: Responsible for part of the idea, most of the experimental work, data analysis and most of the writing of the manuscript.

Paper V: Responsible for part of the idea, part of the experimental work, data analysis and most of the writing of the manuscript.

Paper VI: Responsible for part of the experimental work, data analysis and part of the writing of the manuscript.

NOMENCLATURE

AFEX	Ammonia fibre expansion
AIM	Alkali-insoluble material
ALB	Airlift bioreactor
BC	Bubble column
CDS	Condensed distiller's solubles
CSTR	Continuous-stirred tank reactor
DDGS	Distiller's dried grains with solubles
DHA	Docosahexaenoic acid
DM	Dry matter
EPA	Eicosapentaenoic acid
GHG	Greenhouse gases
GlcN	Glucosamine
GlcNAc	N-acetyl-glucosamine
GRAS	Generally regarded as safe
GYV	Glucose, yeast extract and vitamin
LCAs	Life cycle assessments
PM_{10}	Particulate matter
s.d.	Standard deviation
SHF	Separate hydrolysis and fermentation
SS	Suspended solids
SSF	Simultaneous saccharification and fermentation
SSFF	Simultaneous saccharification, fermentation and filtration
SSL	Spent sulphite liquor
SSL50%	Spent sulphite liquor diluted to 50%
Vvm	Volume of air per volume of medium per minute
WO	Wet oxidation

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PREFACE

I started my academic life within the field of applied biology around nine years ago. I soon realised that I did not want just fundamental research but that I needed an applied component. During the last year of my bachelor's course, I saw an advertisement poster about a master's programme in Bioengineering. The figure on the poster was a reactor, and then I knew which path to follow. In 2009, I started my masters program in Bioengineering at the same university (University of Minho), where I just changed departments (from the biology to the biological engineering department). Even though I felt that it was the right path to follow since I had some kind of "crush" on reactors (I think), there was a reason behind the fact I had earned my bachelor's degree and moved to a master's program at a university 30 minutes away from home; I wanted to stay close to my family and help them in the family business.

However, as time went by, I started to develop a feeling that a needed to go out and see the world. One morning, like magic, when we were having this lecture in molecular biology, a friend of mine said he was going to talk to our Erasmus coordinator. He wanted to perform his master's thesis abroad. Automatically, and without thinking at all, I just said 'OK, I'm going with you'! Well, some months later I was dealing with my Erasmus papers. During the process, and considering that I wanted to work with lignocellulosic materials and ethanol, there were universities in two different countries with which our department had collaboration: Sweden and Spain. There is another funny aspect about the subject I chose: I had never heard about ethanol from lignocellulosic materials at that time. However, one day when I was doing some normal searches on the Internet, I came across some article about it and again it just felt right. The choice of the country was quite easy to make: Spain was too close and similar and Sweden was something completely different.

Since the EURO 2004 (football championship) in Portugal and after seeing the Swedish supporters, I thought that living in a country where it would snow for months seemed quite interesting and right at that moment. And it was true, during the winter of 2010 (my first year in Sweden), the pavements and trees being full of snow made me feel like it was Christmas every day and that I was in one of those "Home Alone" movies. Even the choice of the Swedish university where I would do my master's thesis had something funny about it. The first plan was for me to go to Chalmers University in Gothenburg. However, there was one

more person going to the same university, while his close friend (or they were a couple, I don't remember exactly) was going to the University of Borås. Since we could not all three go to the same university, I was the one sent to the University of Borås.

When I arrived and had the first meeting with my supervisors, Mohammad and Patrik, the word "ethanol" was removed. It made me feel a bit disappointed at that moment, but the expression "fish feed" replaced it, and the interesting aspect came back. I started working with spent sulphite liquor (SSL) from a paper and pulp industry for production of biomass using edible filamentous fungi. The work with SSL continued during my PhD thesis (**Paper I**). During my master's thesis, I had the opportunity to work with an airlift bioreactor of 26 L capacity; I felt like a fish in the sea working on exactly what I wanted. That was something that I always thought would be very difficult to accomplish, namely, to have the opportunity to work with bioreactors, big volumes, and to have collaboration with companies.

During the first year of my PhD thesis, I started by doing a literature review about zygomycetes filamentous fungi, something with which our department has more than 10 years of experience, and their relevance for biorefining (**Paper II**). From the research point of view, conducted in the laboratory, other industrial sectors came into play. I started working with leftovers from the Swedish 1st generation bioethanol industry (Agroetanol, Norrköping). Thin stillage was the first leftover substrate on which I developed further research. At a first stage, the focus was on the use of zygomycetes since they had also been used during the work with SSL. However, it seems that I arrived at the right time since the boundaries were extended to include the ascomycetes and that was a breath of fresh air (well two years had passed since I started with the zygomycetes) (**Paper III**). When I started with this part of the thesis work, I felt a lot of motivation because, among other things, we were working towards the improvement of the industrial process, and I just love the word "improvement". I think it is always possible to make improvements.

During my second year, the work with thin stillage went forward and I switched from bench-scale to bioreactor-scale experiments. Suddenly, I left behind the 50 mL of medium and started to receive 1 m^3 containers of thin stillage; I left behind the 250 mL Erlenmeyer flasks and started to work again with the 26 L airlift bioreactor. At this point, I tested the aeration rate, reactor design and cultivation mode effect on production of ethanol and fungal biomass using the fungus *Neurospora intermedia* (an ascomycete) (**Paper IV**). During this second

year, I also started collaborating with a work towards production of biomass and ethanol using pretreated wheat straw slurry using zygomycetes (**Paper VI**). It was quite interesting since I was working with perfect spherical pellets all the time!

At the beginning of my third year, trial runs had started at the company Agroetanol in Norrköping towards production of ethanol and biomass from thin stillage with *N. intermedia*. I left my "little" bioreactor of 26 L and started to work with bioreactors of 2.3 m^3 and 80 m^3 capacity. That was another thing that I've always wanted to do, that is, to see a process go up in scale. The time I spent there was very interesting; I really learned a lot and the bridge between academia and industry had then been fully established. In parallel, I started a collaboration work with whole stillage (also from the bioethanol industry) for production of ethanol and biomass using ascomycetes fungi (**Paper V**).

This has been my last year and well, it has been about finishing some manuscripts, keeping up with the trials at the company for further insights, some teaching, and the thesis is right here around the corner.

CHAPTER 1

1 INTRODUCTION

1.1 Preface and scope

In an increasingly industrialised world, every single industrial process gives rise to value-added products but also to leftovers that need to suffer further processing before disposal or conversion to other products of lower value. Therefore, huge amounts of industrial sidestreams are available worldwide and steadily increasing. Therefore, cost-effective processes are needed for the treatment of such leftovers. Moreover, it is becoming globally accepted that humans live beyond available resources on Earth; thus, being able to use raw materials efficiently must continuously evolve and improve. Depletion forecasts of oil reserves have also put pressure on society to move away from oil refineries and look for alternative green energy and chemicals provided by biorefineries. Bioethanol has gathered intense research interest over the years, as gasoline substitute for the steadily expanding transport sector. Pressure also exists on production of alternative sources of protein for animal or fish feed that can replace human proteins. Edible filamentous fungi with their high metabolic diversity are good candidates as core or partial biocatalysts in biorefineries. They can contribute for the treatment of those side streams via assimilation of organic matter followed by its conversion to value-added products such as ethanol and fungal biomass for feed applications.

The 1st generation bioethanol plants, which use sugar or starch from agricultural crops, represent a technology that is well established on industrial scale. Nowadays, they are crucial alternative source of ethanol used as a green replacement of gasoline. Beyond ethanol, this industrial sector also produces protein for animal feed (known as DDGS – distiller's dried grains with solubles) from process leftovers. Therefore, 1st generation bioethanol plants have been making an important contribution towards reduction of emissions of greenhouse gases as well as towards the increase of alternative sources of proteins. However, there are some constraints related to 1st generation bioethanol plants, including the energy intensive character of the process, mostly related to the production of DDGS, the overtime feasibility of the facility, and the use of sugar and starch from agricultural crops that could be diverted to

human consumption. The present thesis has addressed all of these constraints. The first two are related, and the approach was to valorise the leftovers, including thin stillage and whole stillage, used to produce the DDGS, via production of additional ethanol and fungal biomass. The proposed biocatalysts were edible filamentous fungi. In order to grow and produce ethanol, the filamentous fungi have to assimilate organic matter from the medium, giving rise to a spent medium with lower content of solids. This will hopefully lower the energy need for production of DDGS. Another strategy was to study the potential of different origins of substrates of lignocellulosic nature, extrinsic to the process (namely, SSL and wheat straw), to be included in the established bioethanol facilities. Such strategy can lead to faster development of biorefineries, as the process is much more troublesome if an entire facility is to be realised. Lignocellulosic materials do not compete with human food, are cheap and abundant, and have been considered as potential substrates for production of the so called 2nd generation bioethanol. However, its industrial realisation faces many constraints, and the present strategy could be a step forward to that industrial realisation. A typical bioethanol facility producing 200,000 m^3 of ethanol per year was used as a model in the present thesis. The flow of thin stillage to the evaporators was considered to be 200 m^3/hour.

The main goals of the present thesis were to highlight the relevance of edible filamentous fungi as biocatalysts in biorefineries and to investigate and develop edible fungal strains and cultivation strategies for production of ethanol and fungal biomass. Various industrial substrates that can potentially lead to improvements of the 1st generation bioethanol plants were considered. In order to accomplish these goals, the work was divided into six parts:

- Utilisation of spent sulphite liquor for production of fungal biomass (**Paper I**).
- Literature review on the relevance of zygomycetes fungi for inclusion into biorefineries (**Paper II**).
- Utilisation of thin stillage for production of ethanol and fungal biomass (**Paper III**).
- Influence of aeration rate, reactor design and cultivation mode on production of ethanol and fungal biomass from thin stillage (**Paper IV**).
- Impact of cellulase addition to whole stillage on production of ethanol (**Paper V**).
- Utilisation of SSFF for production of fungal biomass by assimilation of carbon sources in pretreated wheat straw slurry (**Paper VI**).

1.2 Thesis outline

The present thesis is divided into seven main chapters:

- **Chapter 1** introduces the thesis, explains the motivation, the goals, and provides an overview of oil refineries and biorefineries. Ethical and social aspects are also included in this chapter.

- **Chapter 2** introduces the 1st generation bioethanol plants with its characteristics and potentialities.

- **Chapter 3** approaches the potential substrates, both intrinsic and extrinsic to the 1st generation bioethanol plant, which can be used for further process improvements.

- **Chapter 4** gives an overview of potential fungal biocatalysts for inclusion in the 1st generation bioethanol plants.

- **Chapter 5** deals with the characteristics of fungal biomass, potential feed markets, and reports on results obtained from biomass and ethanol production using shake-flasks.

- **Chapter 6** presents the extent to which the research has progressed using each of the substrates considered in this thesis, giving insights on the results obtained with different bioreactors of different designs and sizes. The results of the scale up of the process of ethanol and biomass production using thin stillage are included in this chapter.

- **Chapter 7** wraps up the main findings of the thesis and gives directions for future research.

1.3 Oil refineries

Refineries using fossil fuels, in particular oil, as feedstocks have been the main source of primary energy and chemicals worldwide [1]. Nowadays, the daily world oil demand is around 84 million barrels, and it is projected to reach about 116 million barrels by 2030. A

major share of the oil consumption of 60% is utilised by the transport sector while the chemical and plastic production accounts for 4% [2, 3]. Nonetheless, a detrimental impact on the environment is associated with the intensive use of fossil fuels via extraction, transportation, processing and particularly during combustion [4]. The problem is exacerbated by the emerging world economies, especially China and India, and growing population and per capita energy demand that contributes to the rapid increase in greenhouse gas (GHG) emissions [5, 6] (**Figure 1.1**). Being of non-renewable character, the increase in oil demand leads to diminished resources. Indeed, there has been an intense debate on whether the maximum production of oil has already been reached and if it will start do decline. The fact is that oil areas that are easily accessible have already been reported as depleted, and the exploitation of lower grade oil and less accessible oil areas has started. Tar sands and oil shales have been considered as possible oil resources and their exploitation has started despite its expensive and environmentally hazardous character [7-9].

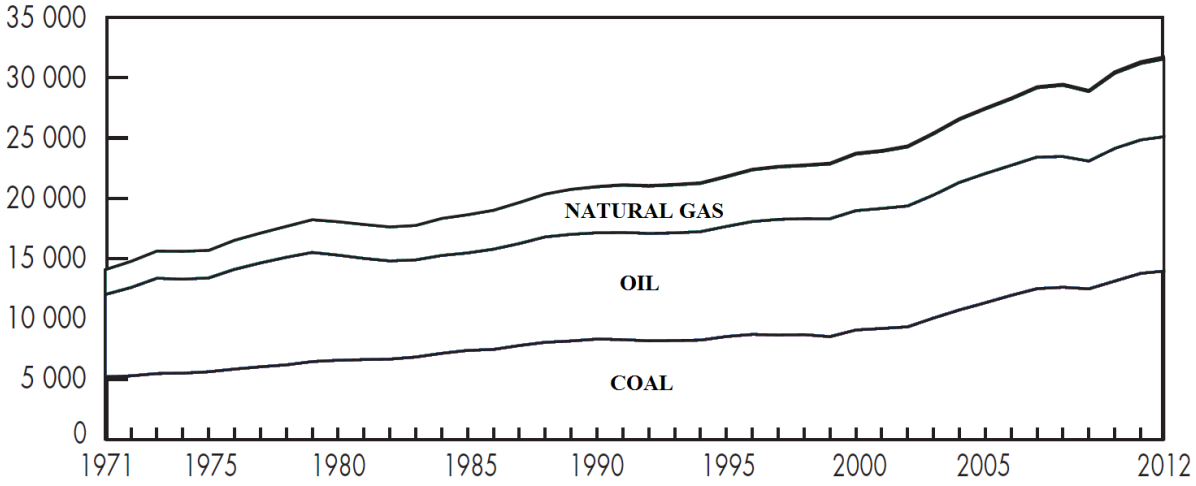

Figure 1.1: World CO_2 emissions in megatons from 1971 to 2012 from combustion of coal, oil and natural gas [10]. Reprinted with permissions.

The oil supply has a tremendous influence on the stability of the world economic and social system. Large price increases can destabilise the world macro economy, similar to abrupt price increases for food and other raw materials [11]. Therefore, strategies to overcome the oil dependence are urgently needed and arguably those should greatly focus on the transportation sector. However, it is defended that inflicting a decrease in oil dependence and climate change within the transport and chemical sectors requires a combined and complex action. Changes in behaviour, changes in vehicle technologies, expansion of the public transport and

introduction of innovative fuels and technologies have been stated as being part of such an action plan [12]. A panoply of fuel substitutes has been suggested including bioethanol, biodiesel, biomethane, dimethyl ether or hydrogen, produced from renewable resources in biorefineries [13]. The selection of biofuels with the highest potential is based on the possibility of being mixed with gasoline, kerosene, diesel and natural gas, taking advantage of the existing infrastructure [14]. Bioethanol is one of the bio-based products on which the present thesis has its focus.

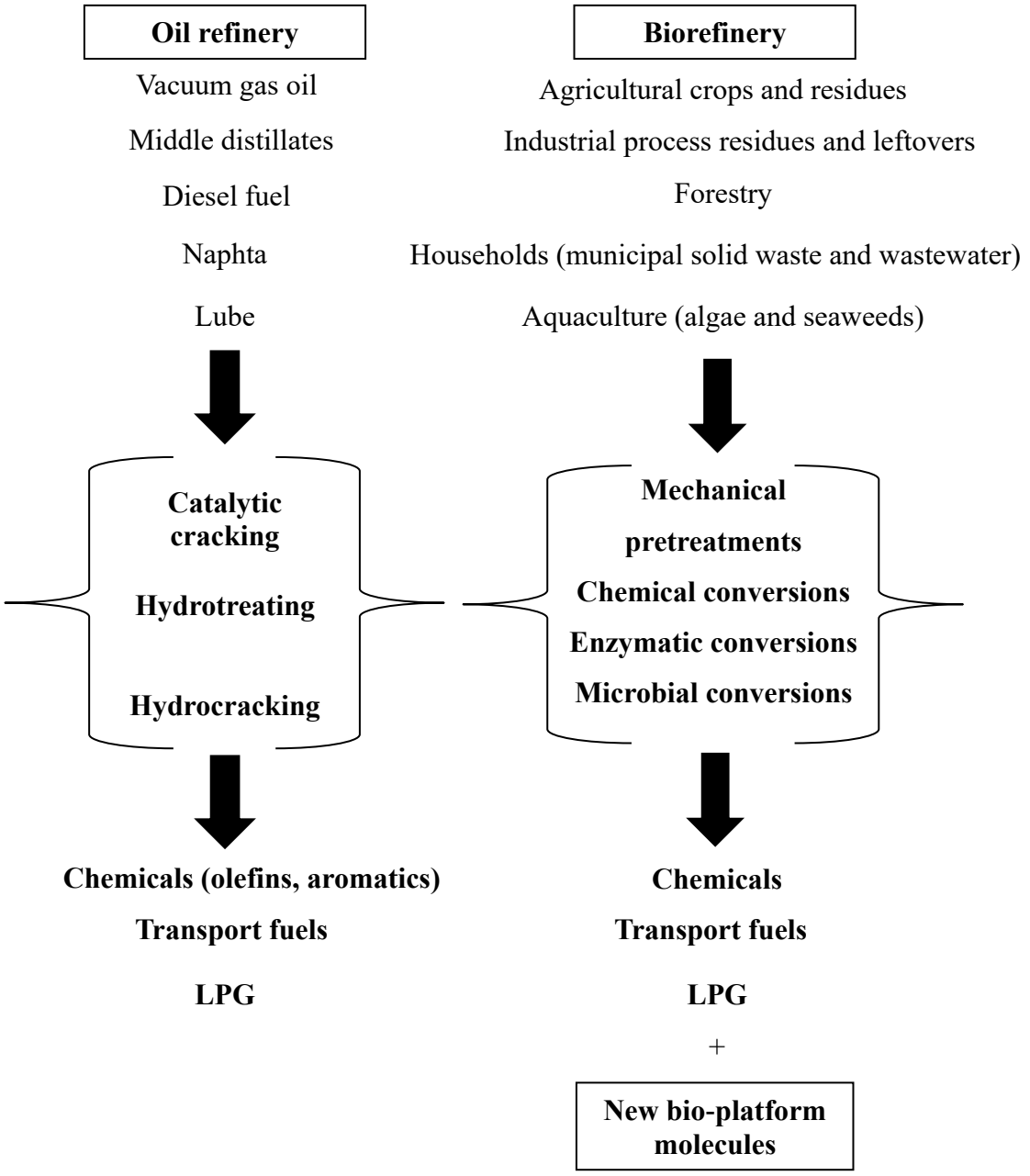

Figure 1.2: Process differences between an oil refinery and a biorefinery. Adapted from [1, 9, 14, 15].

1.4 Biorefineries

Attempting to have a sustainable economy based on renewable sources, worldwide entities have either started to finance or have intensified the financing of R&D projects, for its implementation. Plant-based materials (e.g. biomass) have increasingly been considered worldwide as having the potential to gradually replace the fossil fuels as feedstocks for industrial production of bioenergy, biofuels and biochemicals [16]. Electricity and heat can alternatively be provided from e.g. sun, wind, water and biomass. However, biomass, being the only available carbon-rich source on Earth beyond fossil fuels, is more likely the only one viable alternative for production of transportation biofuels and biochemicals. An implication of such exclusivity, however, concerns competition with possible fertile land for the food and feed industries [1].

According to the IEA Bioenergy Task 42 [17] '*Biorefining is the sustainable processing of biomass into a spectrum of marketable products and energy*'. A biorefinery can be a facility (or network of facilities), a process or a plant integrating the integral upstream, midstream and downstream processing of biomass to transport biofuels, power and chemicals [14]. Therefore, it resembles today's petroleum refinery, which produces multiple fuels and products from petroleum [1]. However, biorefineries have the potential to produce a wider variety of value-added products in comparison to that of oil refineries, in view of the higher compositional heterogeneity of possible feedstocks (**Figure 1.2**).

The future development of biorefineries will be promoted by the need to produce renewable biofuels for the steadily increasing demands of the transportation sector [18]. Therefore, the main challenge regards the efficient and cost-effective production of transportation biofuels, together with the production of biomaterials and biochemicals for additional economic and environmental benefits [1]. This has become the background for the development of the biorefinery classification system in 2008 by the IEA Bioenergy task 42, based on a schematic representation of full biomass to end-products chains (**Figure 1.3**) [19-21]. The classification system is based on four features in order to identify, classify, and describe the different biorefinery systems. Those include platforms, that being intermediates and able to connect different biorefinery systems or final products give an indication of the system complexity (e.g. C5/C6 sugars, syngas, biogas); products including energy (e.g. bioethanol, biodiesel, synthetic fuels) and bio-based materials and chemicals including food and feed; feedstocks

including “energy” crops from agriculture (e.g., starch crops, short rotation forestry) and “biomass residues” from agriculture, forestry, trade, and industry (e.g. straw, bark, wood chips from forest residues, used cooking oils, wastestreams from biomass processing); and conversion processes including biochemical (e.g. fermentation, enzymatic conversion), thermochemical (e.g. gasification, pyrolysis), chemical (e.g. acid hydrolysis, synthesis, esterification), and mechanical processes (e.g. fractionation, pressing, size reduction) [14]. A full overview of the platforms, products, and conversion processes is depicted in **Figure 1.3**.

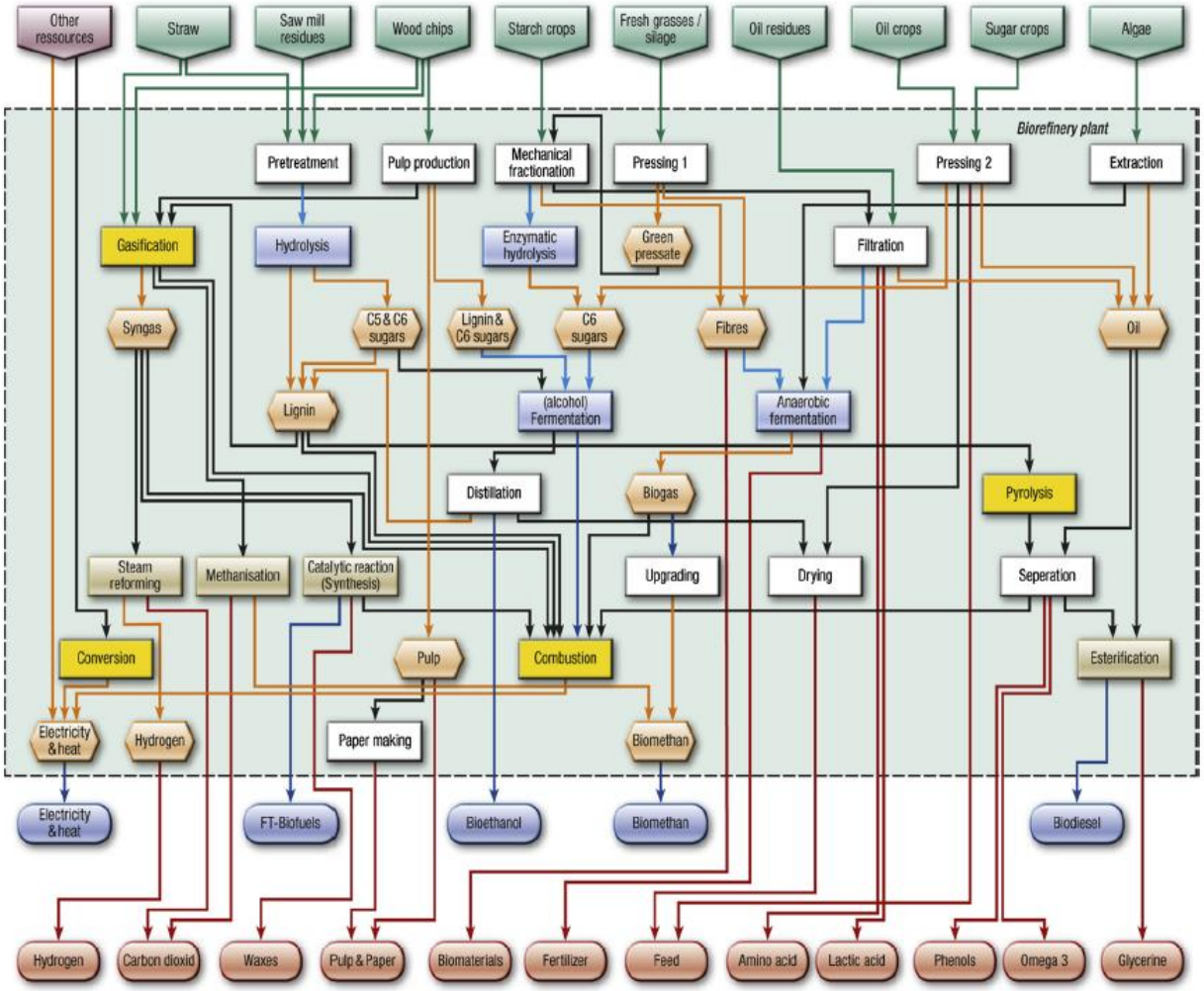

Figure 1.3: Network on which the biorefinery classification system is based [22]. Reprinted with permissions.

Some examples of classifications are the following:

- Oil biorefinery using oilseed crops for biodiesel, glycerine and feed.
- C6 sugar platform biorefinery for ethanol and animal feed from starch crops.

- Syngas platform biorefinery for Fischer-Tropsch (FT) diesel and phenols from straw.
- C5 and C6 sugars, electricity and heat, lignin biorefinery using wood chips for ethanol, electricity, heat and phenols.

A biorefinery, however is not, a completely new concept. Conversion techniques, pretreatment and separation were applied for the production of vegetable oils, beer and wine thousands of years ago; the production of paper started around 100 AD. The sugar, starch and pulp and paper industrial sectors are traditional examples of primordial biomass-converting technologies that have greatly developed through the years and which can be (partly) regarded as biorefineries [14]. Nowadays, in addition to the main bio-based products obtained from the conversion of biomass to basic products such as starch, oil and cellulose, other bio-based products have been produced. Those include lactic acid, amino acids, adhesives, cleaning compounds, detergents, dielectric fluids, dyes, hydraulic fluids, inks, lubricants, packaging materials, paints and coatings, paper and box board, plastic fillers, polymers, solvents and sorbents [1]. Furthermore, the world-scale production of bio-based intermediates such as 1,3-propanediol, isobutanol, succinic acid, and 1,4-butanediol is presently available [23]. However, even for those established industrial bioprocesses, further process improvements within a biorefinery concept are arguably motivated. Those are driven by economic and environmental aspects including global warming, energy conservation, security of supply and agricultural policies. This will likely lead to enhanced integration and optimisation aspects for all biorefinery subsystems [14].

1.5 Ethical and Social Aspects

According to the Global Footprint, a nongovernmental organisation, the resources generated by 1.1 planets were needed to fulfil needs of mankind in 1975, compared to 1.3 planets in 2005. This year 1.5 planets will be needed by mankind. In December, the world conference on climate change will take place in Paris. Unless the 195 participating countries come to an agreement regarding the reduction of 30% in the CO_2 emissions, the use of the Earth's resources can go back to those values of 2005. On the contrary, resources generated by two planets will be needed to meet the needs of mankind by 2030. The use of fossil energy is probably the main culprit of the world rising CO_2 emissions. Evidently, society urgently needs to move towards greener processes. This can be accomplished by using renewable resources and continuously improving the processes, leading to less energy and water consumption and

higher yields of the desired products. In practice, it is necessary to make more from the same or less.

This thesis follows this line of thinking. Taking the 1st generation bioethanol plants as the core case, the present thesis focuses, to a great extent, on the leftovers (thin stillage and whole stillage) after distillation of ethanol. These are normally dried to produce animal feed products. The steps leading to the animal feed production are energy- and cost-intensive. One of the goals of the thesis was to investigate if other products could be produced from the leftovers. Indeed, ethanol and fungal biomass for feed could be produced. Therefore, if the process is included on industrial level, more ethanol can be produced from the same amount of grains. Ethanol can be used as greener transportation fuel, alternatively to gasoline. In addition, one of the premises of the present thesis was to limit the use of biocatalyst to edible filamentous fungi. All filamentous fungi used in this thesis have been used for production of fermented foods for human consumption for a long time. Therefore, they are well known and generally regarded as safe (GRAS) microorganisms. This aspect has some positive consequences: the process of feeding trials is easier and the probability of public acceptance is higher when compared with e.g. genetically-modified microorganisms or bacteria. The produced fungal biomass can be an alternative source of protein for animal or fish feed to the commonly used soybeans-based meals. The latter could be diverted for human consumption instead. Nevertheless, the present thesis also focuses, to a great extent, on the spent medium after fungal cultivation and after the biomass has been harvested. Thus, the removal of solids was a frequent area of analysis throughout the thesis. In order to produce ethanol and biomass, the filamentous fungi assimilate organic matter from the medium. Therefore, it is hypothesised that the drying and evaporation steps needed for the production of animal feed in the 1st generation bioethanol plants as well as the recycling of water back to the process will become easier. The aspect of organic matter is of high importance since most industrial sidestreams have to undergo wastewater treatments before disposal. On the contrary, it would promote serious environmental problems, as was the case before the legislation was tightened. This is how it was for the leftovers from the paper and pulp industry that was studied in this thesis. In addition, the use of agricultural residues for ethanol production is of special importance. Since it is not in competition with human consumption, it would be the perfect alternative to the grains used nowadays for ethanol production. The intensive debate on “Food versus Fuel” in the case of the 1st generation bioethanol plants would be smoothened, to a great extent. Altogether, the present thesis provides insights on valorisation of substrates with

high organic load for production of ethanol and fungal biomass for feed purposes. The ultimate goal was to increase the amount of green fuel for the transport sector from the same amount of raw material used and to diversify the type of products for animal feed, possibly together with easier drying and evaporations steps, leading to the production of DDGS, as well as the recycling of water back to the process.

CHAPTER 2

2 1ST GENERATION BIOETHANOL BIOREFINERIES

2.1 (Bio)Ethanol

Ethanol, or ethyl alcohol, is a volatile, flammable, and colourless liquid. Although its production using renewable feedstocks (bioethanol) seeks the replacement of gasoline in the transport sector, ethanol is also used in a variety of industries including chemical, cosmetic, pharmaceutical and medical as well as the automotive and beverage sectors. Traditional products where ethanol is used include perfumes, deodorants, de-icer and anti-freeze products, paints, thermometers, sanitary wipes and antibacterial hand gels, vodka, gin and ready-to-drink mixes [24].

The transportation sector is responsible for more than 25% of EUs total GHG emissions, with a rising trend. Among the biofuels considered to replace transportation fossil fuels, bioethanol is the most cost-effective and readily available alternative. The use of bioethanol is estimated to reduce transport sector GHG emissions by up to 90%, and it can be further improved if the right policies are in place [24]. Actually, ethanol has long been considered as a fuel for vehicles and it was, for example, one of the options in Henry Ford's Model T cars [25]. Henry Ford saw ethanol as "the fuel of the future" already in 1925 [26]. Traditionally, the car fuel contains up to 5% ethanol, while cars built since 2000 can run on fuel with 10% ethanol known as E10 [24]. The blending of gasoline with ethanol leads to more efficient fuel usage, via boosting of the octane rating [25]. Therefore, the use of bioethanol has the potential to decrease carbon not once, but twice during its production and combustion [24]. Beyond its well-stated beneficial effect when used as alternative to fossil fuels, the bioethanol sector makes an important contribution to the creation of jobs and revitalisation of rural areas. The European renewable bioethanol industry has created and sustained 70,000 direct and indirect jobs, and the potential for creating 1 million new jobs in Europe exists [24].

The production of bioethanol has been steadily increasing in EU since 2003, and values from 2014 point towards a 6.7 billion litres installed production capacity. Therefore, EU is still a

modest player globally when compared to the annual production of 50 billion and 23 billion by USA and Brazil, respectively [27]. Such developments and increases in the bioethanol production were motivated by high stated goals by several countries worldwide. For instance, the European Union set a goal that by 2020, 10% of the transportation fuel should be bioethanol, and the US Department of Energy Office set a goal that by 2030, 30% of gasoline should be replaced by biofuels [28]. The industrial-scale bioethanol production plants can be found in 20 EU member states, where France and Germany are the largest European producers [24].

Currently, transportation fuels based on biomass are identified as 1st and 2nd generation biofuels. The former include bioethanol, biodiesel, starch-derived biogas, vegetable oils, biomethanol and bioethers produced from raw materials in competition with food and feed industries. The latter include Fisher Tropsch (FT)-diesel from biomass and bioethanol from lignocellulosic feedstock produced from non-food crops [1]. Contrary to 1st generation biofuels, technologies for 2nd generation biofuels are currently at a pre-commercial stage.

2.2 1st generation bioethanol production plants

Substrates for production of 1st generation bioethanol include starch-based crops such as wheat, maize, corn, barley, rye, triticale (a hybrid of wheat and rye), or sugar-rich crops such as sugar beet or sugarcane [24, 29]. The leading bioethanol producers USA and Brazil use corn and sugarcane as primary feedstocks, respectively [29]. In Sweden, the commercial production of ethanol is mainly from wheat grains [30].

The production of ethanol from starch-containing crops can be carried out either by the dry-grind or wet-mill process, the main being difference the number of final products obtained [31]. During the wet-mill process, many different components in the grain can be separated, including starch, fibre, gluten and germ, rendering it a more capital- and energy-intensive character but also the title of “true biorefinery” [31].

The dry-grind process, in focus in this thesis, gives rise to two products in addition to CO_2, namely, ethanol and animal feed products known as DDGS. During the dry-grind process, the whole grain is ground by hammer mills into a powder, with a mean particle diameter of approximately 1 mm [32]. The powder is mixed with water to form a mash, which is cooked;

thereafter, enzymes are added to convert starch into sugar monomers. The baker's yeast *Saccharomyces cerevisiae*, the main biocatalyst used for bioethanol production, then converts the glucose monomers into ethanol. An ethanol concentration of typically 10-12% (w/v) is obtained at the end of the fermentation [33, 34]. The hydrolysis step can be eliminated by concomitant addition of saccharifying enzymes and yeast to the fermentation vessel [14]. When the fermentation is finished and the CO_2 has been sequestered, the aqueous slurry of yeast cells and residuals from the ground grain kernels remaining after fermentation goes to the distillation column [31]. The ethanol is then separated, purified and dehydrated to concentrations above 99.7% for fuel applications, according to the European standard EN 15376 [35]. The non-volatile components leave the process at the bottom of the rectifier as a product called whole stillage [31]. Whole stillage contains fibre, oil, protein, other unfermented components of the grain, yeast cells and by-products such as glycerol from the fermentative metabolism of the microorganism. Organic acids such as lactic and acetic acids originated from contamination can also be found. The whole stillage suffers a centrifugation step, giving rise to a liquid fraction known as thin stillage, and a solid fraction known as wet distiller's grains. A fraction of at least 15% of the thin stillage is recycled as backset water [36], while the remaining is concentrated through multiple effect evaporators to produce a syrup called condensed distiller's solubles (CDS) [37]. In order to extend the shelf-life, the wet distiller's grains and syrup are often dried together to produce DDGS, a protein source for animal feed [14, 38] (**Figure 2.1**). In 2011, the bioethanol sector produced 68 million tonnes of DDGS [29], arguably playing a crucial role in the overall process economy.

The main advantages of the 1st generation bioethanol production plants include the high sugar content of the feedstocks as well as their easy conversion to bioethanol. Life cycle assessments (LCAs) performed in order to analyse the bioethanol production chains have unveiled a net reduction in global GHG emissions and fossil energy consumption when bioethanol is used to replace conventional gasoline [39, 40].

Since the feedstocks of the 1st generation bioethanol production plants are agricultural crops, the ethical and social aspects around the food vs fuel issue have intensively been debated. However, according to the State of the Industry Report 2014 [24], the bioethanol production makes excellent use of available space, especially when one considers that the crops and refining process are also used to produce high protein content animal feed. Indeed, according to the UNs Food and Agriculture Organization (FAO), the use of animal feed co-products

from the EU biofuel production (including EU biodiesel) reduces global land use by about 3 million hectares [24]. It is additionally defended that renewable bioethanol has had a tiny impact on global food commodity prices to date and it is not expected to have any larger impact in the future [24].

The 1st generation bioethanol plants are characterised by a low rate of return, being dependent on the cost of the feedstock and the price of process products, namely, ethanol and DDGS [33]. Besides, there is a potential that supply of feedstock becomes a hurdle when compared with the potential demand [41]. Therefore, the expansion of bioethanol sector has been motivated to take place away from food-based feedstocks [33].

2.3 Process diversification

There are many motivators behind the continuous diversification of well-established industrial biorefineries. Those can include economic, environmental and social aspects. The main premise behind biorefining can perhaps be translated as ‘doing more and better from less’. Thus, the R&D department should play an important role in any biorefinery, aiming at making the process of biomass conversion cleaner, more cost-effective and with a gradually increasing diversification of the variety of value-added products produced [14]. The latter can have huge effects on the economic viability of an industry on a short, medium and long term.

A common strategy is to use low-value process sidestreams as substrates for further production of value-added products. This is normally carried out by using microorganisms as catalysts. However, following the premise of biorefinery networking, there is also the potential of including substrates extrinsic to the industrial process. Therefore, in both situations, a good knowledge and understanding of the industrial process with all its sidestreams and external substrates characteristics are arguably needed.

Using an already established biorefinery for further process improvement, via inclusion of substrates from other biorefineries or industrial sectors (e.g. agricultural), is of special interest. It can take advantage of the knowledge and experience gathered during the years when running the plant as well as take advantage of most (if not all) needed process equipment already available at the place. Furthermore, even though new concepts of biomass conversion to value-added chemicals have been developed, their commercial realisation can

be hampered by several factors including relatively low oil prices, credit crisis and recession in parts of the global economy [14]. Therefore, biorefinery networks can be an easier way of keeping a continuous flow of new commercially-available processes for biomass conversion. This can be the case for the 2nd generation bioethanol, industrial realisation of which is difficult to accomplish. Therefore, including substrates of lignocellulosic origin in the process of the 1st generation bioethanol plants could potentially lead to the production of 2nd generation bioethanol [33]. However, such inclusions that lead to changes in the process must ascertain that the quality of the DDGS is not compromised [33].

In this thesis, the 1st generation bioethanol plant based on mostly wheat grains has been considered as the model biorefinery for further process improvements. The proposals studied included the valorisation of the process sidestreams, namely, whole stillage and thin stillage to value-added products. Being composed of unfermented components from fermentation, they are good sources of carbon and nitrogen that microorganisms can use. The design of the process also allows external substrates such as SSL or wheat straw of lignocellulosic origin to be included in the process, with high potential for the establishment of a biorefinery network. The proposal of the present thesis was to evaluate the potential of these four alternative substrates for production of ethanol, biomass for feed, or both products using edible filamentous fungi. As can be seen in **Figure 2.1**, the investment burden with the inclusion of such processes would be lessened since ethanol recovery and drying apparatus are in place. For example, sending back the condensate after the series of evaporations is a common practice nowadays. Therefore, the produced ethanol could just follow the normal stream of condensate back to the process and then towards the distillation column. Thus, the net result would be a flow of fermented mash towards the distillation column, with a higher concentration of ethanol. The harvested biomass could be dried together with the whole stillage solids in order to enhance the quality of the DDGS, or dried separately and sold as a single product. The implementation of the former is simple, while the latter can lead to a product of higher price due to the biomass composition (discussed in detail in **Chapter 5**). Moreover, by assimilating nutrients from the medium to growth and to produce ethanol, filamentous fungi would contribute by lowering the solid loading in the evaporators, making the evaporation and drying steps easier [33]. Concomitantly, the recycling of water back to the process would also be easier.

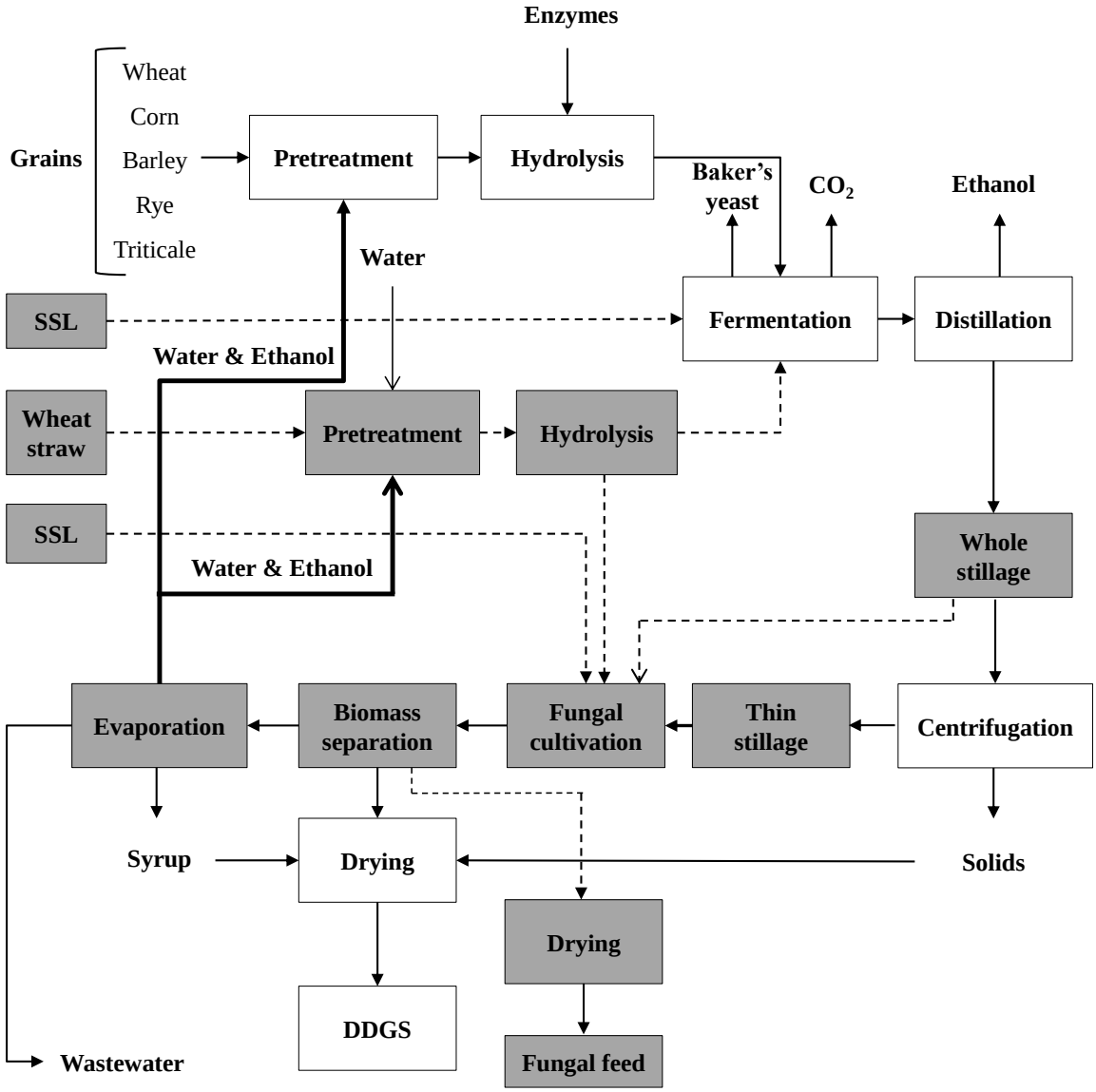

Figure 2.1: General overview of the main starch-based bioethanol process stages leading to the production of ethanol and DDGS. Dashed lines and grey boxes include potential changes in the process including the valorisation of facility's own sidestreams or the inclusion of external substrates such as SSL and wheat straw for production of ethanol and biomass for feed using filamentous fungi. The external substrates can enter the process either in the fermentation step or after the distillation.

CHAPTER 3

3 POTENTIAL SUBSTRATES FOR PROCESS DIVERSIFICATION OF 1ST GENERATION BIOETHANOL PLANTS

The substrates intrinsic to the ethanol dry-mill biorefinery were provided by the Swedish ethanol facility located in Norrköping, mostly based on wheat grains. In this chapter, an overview is provided on the characteristics as well as the potentialities of the four substrates studied, namely, whole stillage, thin stillage, wheat straw and SSL.

3.1 Substrates intrinsic to the process

Wheat (*Triticum aestivum* L.) is the world's most widely grown crop; over 115 nations cultivate this agricultural crop [42]. According to the International Grains Council in 2015, around 883 million tonnes of wheat were produced during 2013–2014, and forecasts point towards a production of 905 million tonnes during 2014–2015, with Asia (43%) and Europe (32%) being the main production regions. The increase in wheat cultivation is promoted by the growing demand of human consumption [43].

As shown in **Figure 2.1**, the milled, saccharified and fermented wheat grains give rise to ethanol, CO_2 and DDGS. However, the steps leading to the production of the DDGS including series of evaporations and drying represent a large fraction of the energy spent by the facility. During the process, some sidestreams (namely, whole stillage and thin stillage) were generated; moreover, based on their composition, they were considered to be potential sources of substrates for microbial growth for further production of ethanol and biomass for feed (**Tables 3.1** and **3.2**). Hence, higher amounts of these two products can be achieved from the same amount of input material together with an easier process of evaporation and drying due to spent media with lower content of solids. Therefore, there is high potential for process improvements.

3.1.1 Whole stillage and thin stillage

During the distillation of ethanol, the slurry that leaves the bottom of the rectifier is commonly regarded as whole stillage. Whole stillage contains oil, protein, fibre and other unfermented components of the grains, as well as yeast cells [44]. Glycerol and organic acids (namely, lactic and acetic acids) can also be found, originating from the fermentative metabolism of the baker's yeast and contaminants, respectively. Per each litre of ethanol produced, up to 20 litres of whole stillage arise when corn or wheat grains are used as feedstocks [45]; thus, more than a billion tonnes per year are globally available. Whole stillage can be composed, to a large extent, of un-degraded wheat bran, rendering it a lignocellulosic character. Therefore, the valorisation of whole stillage to e.g. ethanol could become an important step towards the industrial production of 2nd generation bioethanol.

Table 3.1: Characteristics of whole stillage that was used in **Paper V**.

		Dissolved monomers (g/L)		**Dissolved saccharides (g/L)[c]**		**Sugar polymers (g/L)[d]**	
Parameter	**Value**	**Parameter**	**Value**	**Parameter**	**Value**	**Parameter**	**Value**
pH	4.3 ± 0.0	Acetic acid	0.4 ± 0.1	Arabinose	6.3 ± 0.1	Arabinan	1.8 ± 0.1
Total Solids(% w/w)	15.6 ± 0.1	Arabinose	1.6 ± 0.1	Galactose	1.7 ± 0.0	Galactan	0.3 ± 0.0
SS(%w/w)	8.8 ± 0.0	Ethanol	0.7 ± 0.0	Glucose	12.0 ± 0.3	Glucan	4.7 ± 0.1
Sieved fibres(%w/v)	3.2 ± 0.2	Glucose	1.4 ± 0.1	Mannose	2.4 ± 0.1	Mannan	0.6 ± 0.0
Crude protein(%w/w)[a]	32.0 ± 0.6	Glycerol	12.0 ± 0.1	Xylose	9.7 ± 0.1	Xylan	3.6 ± 0.1
Crude protein(%w/w)[b]	15.1 ± 3.9	Lactic acid	1.7 ± 0.0				
		Xylitol	0.6 ± 0.1				
		Xylose	0.7 ± 0.1				

[a] Based on dry total solids; [b] Based on dry sieved solids; [c] Dissolved monomers included; [d] From dry sieved solids

Research on ethanol production from whole stillage is currently lacking in the scientific literature. Valorisation studies of whole stillage have included production of biogas, in view of its high levels of protein [46, 47]. Whole stillage or processed fractions of whole stillage are currently being used for biogas production in several large-scale biogas plants in Sweden (Mariana Fridfjell, Swedish Biogas, Linköping, Sweden, personal communication 2011). Whole stillage has also been considered for production of carotenoids by submerged cultivation with red yeasts [48]. DDGS for animal feed are normally supplemented with carotenoids, at dosages ranging from 1 to 120 mg/kg feed in order to improve animal health, meat colour and quality, and to increase the vitamin A levels in milk and meat [48]. Animals

cannot synthesise carotenoids, but they can assimilate the ingested carotenoids [49]. Whole stillage has also been investigated for production of biodiesel, following extraction of corn oil. This strategy is claimed to not harm the final product DDGS for animal feed since it normally contains 8–10 wt. % oil, which is at excess [50]. In **Paper V**, whole stillage was used for production of ethanol and fungal biomass, and some of their characteristics are presented in **Table 3.1.**

Table 3.2: Characteristics of thin stillage that was used in **Paper IV** for batch and continuous cultivations.

Parameter	**Batch** [a]	**Continuous** [b]
pH	3.5 ± 0.0	4.3 ± 0.0
Kjeldahl nitrogen (g/L)	4.4 ± 0.2	5.3 ± 0.1
Total solids (g/L)	77.5 ± 3.4	90.1 ± 3.3
Suspended solids (g/L)	26.0 ± 0.9	30.5 ± 0.2
Ash (g/L)	10.3 ± 0.5	8.8 ± 0.4
Dissolved monomers		
Arabinose (g/L)	0.6 ± 0.1	1.0 ± 0.1
Galactose (g/L)	ND [c]	0.7 ± 0.1
Glucose (g/L)	0.5 ± 0.1	0.9 ± 0.1
Xylose (g/L)	0.6 ± 0.2	0.7 ± 0.1
Glycerol (g/L)	7.6 ± 0.5	9.9 ± 0.1
Acetic acid (g/L)	0.9 ± 0.1	0.3 ± 0.1
Ethanol (g/L)	1.7 ± 0.2	0.4 ± 0.1
Lactic acid (g/L)	11.6 ± 0.9	2.8 ± 0.1
Dissolved saccharides [d]		
Arabinose (g/L)	4.4 ± 0.2	6.2 ± 0.0
Galactose (g/L)	1.6 ± 0.2	1.7 ± 0.0
Glucose (g/L)	9.8 ± 0.7	16.4 ± 0.3
Mannose (g/L)	1.4 ± 0.2	1.7 ± 0.2
Xylose (g/L)	6.1 ± 0.4	6.8 ± 0.1
Solid fraction		
Arabinan (mg/L)	242 ± 140	374 ± 46
Galactan (mg/L)	175 ± 42	193 ± 32
Glucan (mg/L)	3182 ± 383	4553 ± 531
Mannan (mg/L)	488 ± 234	979 ± 83
Xylan (mg/L)	907 ± 419	920 ± 129

[a] Thin stillage used for batch cultivation; [b] Thin stillage used for continuous cultivation; [c] Not detected; [d] Dissolved monomers included

Thin stillage has a lower solid content than that in whole stillage, but it is still an interesting substrate for fermentation considering its nitrogen and carbon amounts (**Table 3.2**). The contents of pentose monomers and sugar polymers present in both whole stillage and thin stillage more likely originate from the degradation of bran during several stages of the process as well from the un-degraded bran, respectively.

Production of ethanol from thin stillage is also scarce in literature; only one work, carried out by Gonzalez, et al. (2010), is available where an ethanol yield of 0.42 g/g based on glycerol, maltose and glucose by *Escherichia coli* was obtained. Commercial bioethanol plants based on wheat or corn grains have treatment systems for whole stillage and thin stillage, in contrast with e.g. rice distilleries in developing countries. At those facilities, the thin stillage can represent an environmental problem if discarded [52]. Studies have been carried out for some decades on production of value-added products from the thin stillage originated from rice distilleries. Those include microbial biomass using *Ganoderma lucidum*, a fungus usually used in traditional Chinese medicine [53], or *S. cerevisiae* [54], acid protease with *Aspergillus niger* [55], bacterial cellulose production by *Gluconacetobacter xylinus* [56], single-cell oil by *Rhodotorula glutinis* [57].

Thin stillage from bioethanol plants has also been investigated for production of bio-hydrogen [58, 59], biogas [59], carotenoids [60], butanol [61], malic acid [62], eicosapentaenoic acid (EPA) [63], fungal single-cell oil [64], protein-rich fungal biomass [65] and lactic acid [66]. More recently, a *Lactobacillus panis* PM1, capable of converting glycerol to 1,3-propanediol, was isolated from thin stillage [67]. In this thesis, extensive work was developed with thin stillage and its characteristics are presented in **Table 3.2**.

3.2 Substrates extrinsic to the process

Taking advantage of the long experience and available equipment, the way the 1st generation bioethanol plant is designed provides opportunities for the addition of extrinsic substrates to the process either at the fermentation step or after distillation (**Figure 2.1**).

In view of the intense debate over food vs fuel, triggered by the development of the 1st generation bioethanol production plants, new substrates that do not compete with human food and which were readily available and cheap were sought. In view of their abundance,

lignocellulosic materials have been recognised for many decades [68] as the prime substrates for the production of 2nd generation bioethanol. Around 1.8 ± 10^{11} tonnes of lignocellulosic biomass are produced per year, 10% of which is considered as accessible [69]. Traditionally, lignocellulosic materials for ethanol production included dedicated crops like perennial herbaceous plant species, or short rotation woody crops, waste and residues like straw from agriculture, wood waste from pulp and paper industry and forestry residues [70]. Although intense research has been performed on ethanol production by using lignocellulosic biomass and some demo plants have been built, no commercial cellulosic ethanol is currently available [1]. Such difficulties are mainly related to the highly recalcitrance structure of the lignocellulosic biomass [28, 71-73]. The lignocellulosic biomass is mainly composed of cellulose, hemicellulose and lignin, forming a super complex and resistant structure. Therefore, the use of lignocellulosic materials has to be preceded by a pretreatment step in order to open up the structure and make the cellulose more accessible for further enzymatic hydrolysis [74]. During the process, hemicellulose is degraded, to a great extent, to pentose and hexose monomers. This harsh pretreatment step also gives rise to inhibitors that can negatively impact the fermentation, via inhibition of the microorganism growth. An enzymatic hydrolysis follows in order to convert cellulose into glucose monomers, which together with the monomers from hemicellulose can be converted into ethanol [75, 76]. The production of efficient enzymes to degrade cellulose at competitive prices with that of enzymes used in the 1st generation bioethanol plants has also been a constraint to feasible 2nd generation bioethanol production on a commercial scale [33]. An overview of the diversified methods developed over the years, for conversion of lignocellulosic materials to ethanol is, however, out of the scope of the present thesis. Nonetheless, examples of pretreatment methods used for conversion of wheat straw to value-added products can be seen in **Figure 3.1**. Two substrates of lignocellulosic origin, namely, pretreated wheat straw and SSL from paper and pulp industry were studied.

3.2.1 Wheat straw

Assuming a residue/crop ratio of 1.3, about 1,180 million tonnes of wheat can be produced in 2014–2015. Therefore, wheat straw, left as waste after harvesting, is a renewable, low-cost, and abundant substrate. The straw can be left on the field, ploughed back into the soil, burned or removed from the land. The act of burning has been common for a long time; however, more recently, the practice has been challenged due to health concerns [77]. Large amounts of

air pollutants such as particulate matter (PM_{10}), CO and NO_2 can be released when burning wheat straw [78]. Therefore, other alternatives for wheat straw management are needed. Considering its structure made of cellulose (33%) and hemicellulose (33%), intensive research has been investigating the potential of using wheat straw for production of ethanol [79].

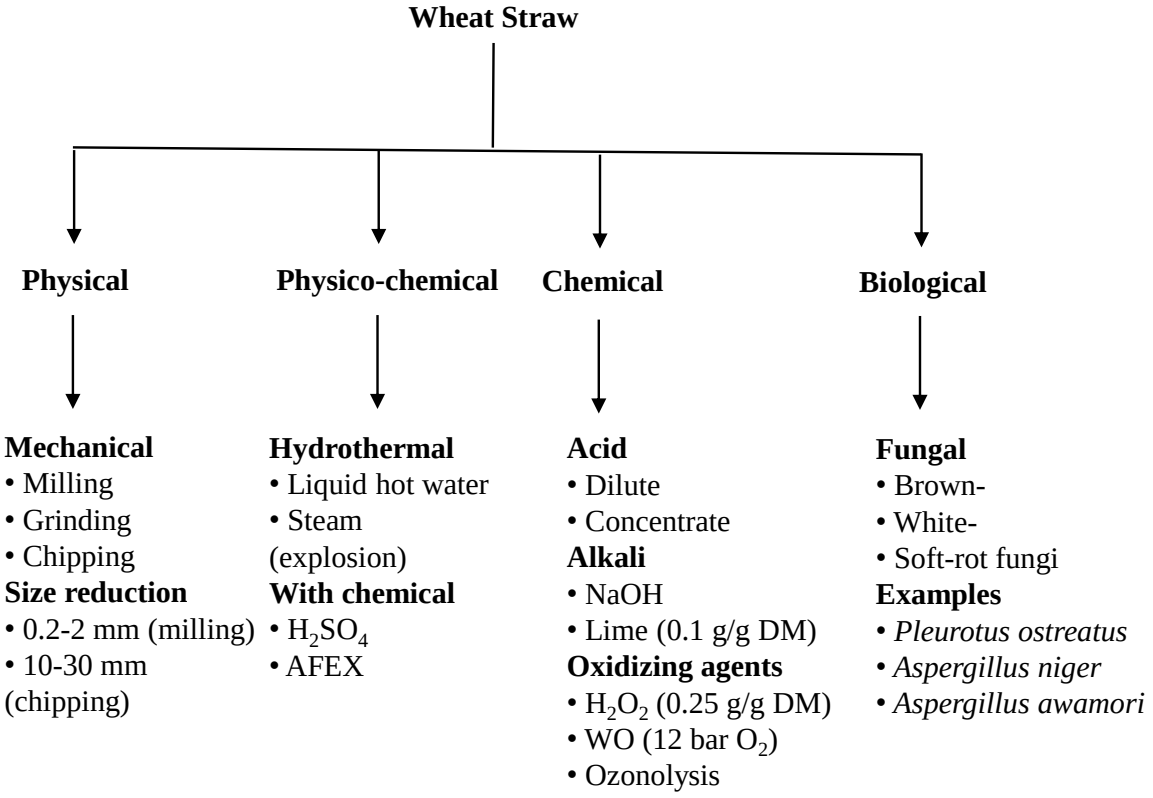

Figure 3.1: Pretreatment strategies used for improvement of the process of ethanol production from wheat straw. Adapted from [42].

Research has been focused on developing more effective pretreatment and hydrolysis processes in an attempt to obtain higher yield of sugars [42]. In **Figure 3.1**, the main pretreatment strategies used for wheat straw are summarised. Focus of research has also been on developing robust microorganisms for ethanol production from wheat straw hydrolysates [42]. Generally, a sugar yield of 74–99.6% of the maximum theoretical yield was reached after enzymatic hydrolysis of wheat straw. Among the bacteria, yeasts and fungi investigated, reaching an ethanol yield ranging from 65 to 99% of the theoretical value, the non-adapted *S. cerevisiae* has gathered the best results. Some recombinant bacteria and yeasts are claimed to be under consideration for commercial scale up [42]. Examples of other

products from wheat straw investigated as well as applications, include oil using oleaginous yeasts [80], biogas [81, 82], super-absorbent polymer [83], lightweight composites [84], gasification [85], adsorption of pollutants [86], biohydrogen [87] and lipid-rich fungal biomass [88].

Table 3.3: Characteristics of the pretreated wheat straw slurry used in **Paper VI**.

Parameter	Value
pH	2.0
Glucose	7.2 g/L
Xylose	22.1 g/L
Galactose	2.3 g/L
Arabinose	4.6 g/L
Acetic acid	5.9 g/L
HMF	2.1 g/L
Furfural	4.2 g/L
Glucan	34.7% w/w
Xylan	4.6% w/w

In **Table 3.3**, the characteristics of the pretreated wheat straw slurry that was used in **Paper VI** are presented. The slurry was obtained after continuous chemical pretreatment with dilute sulfuric acid (0.3–0.5%) at 185 °C and 22 bars for 5–7 minutes. The treatment was carried out at SEKAB E-technology, a lignocellulosic ethanol demonstration plant located in Örnsköldsvik, Sweden. The inclusion of wheat straw in the 1st generation bioethanol plant would represent that the full crop is used for ethanol production, in addition to represent a step towards industrial realisation of 2nd generation bioethanol production.

3.2.2 Spent sulphite liquor (SSL)

In paper and pulp industry, the production of pulp is mainly carried out by two chemical processes, namely, sulphate (Kraft) and acid sulphite pulping. In both cases, lignin is removed from the wood, releasing the fibre material (pulp) used for the papermaking or as a chemical feedstock [89]. More than 90% of the pulp production is done by Kraft pulping [90]. In this process, a solution of NaOH and Na_2S at pH 13–14 impregnates the wood chips, which are further heated up to 160–180 °C for 1–3 h, in batch or continuous digesters [91]. In contrast,

acid sulphite pulping is carried out under acidic conditions (pH 1–2) and high temperatures (135–145 °C) for 8–12 h in batch digesters using aqueous solutions of sulfur dioxide (SO_2) in the presence of bisulphite (HSO_3^-). The pulping bases used include Na^+, Ca^{2+}, Mg^{2+} or NH_3 [90, 91]. Production of pulps by acid sulphite process accounts for about 6% of all chemical pulps, where the most sustainable processes are those using regenerable pulping bases, namely, Na^+ and Mg^{2+} [90]. In addition to papermaking, sulphite pulps are also used for the production of viscose and cellulose esters [89]. One of its main advantages is that the pulp is easier to bleach to get full brightness [92, 93]. During the process, lignin is sulphonated with SO_2 and removed from the wood as salts of lignosulphonates, the hemicelluloses are significantly hydrolysed and removed from the wood to the spent sulphite liquor (SSL), while cellulose is maintained almost intact [90, 91]. A scheme of the acid sulphite pulp production is presented in **Figure 3.2**. After the pulping step, follows the washing of the unbleached pulp and then the concentration of SSL by evaporation [94]. The concentrated SSL is normally burned for energy and recovery of reagents (in case the Na^+ and Mg^{2+} bases are used) or it can also be commercialised [94].

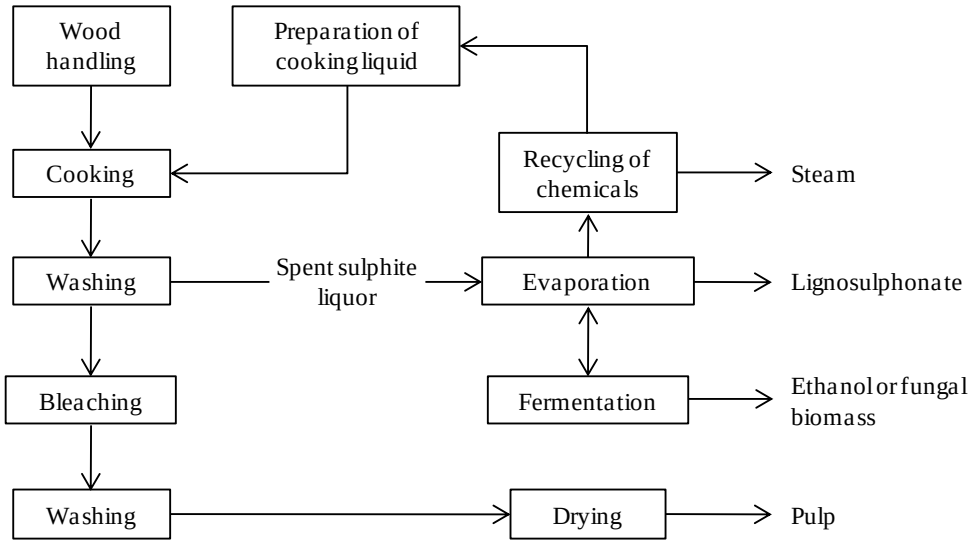

Figure 3.2: Overall scheme of SSL production in a paper and pulp process with details regarding valorisation of SSL to fungal biomass or ethanol [95]. Reprinted with permissions.

SSL is available in large amounts with an yearly worldwide production of 90 million m^3 [94]. More recently, a new pulping method called SEW (SO_2-ethanol-water) has been investigated in which the SSL produced from spruce chips was used for batch and continuous

production of acetone, butanol and ethanol (ABE) [96]. The process is considered to be a hybrid of acid sulphite and organosolv pulping process which main advantages over conventional pulping include: simplified chemical recovery, lower capital costs, rapid impregnation of the feedstocks due to ethanol that eliminates the long heat-up time and the possibility of being applied to all lignocellulosic materials [97].

Naturally, the SSL composition depends on the type of raw material used [98]. SSLs obtained from pulping of softwoods contain a high proportion of hexoses (>70%), while those obtained from pulping of hardwoods contain mainly pentoses (>70%) [91]. The production of other value-added products by the paper and pulp industry within the concept of biorefinery has been investigated, in an attempt to make the acid sulphite mills more competitive [99]. The SSL, containing sugar monosaccharides and lignosulfonate, is a potential substrate for valorisation and thus encourages the building of biorefineries within the sector. Therefore, SSL exhibits a clear advantage over other lignocellulosic materials since the hemicellulose has already been hydrolysed to monomeric sugars, thus, tackling the need for complex and harsh pretreatment processes [100].

The production of ethanol with *S. cerevisiae* from SSL has been studied since 1907 [101]. In 1909, the first sulphite bioethanol plant opened in Skutskär (Sweden), and bioethanol plants were built all around the world [102]. During World War II, SSL was used for production of yeast as a nutritional source of protein and vitamins [101]. However, due to low fossil energy prices, high costs for substrate pretreatment and the inability of non-genetically modified yeast to ferment the highly abundant C5 sugars, xylose promoted the shutdown of many plants [102]. Nowadays, the Borregaard company (Norway) claims to be the largest producer of 2nd generation bioethanol from its sulphite process, with an annual production of 20,000 m^3 [103]. The company DSM Poet (USA) when fully operational will have a capacity of around 95,000 m^3; however, the used substrates are baled corn cobs, leaves, husk and stalk.

Unfortunately, not all spent liquors can be used for fermentative processes; those generated by Kraft pulping are unsuitable for ethanol production [104] since most of sugars/oligosaccharides released from the wood are degraded to C2-C8 hydroxy- and dicarboxylic acids [90, 105]. In contrast to the conventional process of ethanol production from softwood SSL in North America and Europe [101], the ethanol production from hardwood SSL is not implemented in practice due to its high content of pentoses and higher

content of inhibitors where acetic acid plays a crucial role [89, 106]. Therefore, robust microorganisms that are good pentose assimilators, which can withstand the concentration of inhibitors in SSL from hardwood, are needed before industrial realisation [106]. This is really relevant since more than 50% of the SSL produced annually is from hardwood; thus, at least 45 million m^3 are produced per year [100], contributing to economic benefits in South Africa, Portugal and Spain [89]. Alternatively to *S. cerevisiae*, which cannot assimilate pentose-sugars naturally, other microorganisms such as *Pichia stipitis* have been investigated for production of ethanol from xylose and have shown high conversion rates [107, 108]. However, inhibitors are still a problem when *P. stipitis* is used [109], and a detoxification step is still needed. Strategies have included ion exchange chromatography [100], overliming (mainly with $Ca(OH)_2$), treatment with activated charcoal and extraction with solvents [106]. Two genome-shuffled *Scheffersomyces stipitis* strains were developed and exhibited improved tolerance to inhibitors in SSL from hardwood [110].

Table 3.4: Sugar composition of the spent sulphite liquor (received from Nordic Paper Seffle AB, Sweden) used in **Paper I**.

Carbohydrate	Value
Glucose	6.2 g/L
Mannose	19.1 g/L
Galactose	6.5 g/L
Xylose	8.0 g/L
Arabinose	3.0 g/L
Total	**42.8 g/L**

Further research on valorisation of SSL has included the production of bacterial cellulose [111], production of vanillin and syringaldehyde from lignin [112, 113] and PHA (polyhydroxyalkanoates) [102, 114]. Until the 70s, the production of vanillin was relevant for the pulp companies, but the replacement of softwoods by hardwoods made it less attractive [115] due to lower yields of vanillin, in comparison to syringaldehyde, lack of effective recovery techniques for vanillin and lack of awareness of the importance of syringaldehyde as an intermediate for synthesis [113]. Nowadays, the company Borregaard is the only one source of vanillin from lignosulphonates, while 85% of the world's needs are met with petrochemically derived guaiacyl [116]. Gallic acid, pyrogallol and vanillin have

been used for production of syringaldehyde [117]. However, the demand for chemicals from renewable resources promoted renewed interest in production of vanillin and syringaldehyde by the pulp industries [113]. Polyhydroxyalkanoates (PHAs) are a class of aliphatic polyesters produced by organisms that can be a potential alternative to the conventional plastics. PHAs are mostly based on 3-hydroxyalkanoates, which can be of different length and composition of side chains allowing for diversified applications [118]. Complete biodegradability, is another of their advantages in contrast to conventional plastics and other bioplastics [119].

In **Paper I**, SSL produced from softwood was used, and its composition of monomeric sugars is presented in **Table 3.4**.

CHAPTER 4

4 FILAMENTOUS FUNGI AS POTENTIAL BIOCATALYSTS FOR BIOREFINERIES

Baker's yeast is the main biocatalyst in the 1st generation bioethanol plants; however, it is unable to assimilate pentose sugars, and a centrifugation step is needed in order to recover the biomass. These can represent important constraints for the feasible inclusion of a cultivation step for production of ethanol and biomass for feed from low-grade substrates containing pentose sugars. Filamentous fungi, in contrast, can assimilate pentoses, and their biomass is easy to harvest due to their thick mycelial growth in submerged culture. Intense research using these biocatalysts has unveiled their versatility towards the range of substrates they can grow onto as well as the variety of products they can produce. The mycelial growth of filamentous fungi can give rise to cultivation challenges when grown in reactors; airlift and bubble column bioreactors of simple design have been developed for cultivation of filamentous fungi, alternatively to continuous-stirred tank bioreactors. Depending on the general characteristics of a cultivation set up, different cultivation modes can be applied such as batch, fed-batch and continuous.

The present chapter gives an overview on *Zygomycetes* and *Ascomycetes* fungal groups, with special focus on the fungal strains used in this thesis (**Figure 4.1**), where their biotechnological diversity regarding substrates and products are reviewed (**Figure 4.3**). The reasons behind choosing filamentous fungi as potential 2nd biocatalysts for the 1st generation bioethanol plants, despite of baker's yeast, are tentatively provided. The implications of fungal morphology and reactor design on the performance of the cultivation are also discussed, and the general aspects of different cultivation modes are provided.

4.1 The kingdom Fungi

Classifying living forms has always been a matter of huge and constant scientific debate. Decades ago, fungi were considered to belong to the plant kingdom due to the lack of mobility, or to bacteria and microscopic algae [120]. However, later findings unveiled that from a nutritional standpoint, fungi were more closely related to animals than to plants since

the former are absorptive heterotrophs while the latter are photosynthetic autotrophs [121]. Such similarity has been supported by DNA-based phylogenetic studies [122]. The kingdom system suggested by Wittaker (1969) comprises five kingdoms: plants, animals, fungi, eubacteria and archaebacteria. In turn, the kingdom *Fungi* is considered to include four phyla, namely, *Chytridiomycota*, *Ascomycota*, *Basidiomycota* and *Zygomycota* [124]. The kingdom *Fungi* is monophyletic, that is, the species have evolved from a common ancestor, which might have happened between 400–500 [120] to 1,000 million years ago [125]. Common properties to all fungi, in addition to the fact of being absorptive heterotrophs, include lack of photosynthesis and phagotrophy, spores normally chitinous and filamentous growth with several nuclei, the only exception being yeasts [121]. Although the total number of species of *Fungi* is unknown, a common estimate points towards 1.5 million, including fungi-like species from other kingdoms [126]. Fungi can be found worldwide growing as saprobes, parasites or mutualists [127], playing crucial, or even essential, roles in the ecosystem [126] such as lignin degradation [128], formation of mycorrhizae [129], and possibly played some role in the first colonisation of land by plants [130].

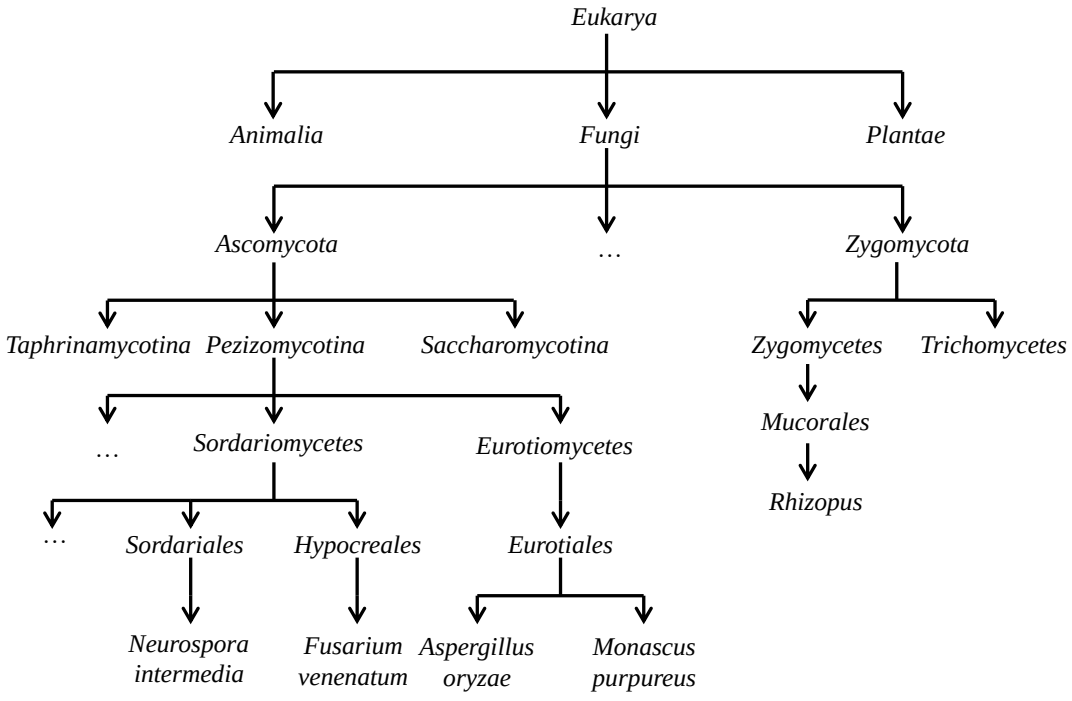

Figure 4.1: Overview of the organisation within the fungal kingdom with special emphasis on the filamentous species used in this thesis. Suspension points represent the clades that were omitted for being out of the scope of the present thesis.

4.2 Baker's yeast

Among the microorganisms used for production of ethanol, *S. cerevisiae*, also known as "baker's yeast", is the dominant biocatalyst on the industrial scale. Distinctive characteristics include the status of being generally regarded as safe (GRAS) microorganism due, to a large extent, to its use in baking and alcoholic beverage production as well as its intensive use in genetic research. Moreover, *S. cerevisiae* can ferment hexoses at high rate, furthermore, it has high tolerance to ethanol, inhibitors and acidity [131, 132]. *S. cerevisiae* can convert hexoses sugars such as glucose, mannose and galactose and the disaccharide sucrose to ethanol. Nevertheless, the wild-type *S. cerevisiae* is unable to use pentose sugars such as xylose and arabinose [133]. This aspect knocks out the *S. cerevisiae* as the core biocatalyst for the research this thesis has its focus on, based mainly on two aspects: the substrates whole stillage, thin stillage, wheat straw and spent sulphite liquor being of lignocellulosic origin, all contain those pentose sugars; in addition, since one of the products desired is biomass for feed applications, the use of genetically-modified *S. cerevisiae* that can consume pentose sugars is not of interest due to possible future public acceptance issues.

4.3 Filamentous fungi

Filamentous fungi have been making an important contribution to the global economy via production of a plethora of commercially interesting products such as antibiotics, enzymes, organic acids, drugs, food components, among many others [134]. The group of filamentous fungi is diverse and so are their metabolic capabilities. That is clearly represented by the increasing range of substrates they can grow onto as well as the products they can produce. Therefore, filamentous fungi are increasingly seen as the right biocatalysts in order to close the loop between the generation of waste streams by human processes and their conversion to value-added products that mankind and industries can use (**Figure 4.2)**. In the present thesis, the choice of filamentous fungi, as core biocatalysts, is mainly based on the following aspects: filamentous fungi with ethanol yields similar to those of S. *cerevisiae* have been isolated [135]; they are able to consume pentose sugars and even produce ethanol from xylose; their filamentous growth allows for easy separation of the fungal biomass from the medium; thus, a centrifugation step is not needed as it would be if *S. cerevisiae* was used. Many of them have been used for centuries for production of human food; moreover, they have been reported to produce a panoply of enzymes which can be of special value if substrates like whole stillage, thin stillage and wheat straw containing sugar polymers

(e.g. xylan, glucan and arabinan) or saccharides of different complexity, are used. In following sections, an overview is provided on two fungal groups (*Zygomycetes* and *Ascomycetes*), with particular focus on the fungal strains used in this thesis. The biotechnological potential of these fungal groups is well documented in **Figure 4.3**.

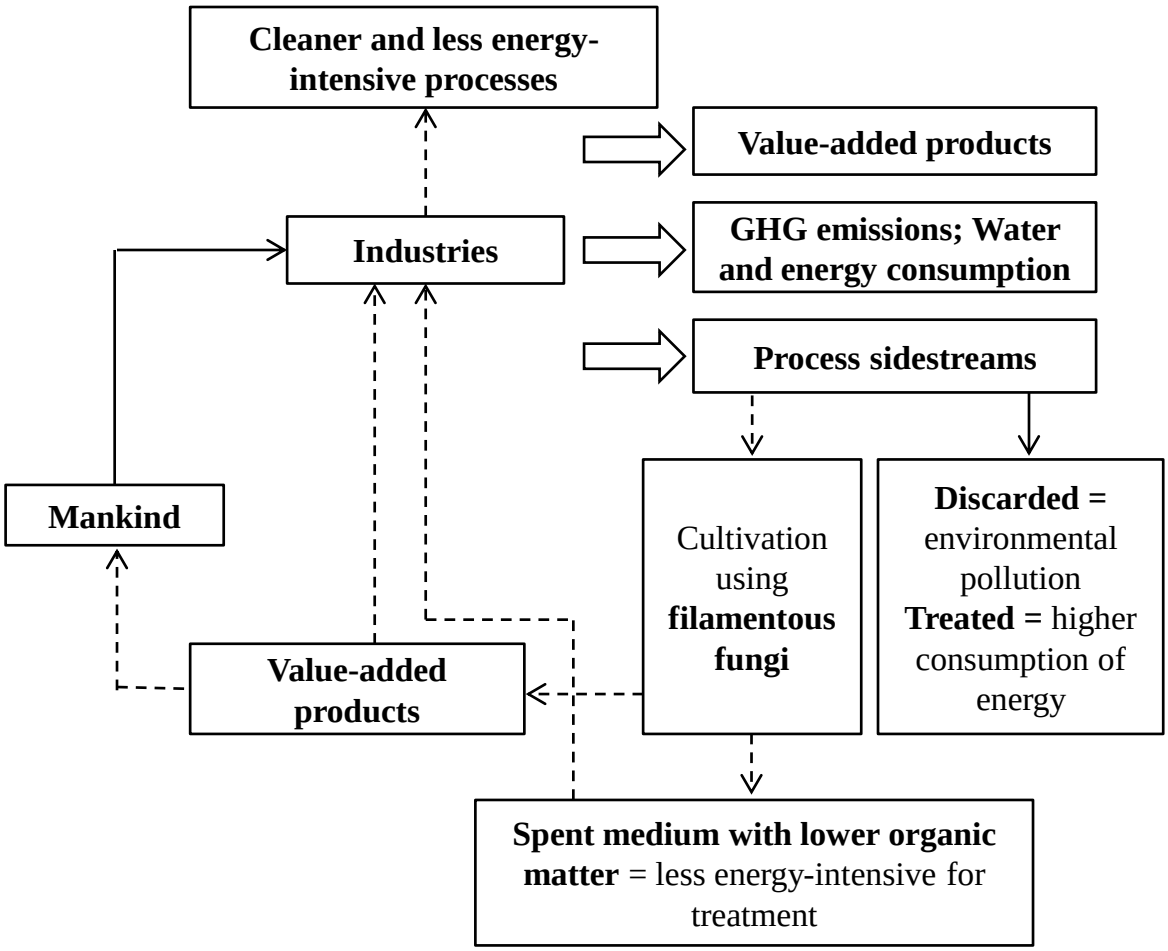

Figure 4.2: General scheme displaying the potential of filamentous fungi to close the loop between the generation of waste streams by human processes and their conversion by filamentous fungi to value-added products that mankind and industries can use. The "mankind" box is the starting point of the scheme.

4.3.1 An overview of Zygomycetes

The phylum *Zygomycota* comprises two sub-phyla: *Trichomycetes* and *Zygomycetes*. The microorganisms belonging to the former are mainly living in the guts of arthropods, while the microorganisms belonging to the latter are found worldwide as saprobes growing on dead organic matter, as part of mycorrhizae, and as parasites of other organisms like mushrooms,

amoebae, nematodes, rotifers and arthropods [136]. *Zygomycetes* can grow either as filaments (usually without septa) or as yeasts. Their asexual reproduction is carried out via production of spores or conidia, while the sexual reproduction (and sometimes also asexual reproduction) occurs by production of zygospores [136]. Zygospores are the structure after which the sub-phylum *Zygomycetes* is named and have a thick wall and a significant survival potential [137]. Based on differences in the morphology and nutrition, the sub-phylum is further divided into ten orders: *Basidiobolales*, *Dimargaritales*, *Endogonales*, *Entomophothorales*, *Geosiphonales*, *Glomales*, *Kickxellales*, *Mortierellales*, *Mucorales* and *Zoopagales* [136]. As common to all taxonomic studies, the classification of the *Zygomycetes* has been under discussion by the scientific community [120, 124, 138].

Modern phylogenetic studies have unveiled *Zygomycetes* and the entire phylum *Zygomycota* (closely associated with *Chytridiomycota*) as the earliest emerging Fungi [139]. This is also reflected by their relatively simple structure, rendering them the title of "evolutionary primitive". The simpler structure allows *Zygomycetes* to rapidly extend their hyphae and quickly colonise new areas in search of substrates. The fast growth is more likely due to the lack of septa, leading to faster cytoplasmic movement or intra-hyphal transportation [136, 137]. It should be noted, though, that some orders, including the *Mucorales*, possess the ability to produce septa under certain conditions [140].

The group of *Zygomycetes* is diversified where its species can grow in a variety of environments. Some are thermophilic and can grow at above 50 °C, e.g. *Rhizomucor pusillus*, while others, for instance, *Mucor hiemalis*, can grow at temperatures below 0 °C [137]. Some species of the order *Mucorales* are able to grow under anaerobic conditions, while others require aerobic conditions. Additionally, although most of the *Zygomycetes* only grow at high water activities, some of them are able to grow in salt concentrations of at least 15% [137].

In the present thesis, three different strains of zygomycetes, belonging to the *Rhizopus* genus (order *Mucorales*), were studied. The *Rhizopus* sp. used in **Paper I** was isolated from Indonesian tempe [141] while the *Rhizopus* spp. used in **Papers III** and **VI** were isolated from Indonesian *Tectona gandis* (Laru) and *Hibiscus* (Usar) leaves that are traditionally used for tempe preparation [135].

a) *Rhizopus* sp.

Zygomycetes from the *Rhizopus* genus are well known fungi to humans, as they have been used for production of fermented foods e.g. tempe and tofu in Southeast Asia and China for hundreds of years [142]. Over the years, *Rhizopus* spp. have been intensively studied for production of a wide range of commercially-relevant products, including organic acids, for example, lactic and fumaric acids, ethanol and enzymes using an array of substrates and fermentation set-ups [143].

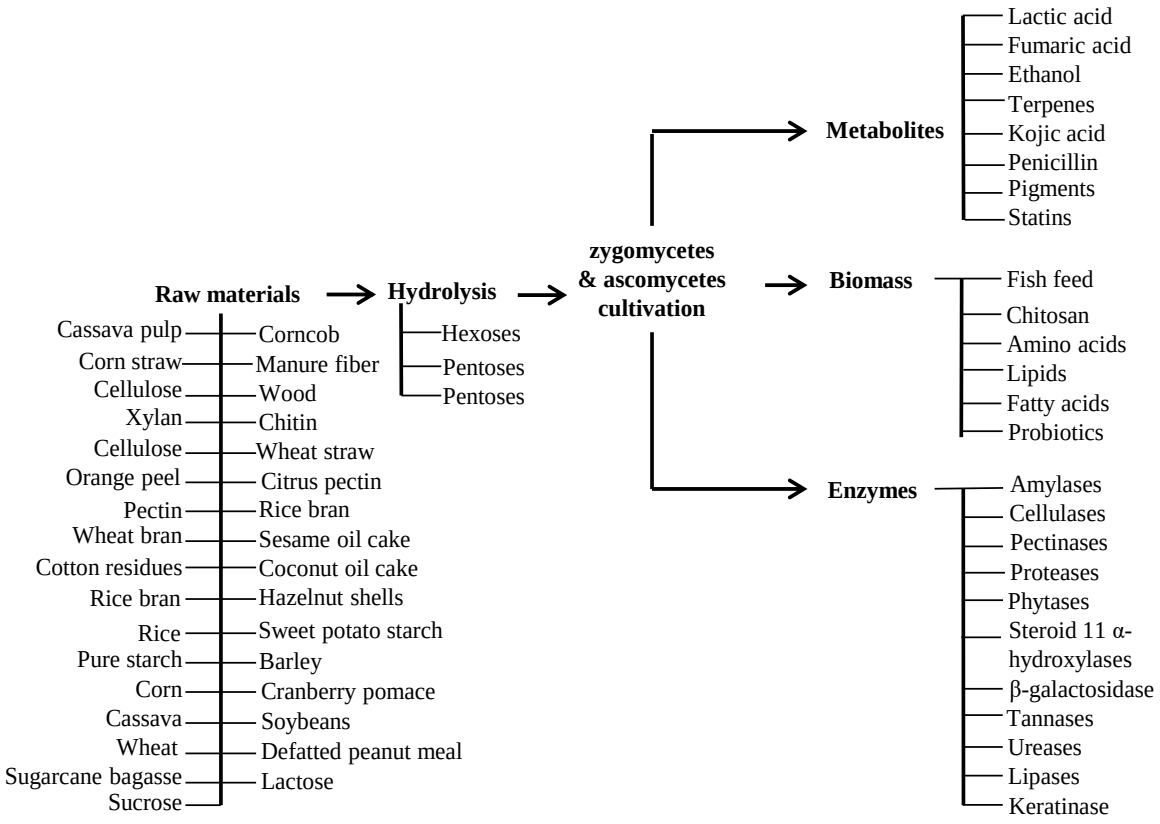

Figure 4.3: Overall scheme of a fungi-based biorefinery, illustrating the alternative feedstocks inputs as well as the product outputs, including metabolites, enzymes and potential cell mass applications. Adapted from **Paper II.**

The qualitative and quantitative production of different organic acids and ethanol is strain-specific and is regulated by the activity and substrate affinity of *Rhizopus* metabolic enzymes [144]. The final glycolytic product pyruvate can follow different pathways: the shortest route leads directly to the production of lactic acid via lactate dehydrogenase; fumaric acid can be produced via the cytosolic reductive TCA pathway, where fumarase plays an

important role, and ethanol can be produced via decarboxylation of pyruvate to acetaldehyde via pyruvate decarboxylase, which is further converted to ethanol by alcohol dehydrogenase [145]. Stress has been found to be a requirement for production of organic acids and ethanol where nitrogen-limiting conditions trigger the production of the former while oxygen-limiting conditions or anaerobic conditions trigger the production of the latter [95]. *Rhizopus* spp. are divided into lactic and fumaric acid producers based on the yields of the produced organic acids (and other metabolites). The species mainly producing lactic acid have been found to possess two lactate dehydrogenase genes, ldhA and ldhB, while the fumaric acid producers only had the ldhB gene [146]. Additionally, a phylogenetic analysis of DNA sequences has also confirmed the metabolic differences [147]. Strategies for production of organic acids include a two-stage cultivation [148], where a cell-growth stage in a relatively rich medium is followed by an acid production stage with nitrogen at limiting levels, oxygen-rich conditions, and a neutralising agent for medium pH-value maintenance [149].

L(+)-lactic acid is the isomer required by the food industry as well as the preferred for polylactide manufacturing. Polylactide is the building block in the production of bioplastics [150, 151]. The intense research on production of the organic acid from *Rhizopus* spp. has been seeking to replace the chemical route of fermentation using lactic acid bacteria. Main advantages when using the former include the possibility of producing lactic acid using less expensive media as well as the production of just the intended L(+)-isomer. In contrast, bacteria normally produce a mixture of both isomers [149]. Research trends have been including glucose as the most studied carbon source, but relevant research has also been carried out with sucrose or starch as well as using lignocellulose-based substrates (**Paper II**). The scale up of the process of lactic acid production from glucose using 5 m^3 ALBs has been reported, together with prospects of designing ALBs larger than 3,000 m^3 [152]. Lactic acid produced from *Rhizopus oryzae* is nowadays commercially available from common suppliers.

Due to its structure, a double bond and two carboxylic groups, fumaric acid is suitable for production of biodegradable polymers, synthetic resins and intermediates for chemical syntheses. Moreover, in view of its non-toxic and non-hygroscopic properties, fumaric acid finds applications in food and pharmaceutical industries; it has also been considered for treatment of psoriasis, a skin condition, and as feed additive for cattle [153]. The production of fumaric acid using *Rhizopus* spp. seeks to replace the usual chemical route from maleic

anhydride produced from butane [153]. Research on fumaric acid production has been marked by the absence of high-performance *Rhizopus* strains as well as by the scarcity of investigations using other substrates than monomers such as glucose. However, the fact that fumaric acid has been identified as one of the top ten chemicals to be produced via fermentation on an industrial scale, further intense research is expected to take place (**Paper II**).

The biotechnological potential of *Rhizopus* spp. as well as their contribution to the global economy is well highlighted by the variety of enzymes they can produce. *Rhizopus* spp. have been studied for production of amylases, enzymes with cellulolytic activities, pectinases, proteases, steroid 11 α-hydroxylases, tannases, ureases and lipases [143]. Further interest on *Rhizopus* spp. has been on their biomass, including the protein content rich in essential amino acids, the lipid content rich in long-chain polyunsaturated fatty acids and the chitosan present in their cell walls (**Paper II**).

4.3.2 An overview of Ascomycetes

Ascomycota are the largest phylum of the kingdom Fungi [154], composed of nearly 75% of all described fungi [155]. Similar to the *Zygomycota*, the classification of the entire phylum is under discussion by the scientific community [156, 157]. The *Ascomycota* produce their sexual spores (ascospores) in tubular sacs called asci, where some can act as small water cannons and expel the spores into the air [155]. This mechanism is crucial for spore dissemination by nonmotile propagules of many fungal plant disease organisms and saprophytic fungi [158]. The *Ascomycota* include three monophyletic subphyla: the *Taphrinamycotina* and the *Saccharomycotina*, lacking a complex fruiting body, and the *Pezizomycotina*, producing the multi-tissue fruiting bodies. The former is the earliest diverging clade [159]. *Pezizomycotina*, to which all ascomycetes strains used in thesis belong to, is further divided into seven classes: *Dothideomycetes*, *Eurotiomycetes*, *Lecanoromycetes*, *Leotiomycetes*, *Lichinomycetes*, *Pezizomycetes* and *Sordariomycetes* [158]. *N. intermedia* and *Fusarium venenatum* belong to the group of *Sordariomycetes*. The class usually forms minute fruiting bodies (ascomata) containing hymenial layers, commonly composed of sterile hyphae intermixed among asci (with single wall layers) possessing ascospores [160]. Ascomata can be composed of single- or multi-layered walls as well as different types of outside coverings such as tomentum, hairs or setae [161]. *N. intermedia* belongs to the order *Sordariales*, which

is one of the most economically and ecologically important within the *Ascomycetes*. The species is responsible for the destruction of paper and fabrics, and the "fruit flies" of the fungal world are included in this order. *Sordariales* occur worldwide as saprobes on dung, decaying wood, leaf litter and soil [162]. The ascospores within the order range from cylindrical, hyaline ascospores to ellipsoidal, brown ascospores. Intermediates exist where ascospores are two-celled with cylindrical to ellipsoidal, brown cells and different degrees of cylindrical (often basal), hyaline cells [162]. *F. venenatum* belongs to the order *Hypocreales*. *Hypocreales* include plant pathogens, antibiotic producers, mycotoxigenic species and endophytes [163]. *Aspergillus oryzae* and *Monascus purpureus* belong to the order *Eurotiales* in the class *Eurotiomycetes*. *Eurotiomycetes* generally produce prototunicate asci in enclosed ascomata [164]. They are found on all types of decaying material and are one of the most ubiquitous type of fungi [165]. They have also been identified as human allergens [166]. The species belonging to *Eurotiales* have spherical or obovate-saccate, thin-walled and unitunicate asci as well as aseptate, hyaline or pale ascospores without germ pores, and absence of ostiolate or discoid ascomata [167].

Similar to the *Zygomycetes*, *Ascomycetes* have also been intensively produced for a wide range of value-added products using a wide range of substrates as cultivation medium. In further sections, research directions with the four ascomycetes used in this thesis are provided.

a) *Aspergillus oryzae*

Aspergillus species have been applied in industry for the production of enzymes and metabolites; thus, much is known about its behaviour, from a metabolic point of view as well as from a morphological point of view [134, 168]. *A. oryzae* has played an important role in brewing, distilling and baking industries [169]. It has been used for the production of indigenous Japanese foods such as sake, shoyu, miso and vinegar [168]. Its conidia are essential for the food industry as starters in the first step of fermentation to digest ingredients such as steamed rice, soybean and wheat, and its conidiation regulatory pathway has been a matter of research [170]. Due to its long history of extensive use in the food fermentation industry, its safety is well established, and it has been approved by the Food and Drug Administration (FDA) and by the World Health Organization (WHO) [171]. The ascomycete has also been used as probiotics [172]. Probiotics are live microbial feed supplements that benefit the host animal by enhancing its intestinal microbial balance [173], and they have

been used as replacements to antibiotics [174]. More recent research with *A. oryzae* has included the production of different products. An example is the production of fungal lipids using wheat straw [88] or potato processing wastewater [175] as substrates. The production of various enzymes has kept going including tannase for drink, food, brewing and animal feed industries [176], proteases [177, 178] for hydrolysis of defatted peanut meal, the main by-product during peanut oil production, or for improving the baking process of gluten-free bread from rice flour, milk-clotting enzymes as substitutes of calf rennet in the cheese industry [179], β-galactosidase for processing of lactose-containing products [180], polygalacturonases that are pectin hydrolases with use in juice and wine clarification [181], α-amylases [182] and keratinase produced during aerobic cultivation in a medium containing chicken feathers [183]. Other applications of *A. oryzae* studied include production of indoloditerpenes with insecticidal and antimicrobial activities [184], kojic acid for the cosmetic industry [168], penicillin [171] and swollenin, an expansin which is beneficial to the cellulose decomposition by cellulase [185]. Other researches have included biosorption of dyes [186] and extraction of antioxidant compounds from soybeans [187].

b) *Fusarium venenatum*

F. venenatum is most well-known or even famous for its use in commercial production of myco-protein for human consumption [188]. The myco-protein is commercialised under the trade name Quorn®. *F. venenatum* was investigated as a potential protein source for human consumption during the late 1960s by the British company Rank Hovis McDougall (RHM). After intensive research on safety of the microorganism and on the final product (12 years), the myco-protein from the ascomycete was approved by the Ministry of Agriculture, Fisheries and Food (MAFF) for sale in United Kingdom in 1984 [188, 189]. Industrially, the myco-protein is produced in 150,000 L pressure-cycle bioreactors in a continuous flow process [190]. During preparation, the myco-protein paste is mixed with a binding agent (egg albumin) to align the mycelia into a fibrous network with a similar texture to that of meat. Quorn® products range from chunks and mince to sausages, burgers, fillets and steaks [188].

c) *Monascus purpureus*

Monascus species have been traditional Chinese fermentation fungi, used in food and wine making for over thousands of years. The red mould rice, containing a large amount of γ-

aminobutyric acid and having anti-hypertensive effects on humans, is usually obtained by cultivation of *Monascus* spp. on the rice grain [191]. The most widely known interest on *Monascus* species is related to its pigments. Those can be used as natural colorants, food additives (traditionally in East Asia), and in meat processing industry in Western countries [192, 193]. Three different categories of pigments have been produced by *Monascus* spp. and are structurally well characterised, including orange, red and yellow pigments, each with two components of polyketide origin. Those relate to secondary metabolites with a common azaphilone skeleton [194, 195]. The orange pigments include monascorubrin and rubropunctatin, possessing the oxo-lactone ring; the red pigments include monascorubramine and rubropunctamine that are the nitrogen analogues of the orange pigment; and the yellow pigments include monascin and ankaflavin [196]. The red pigments are of high relevance due to their use in meat products, replacing nitrites [193] and their potential therapeutic activities particularly when produced in red rice [197]. However, there are several components still unknown in the *Monascus* product, furthermore, new pigments are frequently isolated and their structures tentatively characterised [198-200]. Various biological activities such as embryotoxicity, teratogenicity, immunosuppressive, antioxidant, antibiotic, anti-inflammatory, anti-diabetic and cytotoxic activities have been evaluated for *Monascus* pigments [198, 201, 202]. *Monascus* are also relevant producers of other functional metabolites such as statins, cholesterol-lowering drugs, such as lovastatin also known as mevinolin, monacolin K and mevacor® [203]. Lovastatin has made important contribution for the treatment of hypercholesterolemia, and its positive therapeutic properties have also been useful for the positive prevention of several diseases such as atherosclerosis, sepsis, peripheral arterial disease, peripheral vascular disease, cerebro vascular disease, ischemic disease and bone fracture [191]. Nonetheless, *Monascus* can also co-produce toxic compounds such as the hephrotoxic and hepatotoxic azaphilone citrinin via fermentation [204]. Citrinin has been classified by the International Agency for Research on Cancer as a potential human carcinogen [205], resulting in a safety concern [206]. Therefore, its content has to be closely controlled during fermentation [207].

Accordingly, studies have been performed towards higher yields of production of pigments, other functional *Monascus* pigments as well as towards the minimisation of citrinin production. Kang, et al. (2014) have produced citrinin-free orange pigments at extremely low initial pH by using $(NH_4)_2SO_4$ and monosodium glutamate. Seraman, et al. (2010) have shown carbon and nitrogen sources as well as salts where the main factors influencing the

production of lovastatin and their optimisation has been carried out. Silveira, et al. (2013) have obtained the best red pigment yields when using sugarcane bagasse as carbon source, in combination with peptone or soy protein isolate. Optimisation of red pigment production using potato powder as carbon source has also been carried out [210]. In contrast to solid-state fermentation, where the pigments are accumulated on the substrate used, in submerged cultivation, the pigments are accumulated in the mycelia, leading to low pigment concentration [211]. The addition of surfactant Triton X-100 could greatly enhance the production of pigments, probably by facilitating the secretion of intracellular pigments [212]. *Monascus* spp. are also relevant sources of proteases. An acid protease from *M. purpureus* was investigated for the reduction of antigenicity of bovine milk whey protein with positive results (Lakshman et al. 2011). A wide array of substrates has been used for production of pigments by *M. purpureus* under solid-state fermentation. However, their review is beyond the scope of the present thesis.

d) *Neurospora intermedia*

Since 1843, *Neurospora* species have been identified in sugarcane refineries, on burned trees and in soil samples [213, 214]. *Neurospora* was first isolated from spoiled bread from bakeries in Paris in 1842 [215]. *Neurospora crassa* is among the most studied organisms in genetics and biochemistry [216]. They have also been used for numerous cytogenetic, molecular and population biology studies [217]. *Neurospora* spp. can be easily spotted in the environment due to their orange colour, fast growth and profuse production of powdery conidia [214]. During all this time, the genus has never been implicated in human disease or observed to cause disease in animals or plants [214]. This aspect might be related to the fact that *Neurospora* species are obligate aerobes, unable to grow in the gut or bladder, in tissues or systemically [214]. Moreover, no dangerous secondary metabolites (e.g. mycotoxins) are known to be produced by strains of any *Neurospora* species [214]. *Neurospora* has been used for production of food and beverages [214]. For instance, *Neurospora* is used for production of the human food oncom, a highly nutritious soybean-based presscake produced in a cottage industry in west Java, where it is distributed on a daily basis to marketplaces and shops [214, 218]. It is also used for production of koji, an oriental food [214]. Other interesting aspects about *Neurospora* include its use by indigenous tribesmen in Brazil to process cassava and prepare a fermented beverage [219]; the Iban people in Borneo collect the orange fungus called kulat amau from burnt-down hilly rice fields and use it as

food [220]; and it was frequently present in Roquefort cheese in Southern France [214]. Beyond its continuous use for genetic studies, *Neurospora intermedia* has started to be used for applications other than fermented food and beverages. Those include production of ethanol from pretreated wheat bran with phosphoric acid [221], adsorption of colour pollutants in distillery spent mash originated from bioethanol production from molasses [222, 223], extracellular production of silver nanoparticles with anti-bacterial properties [224] and production of pigments [225].

4.4 Use of filamentous fungi in biotechnology – challenges

Filamentous fungi can adopt different morphologies when grown in submerged culture. Those can range from uniform filaments evenly distributed throughout the medium, giving rise to "heavy mycelial suspensions" to entangled filaments in clumps or pellets (**Figure 4.4**) [134]. A variety of culture conditions can influence the way the filamentous fungi grow, including medium composition, inoculum size, pH, temperature, aeration rate and agitation speed [134]. The fungal morphology will influence the medium's rheological properties and consequently, the overall process performance and final product yields [134]. The growth of filamentous fungi as pellets gives rise to media with lower viscosity, higher potential for cell-reuse and higher productivity due to the possibility of using high-density cultivations [226]. However, even in the pellet form, there are some critical characteristics such as size and compactness versus fluffiness that can influence oxygen and substrate transfer rates. The trend in research works has been towards the production of small fluffy pellets. Therefore, the development of strategies for the control of fungal morphology has been extensively under scrutiny by the scientific community (**Paper II**).

Figure 4.4: Example of fungal pellets.

The fungal morphology can make the growth in bioreactors troublesome. If grown in CSTRs, the filaments can entangle with the inner parts of the reactors such as temperature and pH probes, baffles and stirrers, ending in low performance cultivations. On the other hand, fungal pellets can suffer from shear stress when grown in CSTRs [134]. As an alternative, BCs have been developed which lack of internal mechanical parts (**Figure 4.5**). ALBs have also been developed for fermentation, and studies have shown that they enhance the oxygen and mass transfer [227]. The difference between ALBs and BCs is that the former has an internal-loop tube (this was the kind of ALB used in this thesis) that gives rise to a different mixing pattern as shown in **Figure 4.5**. In both BCs and ALBs, the supplied air at the bottom promotes the medium mixing.

The supplied air plays crucial roles during cultivation of the filamentous fungi in the substrates studied in this thesis. Beyond promoting the mixing of the medium (which leads to a low energy demand for mixing, in comparison to that needed when CSTRs are used), the supplied air is needed for the filamentous fungi to be able to assimilate xylose from the medium, a pentose common to all substrates studied. Moreover, as it will be discussed in **Chapter 6**, the filamentous fungi used in this thesis were able to degrade more complex substrates, showing that they were able to produce the suitable enzymes. The production of enzymes entails higher energy expenditure by the cells; thus, good access to ATP generating processes is required, stressing further the importance of the supplied air [33].

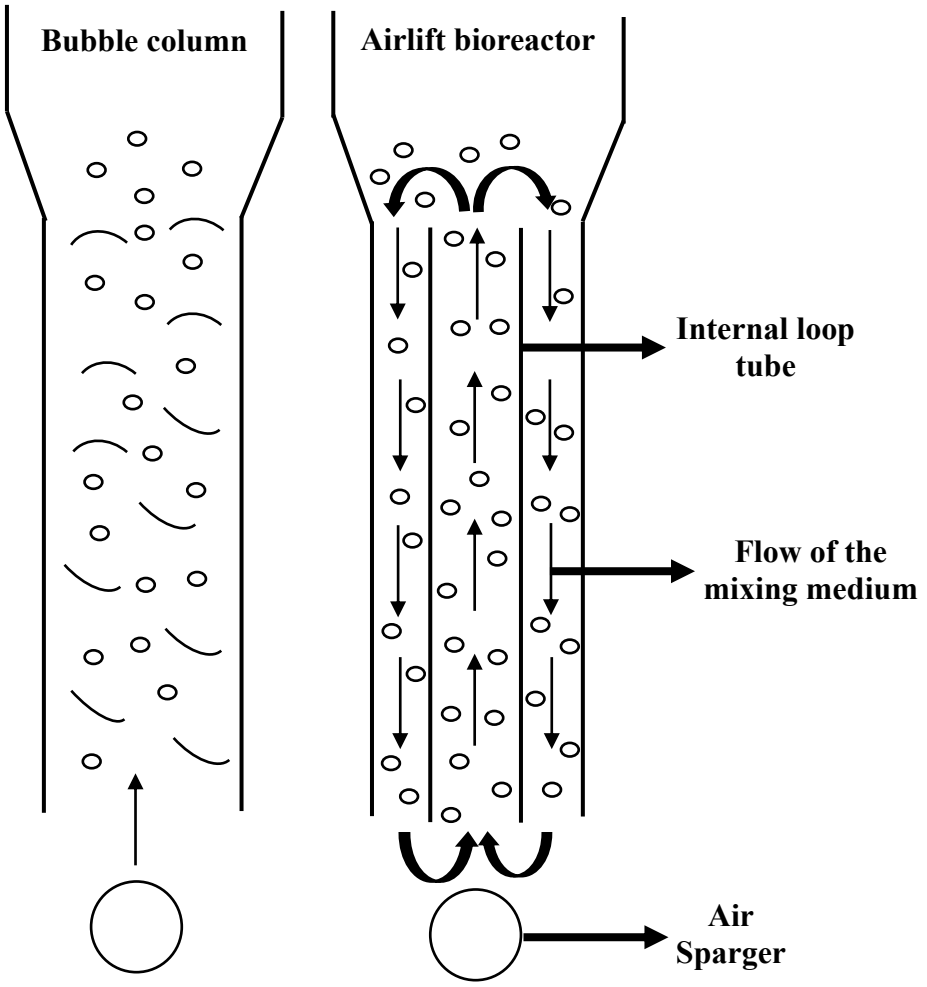

Figure 4.5: Schematic difference between BC and ALB as well as the details of the different mixing patterns in the ALB due to presence of an internal-loop tube.

4.5 Cultivation modes – batch, fed-batch and continuous

Different strategies have been developed over the years for cultivation of microorganisms. Those include cultivation under batch, fed-batch and continuous mode.

Great attention has been paid to the batch fermentation method in the past due to its simplicity and cheap cost to construct and use in action. That is why alcoholic beverages are often produced under this cultivation mode. Disadvantages of the process are that it is time consuming and labour intensive (cleaning, sterilisation, filling, cell lag phase and harvesting for each run) leading to low productivities. One strategy in order to increase productivity has been to set the cultivation at repeated-batch via recycling of the cells [228]. Cultivations in batch mode dominated in this thesis (**Papers I**, **III**, **IV** and **V**).

Under continuous cultivations, fresh medium is continuously fed to the reactor at the same rate as the fermented medium is withdrawn, resulting in a constant volume inside the reactor. In order to avoid the occurrence of cell wash-out, the dilution rate cannot be higher than the cell growth rate. In these optimal conditions, the continuous mode should be able to run for a long time [34]. Product productivity in continuous processes can be arguably enhanced if higher dilution rates are used. To reach that goal, cell retention strategies, including flocculation, encapsulation, or submerged membranes can be used [229-231]. However, these strategies work for unicellular cells such as bacterial and yeast cells. Cell retention systems for filamentous fungi would have to include some sort of cell harvesting and proper pumping back system of the harvested cells due to their growth morphology. Continuous fermentations were carried out in **Papers IV** and **VI**. In **Paper VI**, the fermentation unit of SSFF can be considered as a continuous process since the sugar-rich stream is continuously supplied into the fermentation bioreactor, and the fermented medium is continuously withdrawn into the hydrolysis reactor.

In fed-batch mode, the catalysts are first added to a small amount of medium after which the total volume of the reactor is reached by stepwise addition, without any withdrawal of medium [232]. The production of baker's yeast has been carried out in this mode since 1910s [233] due to higher biomass yield, compared with batch processes via inexistence of substrate repression [234]. Production of ethanol at high productivities is carried out, to a great extent, under fed-batch mode coupled with cell recycling [235]. Moreover, operating problems such as contamination and cell wash-out encountered in continuous processes are avoided in fed-batch [34].

CHAPTER 5

5 FILAMENTOUS FUNGI FOR BIOMASS AND ETHANOL PRODUCTION

In addition to their ability to consume pentose sugars and the harvesting of the biomass being a rather easy process, many of the filamentous fungi have been traditionally used for production of fermented foods. This aspect renders those strains the status of GRAS microorganisms, which is highly relevant if the biomass is considered for feed applications. Their biomass has gathered wide scientific attention due to its high content in protein and lipids with respective relevant profiles of essential amino acids and long-chain fatty acids, and in chitosan present in their cell walls. Thus, the fungal biomass has high potential to be used for feed purposes such as for fish feed or pets. The extracted chitosan can be used for a variety of applications such as in biomedical, food, biocatalysis and wastewater treatment. However, if the different fractions of the fungal biomass are desired, it cannot be dried together with the solids for DDGS; the same being true if the fungal biomass as a single product is desired (**Figure 5.1**). Naturally, the decision on the process design will depend on economic studies of feasibility. Beyond biomass, filamentous fungi can also produce ethanol when assimilating nutrients from the culture medium.

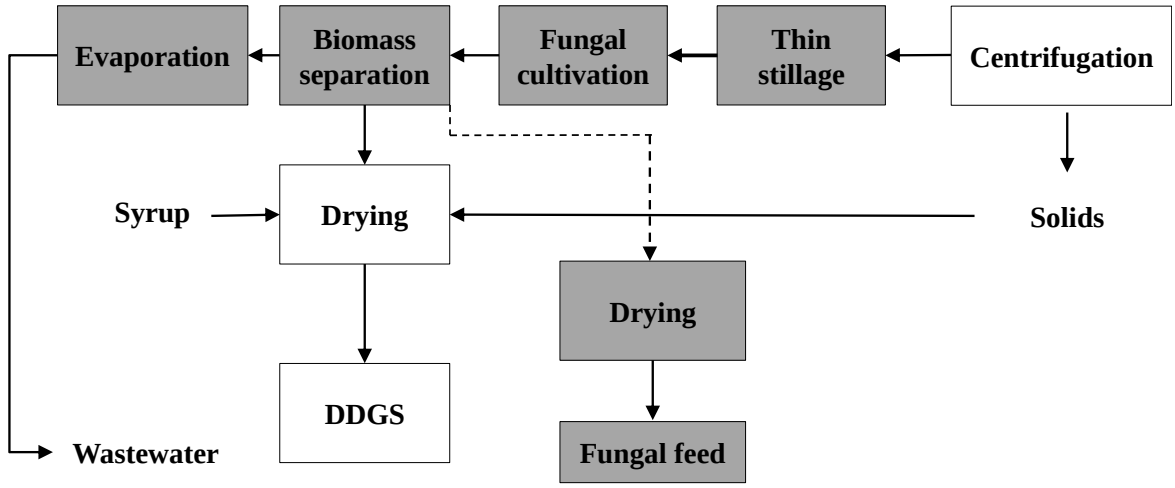

Figure 5.1: Details of the process flow leading to biomass separation and drying. Adapted from **Figure 2.1**.

The present chapter deals with the production of biomass and compositional characterisation and ethanol using the edible filamentous fungi discussed in **Chapter 4**. In the first part of the chapter, fungal biomass characteristics and potential feed markets are overviewed and complemented with preliminary data obtained during culture in SSL and thin stillage using shake-flasks. In the second part of the chapter, preliminary results of ethanol production when the filamentous fungi were grown in thin stillage and whole stillage, also using shake-flasks, are provided.

5.1 Fungal biomass

The interest on fungal biomass goes back to the time when filamentous fungi started to be used for production of human food (**Section 4.3**). Consequently, it has provided those filamentous fungi with the status of GRAS microorganisms. If their fungal biomass is considered for feed purposes, their classification as GRAS can significantly reduce the amount of testing required, prior to commercialisation [95]. The composition of the fungal biomass is arguably behind the increased interest. Depending on cultivation conditions, fungal biomass can reach values of ≥50% crude protein and ≥10% lipids (data given by the research in this thesis). However, if oleaginous microorganisms are used, lipid contents of up to 60% can be reached [64]. Filamentous fungi can be valuable sources of fatty acids such as unsaturated omega-3 (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)), omega-6 (linoleic and γ-linolenic acids), omega-7 (palmitoleic acid) and omega-9 (oleic acid). Saturated C16 (palmitic) and C18 (stearic) fatty acids can also be found in filamentous fungi (**Paper II**). Particularly, γ-linolenic acid is of special nutritional and pharmaceutical importance [236]. Essential amino acids to humans, namely, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan and valine can also be found in the fungal biomass [237]. Lysine, methionine, tryptophan and threonine are of high relevance for non-ruminants, where lysine and methionine are usually the foremost limiting factors in corn and soybean meal diets for swine and poultry [237].

Studies were carried out using shake-flasks to evaluate the potential of biomass production from SSL (**Paper I**) and thin stillage (**Paper III**).

5.1.1 Fungal biomass: production, morphology and protein content

a) *SSL as culture substrate*

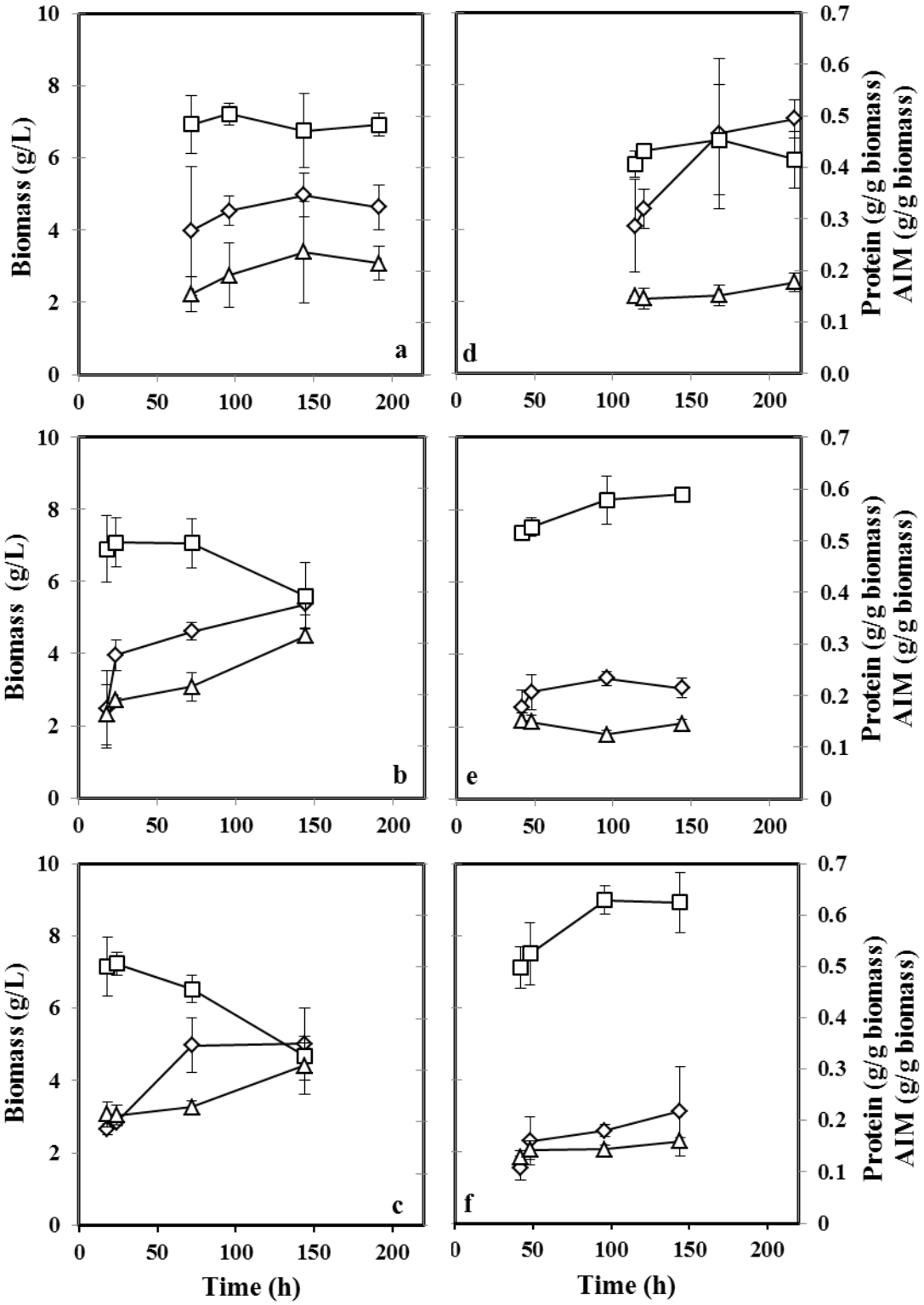

Figure 5.2: Biomass concentration (diamonds), protein (squares) and AIM (triangles) profiles during cultivation of *Rhizopus* sp. in GYV (a-c) and SSL 50% (d-f) at 20 (a, d), 32 (b, e) and 40 °C (c, f). Error bars represent ± 2 s.d. (**Paper I**).

A *Rhizopus* sp. isolated from Indonesian tempe was cultivated in SSL diluted to 50% (SSL50%), supplemented with ammonia and ammonium dihydrogen phosphate. A comparison of performance was made with glucose, yeast extract and vitamin (GYV) medium based on glucose, yeast extract, salts, trace metals and vitamins [141] (**Figure 5.2**). The zygomycete was able to grow within the range 20–40 °C where different lag phases were observed; the fungus grew slower in both media when incubated at the lowest temperature. In GYV medium, a biomass concentration of 5 g/L (0.17 g/g glucose) was obtained regardless of the temperature. In SSL50% the highest biomass concentration of around 7 g/L (0.33 g/g sugars) was obtained at the lowest temperature, while around 3 g/L of biomass (0.15–0.16 g/g sugars) was obtained when *Rhizopus* sp. was grown at 32 and 40 °C. However, the starting of the fungal growth was found to be irregular at the lowest temperature, in successive series of replications.

The protein content (determined by the Biuret method) of *Rhizopus* sp. biomass was followed during cultivation. In GYV medium, the protein content represented around 50% (w/w) of the biomass dry weight and assumed a decreasing trend at 30 and 40 °C. During growth in SSL50%, the protein content of the biomass assumed an increasing trend at the same range of temperatures, reaching around 60% (w/w) of *Rhizopus* biomass dry weight. The presence of sugars throughout the cultivation might explain the constant high values of protein, rendering *Rhizopus* sp. metabolically active when grown in SSL50%.

b) *Thin stillage as culture substrate*

The growth of a *Rhizopus* sp. isolated from Indonesian leaves used for preparation of tempe was studied in thin stillage containing 9% solids using also shake-flasks at different temperatures (**Figure 5.3**). The production of biomass was found to be similar at the beginning of cultivation within 30–40 °C (around 6.5 g/L); a lower value was achieved at 45 °C (ca 4 g/L); in addition, a longer lag phase was observed at 25 °C. The highest maximum produced biomass value (ca 15 g/L) was obtained at 30 °C, while the lowest was obtained at 45 °C (<10 g/L). The growth morphology of the zygomycete was dissimilar at different temperatures. It grew as small mycelial clumps within 30–40 °C and as small mycelial pellets at 25 and 45 °C.

The protein content of *Rhizopus* sp. biomass was reported as crude protein using a conversion factor of 6.25 from the Kjeldahl nitrogen. At the end of cultivation, the zygomycete biomass contained 40–55% (w/w) protein on a dry weight basis, being similar to that obtained at the beginning of the cultivation (52–54% (w/w)). Thin stillage is a complex medium regarding composition, and the constant high protein content points out that the zygomycete made nutrients available throughout the cultivation similarly to that of the *Rhizopus* sp. used in SSL.

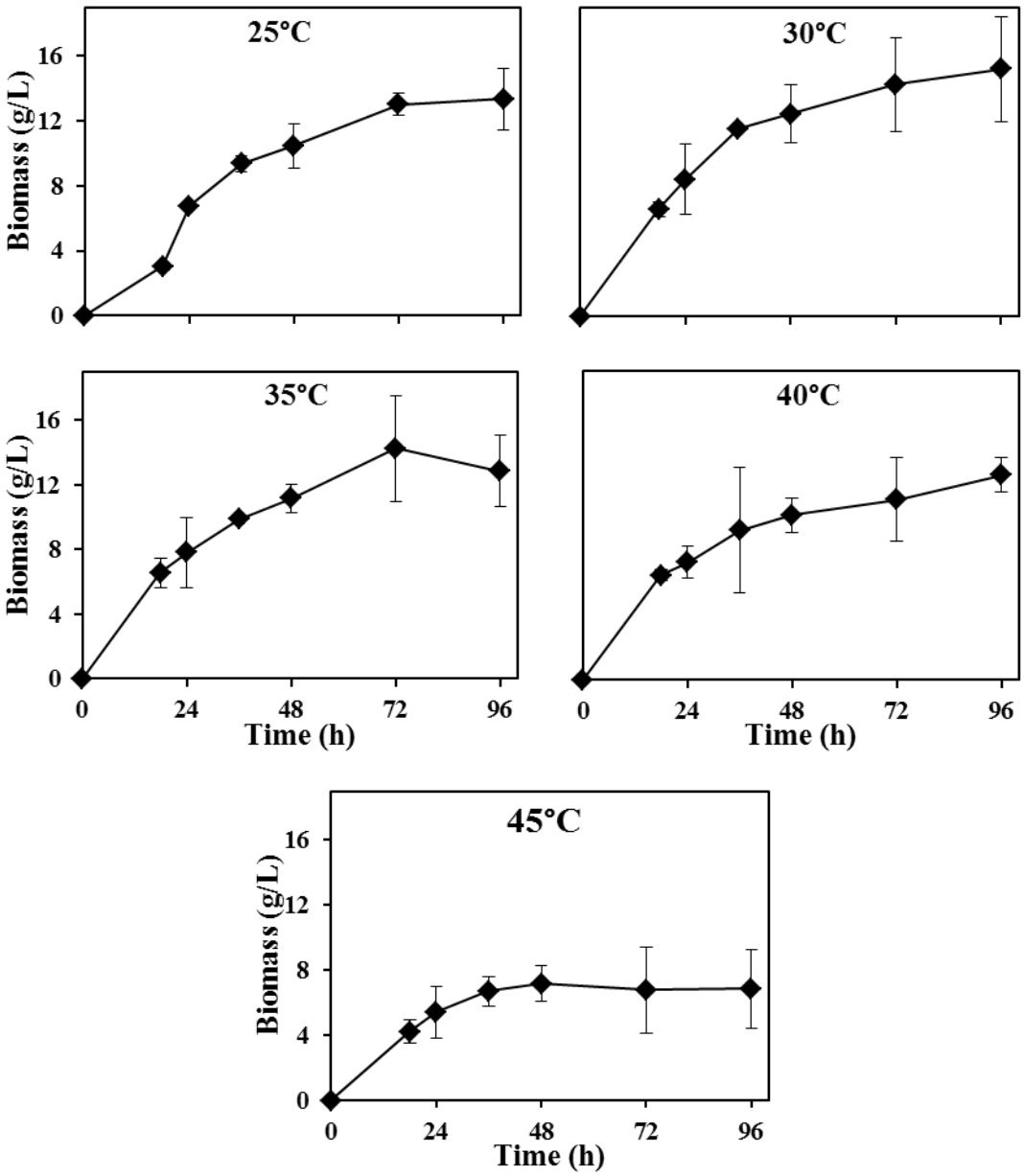

Figure 5.3: Biomass profiles of *Rhizopus* sp. grown in 9% solids thin stillage at different temperatures. Error bars represent ± 2 s.d. (**Paper III**).

The growth of the four ascomycetes fungi, namely, *A. oryzae*, *F. venenatum*, *M. purpureus* and *N. intermedia* were also studied in 9% solids thin stillage (**Figure 5.4**). The highest amount of biomass of 19 g/L was achieved with *A. oryzae*; *N. intermedia* biomass reached 16 g/L while *F. venenatum* and *M. purpureus* exhibited longer lag phases, and their biomass production reached ca 14 and 12 g/L, respectively. The growth morphologies also differed among ascomycetes: *A. oryzae* and *F. venenatum* grew in a well dispersed mycelial form, whereas *N. intermedia* and *M. purpureus* grew as dense mycelial suspensions.

Similar to the growth of *Rhizopus* spp. in SSL and thin stillage, the crude protein contents of the biomass of the ascomycetes were found to be high and slightly varied during cultivation. Crude protein ranges of 44–56% (w/w) of the biomass dry weight were found. *A. oryzae* and *M. purpureu*s exhibited a slight decrease in their crude protein during cultivation (52–48 and 49–44%, respectively). In contrast, the crude protein of *F. venenatum* increased slightly during cultivation (53–56%), while that of *N. intermedia* biomass remained somewhat constant.

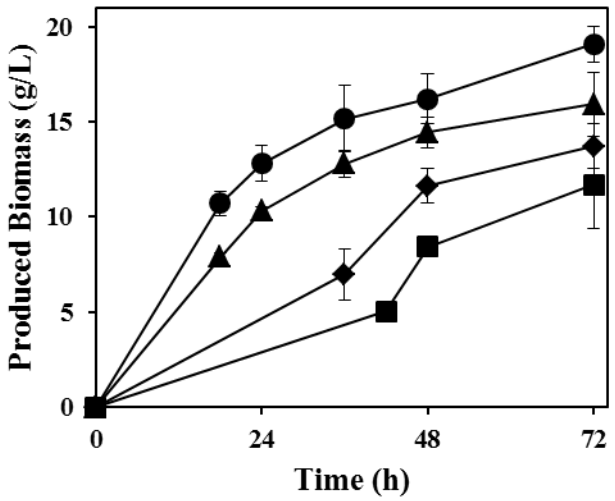

Figure 5.4: Profiles of biomass production during cultivation of *A. oryzae* (circles), *F. venenatum* (diamonds), *M. purpureus* (squares) and *N. intermedia* (triangles) in thin stillage. Error bars represent ± 2 s.d. (**Paper III**).

5.1.2 Cell wall

The cell wall of filamentous fungi has also been of intensive interest by the scientific community due to its composition, including chitin and chitosan. These are similar compounds, where the chitin is mainly based on N-acetyl-glucosamine (GlcNAc) residues, while chitosan is mainly based on glucosamine (GlcN) residues (**Figure 5.5**) [238].

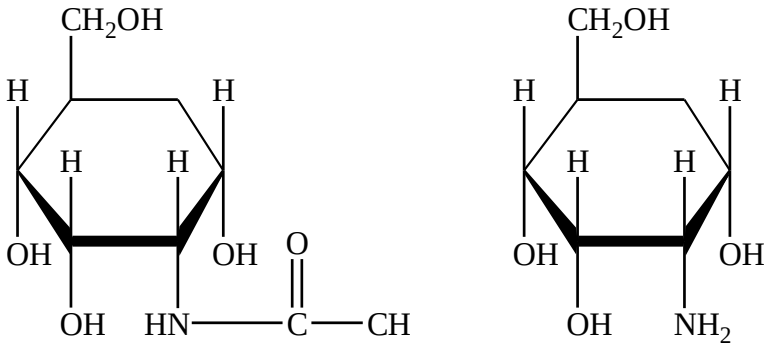

Figure 5.5: Structure of GlcNAc (left) and GlcN (right) that compose chitin and chitosan, respectively [238].

The biosynthesis pathway is common to both chitin and chitosan. The former is produced from the precursor uridine-N-acetyl-glucosamine by a group of different trans-membrane chitin synthetases [239], associated with the micro-vesicle chitosomes [240]. Chitosan is produced via deacetylation of the newly produced chitin by chitin deacetylase before crystallisation [241, 242]. Therefore, chitosan is produced by the "tandem action" of chitin synthetase and chitin deacetylase [241].

The extraction of chitosan from the cell walls from filamentous fungi could be an important alternative to that produced by deacetylation of chitin from marine crustacean shells, a by-product from the food industry. The process of deacetylation is normally carried out by harsh alkaline hydrolysis at high concentration and temperature, resulting in long processing times, environmental pollution and inconsistent physicochemical properties of the produced chitosan. Due to its cationic character, chitosan possesses interesting properties such as nontoxicity, biocompatibility, biodegradability, haemostatic, analgesic, tumour inhibition, mucoadhesive, anticholesterolemic, antimicrobial and antioxidative. Chitosan is of special importance for application in the biomedical, food, biocatalysis and wastewater treatment areas [243]. More recently, chitosan extracted from zygomycetes fungi has been used for

production of superabsorbents with potential use in e.g. single-use medical and personal-care products [244] (**Paper II**).

The cell wall fraction of *Rhizopus* spp. biomass produced during cultivation in SSL50% (**Paper I**) and thin stillage (**Paper III**), denoted here as alkali-insoluble material (AIM), as well as its compositions in GlcN and GlcNAc were characterised.

a) *Cell wall characterisation of the biomass produced from SSL*

The AIM content of *Rhizopus* sp. generally adopted an increasing trend during cultivation in GYV and SSL50% media, being more prominent, where the highest values were obtained, during cultivation in the former at 32 and 40 °C (**Figure 5.2**). The AIM fraction corresponded to 20–30% (w/w) of *Rhizopus* sp. biomass dry weight. Different patterns of GlcN were found at different conditions. It assumed a decreasing trend when *Rhizopus* sp. was grown in GYV medium at 32 and 40 °C, while it increased at other conditions studied. Concurrently, the GlcNAc content generally increased during cultivation, regardless of temperature or cultivation media (**Figure 5.6**). Together, GlcN and GlcNAc made up to 70% (w/w) of the AIM dry weight.

In view of the obtained data, it seems that an increase in the AIM content is mostly due to the production of GlcNAc similar to other zygomycete strains [245], indicating that it is not strain specific. According to Davis and Bartnicki-Garcia (1984), for ageing cells, the degree of deacetylation is lower, since chitin deacetylase cannot access the acetylated growing chains due to higher crystallisation. This leads to higher levels of GlcNAc levels in the cell walls. The data also support that the growth in the harsher SSL50%, and growth at a generally unfavourable temperature, might lead cells to produce more GlcN than under more favourable conditions.

The phosphate content, which has been reported to be associated with chitosan [246], was also followed during cultivation (**Figure 5.6**). It decreased during cultivation, except during cultivation at 20 °C in GYV medium, in which the trend was opposite. The phosphate content of fungi ranges from 0.001 to 0.02 g/g of the cell wall dry weight up to 0.23 g/g in *Mucorales* [238]. In view of the results obtained, it is hypothesised that the content of phosphate in the cell walls is influenced by the microorganism and cultivations conditions

used. The remaining fraction of the fungal cell walls is considered to contain sugars, proteins, glucuronic acid and ash [246].

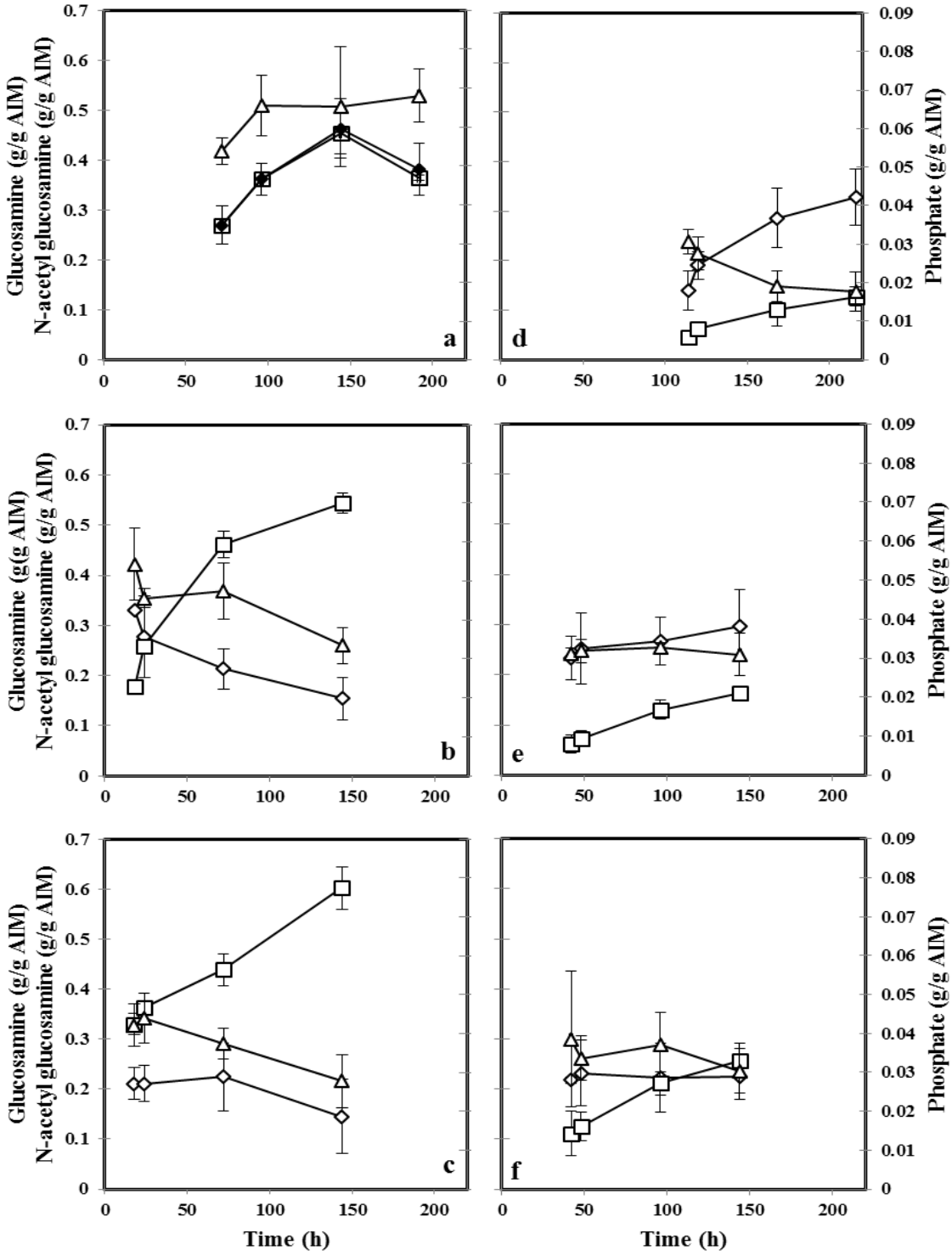

Figure 5.6: Profiles of GlcN (diamonds), GlcNAc (squares) and phosphate (triangles) present in AIM during cultivation of *Rhizopus* sp. in GYV (a-c) and SSL 50% (d-f) at 20 (a, d), 32 (b, e) and 40 °C (c, f). Error bars represent ± 2 s.d. (**Paper I**).

b) *Cell wall characterisation of biomass produced from thin stillage*

When *Rhizopus* sp. was grown in thin stillage, an increasing trend in the AIM content was observed at all tested temperatures (**Figure 5.3**). Overall, the cell wall fraction of *Rhizopus* sp. biomass was in the range 10–15% (w/w) of the biomass dry weight. Both GlcN and GlcNAc present in AIM increased by 45–50% (w/w) during cultivation in thin stillage. These monomers were present at about the same fractions (10–25% each), corresponding to 40–50% of the *Rhizopus* sp. biomass cell wall. Together, GlcN and GlcNAc made up to 6% of the *Rhizopus* sp. biomass.

5.1.3 Feed markets

The biomass of filamentous fungi has many positive aspects for its use for feed production. There are some more positive aspects other than its GRAS character, for example, high contents on protein and comparatively low levels of nucleic acids, high contents of lipids and respective relevant fatty acids and amino acids profiles. The fungal biomass can be easily recovered from the medium, and the taste and smell are generally pleasant [247].

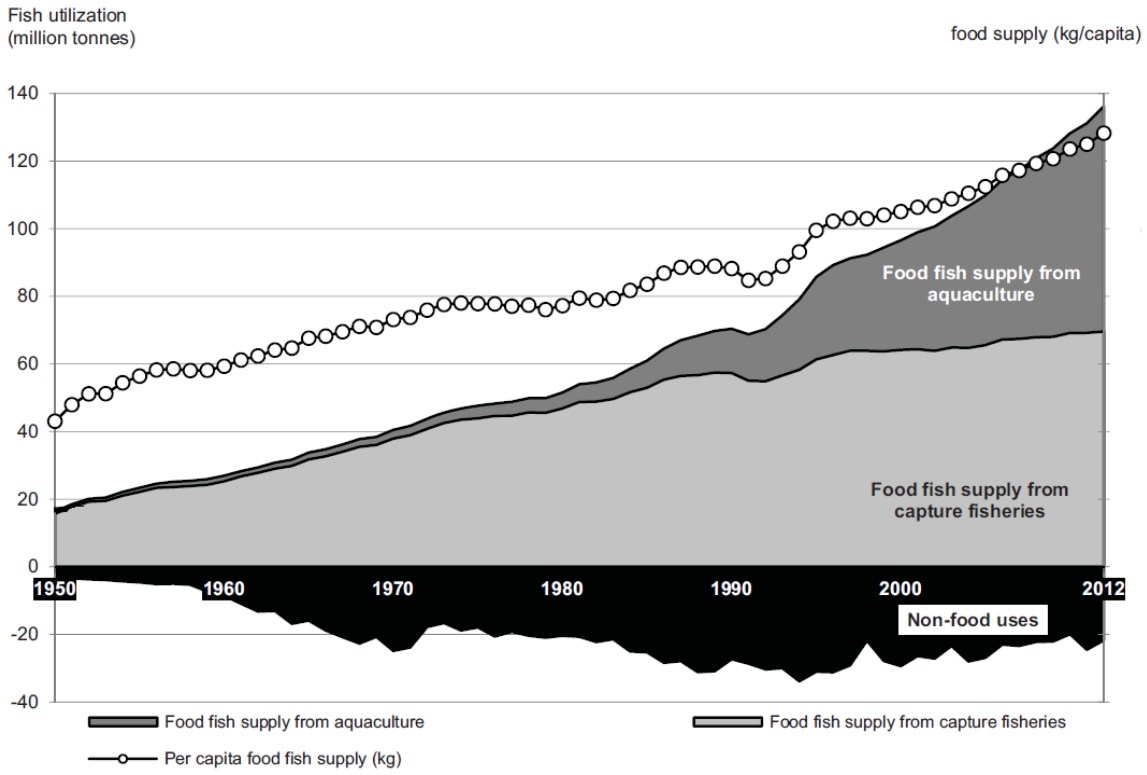

Figure 5.7: World fish utilisation and supply. Adapted and reprinted with permissions from [248].

Potential markets for fungal biomass can include fish, chicken and pet (i.e. cats and dogs) sectors [95]. The fish production in aquacultures has steadily been increasing since 1950 (**Figure 5.7**). However, the increase in fish production in aquaculture is concomitant with the increase in fish feed, namely, fishmeal and oil [249]. The increase of the former translates in a higher pressure on wild fish populations [250]. For example, when producing carnivorous species, more fish is used as feed than it is produced; the ratio of fish used/fish produced is on average 1.4 [251]. The aquaculture sector is responsible for the consumption of ca 70% and 90% of the world fishmeal and fish oil production, respectively [252]. Therefore, research on alternative sources should be intensified in order to reduce the pressure on the wild species [253]. Alternatives studies have included feed sources based on plants (e.g. soy) or terrestrial animals [254].

The production of feed for animals such as for chicken has special requirements, mostly at the levels of specific proteins for satisfactory animal growth. The protein source for chicken originates from plant sources, mainly in the form of soy [255]. Thus, an alternative feed source for chicken is not as essentially needed as for aquacultures, and it would compete just from an economical point of view [95].

The production of products for the pet group has been growing exponentially since 2000, with prospects of continuous growth at high rate [256]. Commonly produced products include those for prevention and treatment of osteoarthritis, where GlcN is normally used. According to the limited studies, the alleviation of the disease is promoted by the administration of GlcN as a preventive measure [256]. Therefore, an alternative source of GlcN can potentially play an important role in this sector.

5.2 Ethanol

The study of ethanol production using filamentous fungi has been more prominent using strains from the *Mucorales* order, more particularly those belonging to *Rhizopus*, *Mucor* and *Rhizomucor*. Ethanol yields per glucose consumed at 0.43 g/g and 0.47 g/g were reported under aerobic and micro-aerobic conditions, respectively. Ethanol yields from xylose of up to 0.28 g/g were achieved under aerobic conditions. *Mucorales* have also been studied for production of ethanol using lignocellulosic substrates. Those included spent sulphite liquor

(ethanol yield of 0.16 g/g sugar), dilute-acid hydrolysate from spruce (ethanol yield of 0.44 g/g sugar) and orange peel hydrolysate (ethanol yield of 0.40 g/g hexoses) (**Paper II**).

Studies using shake-flasks in production of ethanol were carried out using thin stillage (**Paper III**) and whole stillage (**Paper V**) as substrates.

5.2.1 Thin stillage for ethanol production

The five different filamentous fungi used for production of biomass from thin stillage were also evaluated for production of ethanol, and the results are depicted in **Figure 5.8**. During cultivation of *Rhizopus* sp. at 30, 35 and 40 °C, the production of ethanol was around 1 g/L. Concurrently, when the ascomycetes were used for production of ethanol at 30 °C, the production was around 0.5 g/L with *M. purpureus*, 2 g/L with *A. oryzae* and *F. venenatum*, and 5 g/L with *N. intermedia*. Therefore, *N. intermedia* was found to be a biocatalyst with high potential for production of ethanol from thin stillage.

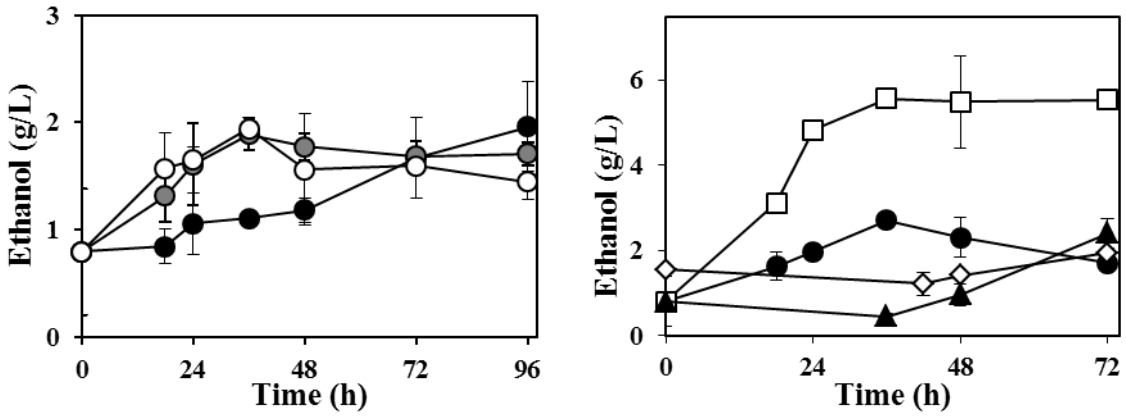

Figure 5.8: Ethanol profiles during cultivation of *Rhizopus* sp. (left) at 30 (black), 35 (grey) and 40 °C (white) and the ascomycetes (right) *A. oryzae* (circles), *F. venenatum* (triangles), *M. purpureus* (diamonds) and *N. intermedia* (squares) at 30 °C. Error bars represent ± 2 s.d. (**Paper III**).

5.2.2 Whole stillage for ethanol production

The effect of enzyme addition on ethanol production was studied using whole stillage as substrate. The enzyme used was the commercial cellulase Celic®Ctec2 (Novozymes, Denmark) and when the enzyme was added, the shake-flask cultivations were carried out under simultaneous saccharification and fermentation (SSF) with *N. intermedia*.

In contrast to separate hydrolysis and fermentation (SHF), in SSF, both hydrolysis and fermentation are performed in the same vessel, leading to investment savings and the end-product inhibition and contamination being avoided due to the immediate consumption by the fermenting microorganism of the released glucose and the ethanol produced, respectively [257].

As depicted in **Figure 5.9**, the addition of enzyme led to improvements in the ethanol production. The highest amount of ethanol of 11.6 ± 0.8 g/L and the highest production rate of 232 ± 6 mg/L/h were achieved at the highest enzyme loading. Nonetheless, the positive effect of cellulase addition was more strikingly observed between no addition of enzyme and when it was added at 1 FPU/g SS. An increase of 85 and 98% was achieved in the ethanol production and production rate, respectively. When needed in the fermentation processes, the use of enzymes represent a large share of overall process costs, and it must therefore be minimised. At gradually higher enzyme concentrations, its effect on ethanol production diminished since 0.35 ± 0.02, 0.13 ± 0.00 and 0.08 ± 0.00 g ethanol/L/FPU were obtained when 1, 5 and 10 FPU were used, respectively. Therefore, the enzyme loading of 1 FPU/g SS was used for further shake-flask studies with whole stillage.

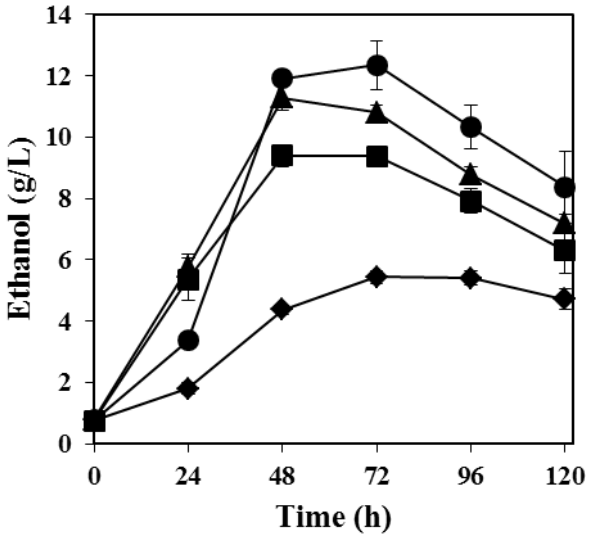

Figure 5.9: The ethanol profiles during cultivation of *N. intermedia* in whole stillage with 0 (diamonds), 1 (squares), 5 (triangles) and 10 (circles) FPU of cellulase per g of SS. Error bars represent ± 2 s.d. (**Paper V**).

By separating the whole stillage into three distinct fractions, namely, the supernatant, large particles (sieved particles) and small particles (centrifuged particles), interesting aspects were unveiled. The supernatant gave the highest contribution to the ethanol production (75%), while the ethanol produced from the "large particles" and "small particles" accounted for 20 and 5% of the total amount when no enzyme was added to the medium, respectively. Addition of cellulase to the supernatant led to a similar maximum of ethanol production, but at a higher production rate. Moreover, addition of cellulase led to no further improvement in the ethanol production from "large particles", while three times more ethanol was produced when "small particles" were used (**Figure 5.10**). The addition of yeast extract (5 g/L) to the medium containing "large particles" did not lead to further improvements on ethanol production.

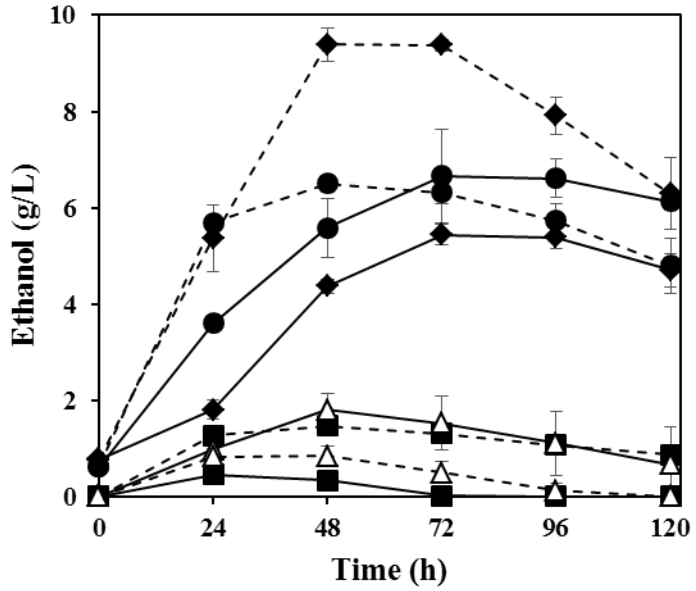

Figure 5.10: Ethanol production profiles during *N. intermedia* cultivation in undiluted whole stillage (diamonds) and its fractions (supernatant (circles), "small particles" (squares) and "large particles" (triangles)) with (dashed lines) and without (straight lines) cellulase addition. Error bars represent ± 2 s.d. (**Paper V**).

CHAPTER 6

6 PROCESS DEVELOPMENTS IN PILOT SCALE BIOREACTORS

In order to obtain further insights on the valorisation of intrinsic and extrinsic substrates to the industrial 1st generation bioethanol plant, studies are needed using larger culture volumes. The robustness of a process under development is evaluated by its gradual successful scale up.

The production of ethanol, fungal biomass or both products when using the four substrates considered in this thesis for diversification of 1st generation bioethanol plants (**Figure 2.1**) was also studied using bioreactors. Those were of different design, where CSTR, BC and ALB were used and of different sizes ranging from 0.75 L to 80 m^3. The stages reached regarding the working volume for the different substrates are presented in this chapter. Furthermore, in view of the obtained data, the suitability of the tested substrates for inclusion in the 1st generation bioethanol plant is scrutinised. As will be presented, the research with thin stillage led to the biggest milestone of the present thesis, since the process scale up using a 80 m^3 bioreactor containing up to 30 m^3 of medium was achieved. Therefore, the challenges faced during scale up of the process are also discussed as well as the implications of the process inclusion in the plant regarding biomass and ethanol production as well as the influence on the steps leading to production of DDGS and recycling of water back to the process.

6.1 Whole stillage

The production of biomass as a single product from whole stillage is hampered by the high content of solids. In such situations, where the production of two single products (in this case ethanol and biomass for feed) is desired, a two-stage process might represent a strategy. At the first stage of the process, *N. intermedia* produced ethanol from whole stillage with addition of 1 FPU/g SS, after which the medium is distilled, the solids sieved and the liquid is used at a second stage for production of biomass with *A. oryzae* (**Figure 6.1**). In such a process, the production of 7.6 g/L of ethanol and 5.8 g/L of biomass containing 42% w/w crude protein could be reached. During the second stage of the cultivation, total consumption

of the arabinose and xylitol as well as 63% of xylose was achieved. The lactic acid and glycerol concentrations were reduced by 13 and 9%, respectively (**Figure 6.1**).

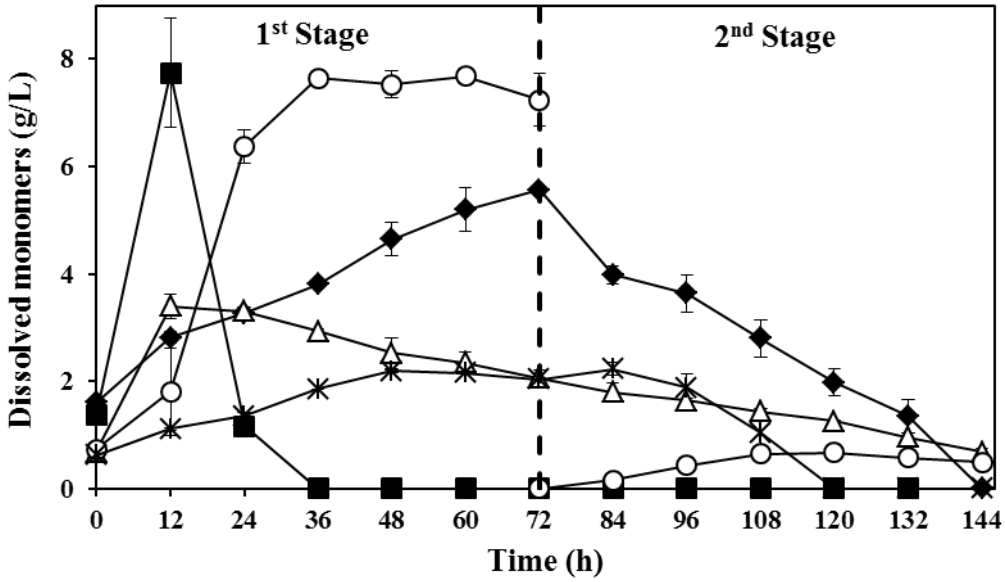

Figure 6.1: Concentration profiles of arabinose (diamonds), ethanol (circles), glucose (squares) and xylose (triangles) during a two-stage cultivation in a 2.5 L CSTR. The first 72 h stage corresponds to the production of ethanol by *N. intermedia* with 1 FPU cellulase/g SS, and the second 72 h stage corresponds to the production of biomass by *A. oryzae* after the medium had been distilled and its solids sieved. Error bars represent ± 2 s.d. (**Paper V**).

A. oryzae was chosen for this second stage of the process based on its good capability to consume glycerol in thin stillage (**Paper III**). The low reduction of glycerol might be related to the C/N ratio or the existence of other more preferable carbon sources in the medium for *A. oryzae*. Also, the aeration rate and agitation applied in the 2.5 L CSTR might have had some effect on these observations. Both fungi degraded complex substrates, including arabinan, glucan, mannan and xylan where reductions of 91, 73, 38 and 89% (w/v) were achieved, respectively.

Since the process of production of ethanol and biomass from whole stillage requires a two-stage cultivation, which means higher investment, thin stillage is more likely the right leftover for improvement of the process and application in a shorter time span.

6.2 Wheat Straw

Disadvantages of using SSF relate to the impossibility of having optimum conditions for both enzymes and fermenting microorganism and the impossibility to reuse the cells. Cellulases have their optimum temperature within 45–50 °C, in contrast with the fermentation that is usually carried out at temperatures below 37 °C [258, 259]. More recently, a new process design has been developed for ethanol production from lignocellulosic materials known as Simultaneous Saccharification, Filtration and Fermentation (SSFF) [260]. SSFF involves a membrane reactor system, where a membrane unit filters the enzyme-slurry mixture in the hydrolysis bioreactor with the sugar-rich permeate continuously reaching the fermentation bioreactor. The fermentation liquid is pumped back to the hydrolysis bioreactor for volume balance in both bioreactors. Thus, SSFF combines the advantages of both SHF and SSF at the same time that it circumvents their limitations: both hydrolysis and fermentation can be performed at optimal conditions. As a result, the end-product inhibition is avoided, and there is also the possibility to reuse the cells in case the ultimate goal of the fermentation is ethanol production [260].

In **Paper VI**, the use of an edible *Rhizopus* sp. filamentous fungus, as a substitute to the often-used baker's *S. cerevisiae*, was investigated for production of ethanol and biomass for feed under SSFF. The used substrate was acid pretreated wheat straw slurry. Two bioreactors, one CSTR (5 L capacity) for the enzymatic hydrolysis of 3.5 L of 5% SS slurry and one CSTR (0.75 L capacity) for production of ethanol and biomass by the fungus, were integrated by a cross-flow membrane for separation of sugars and other organic compounds. The continuously generated filtrate was provided to the aerated fermentation bioreactor for production of biomass and ethanol.

When the integration was carried out after 24 h hydrolysis, a maximum ethanol of around 8 g/L was obtained between 24 and 48 h of cultivation. The recirculation speed was found to be suitable for assimilation of carbon sources by *Rhizopus* sp. since glucose and acetic acid concentrations were not detected in the outgoing feed after 24 and 72 h of cultivation, respectively, after which a more abrupt decrease in the xylose concentration was observed (**Figure 6.2**). A yield of 0.11 gram of biomass per gram of consumed carbon sources was reached.

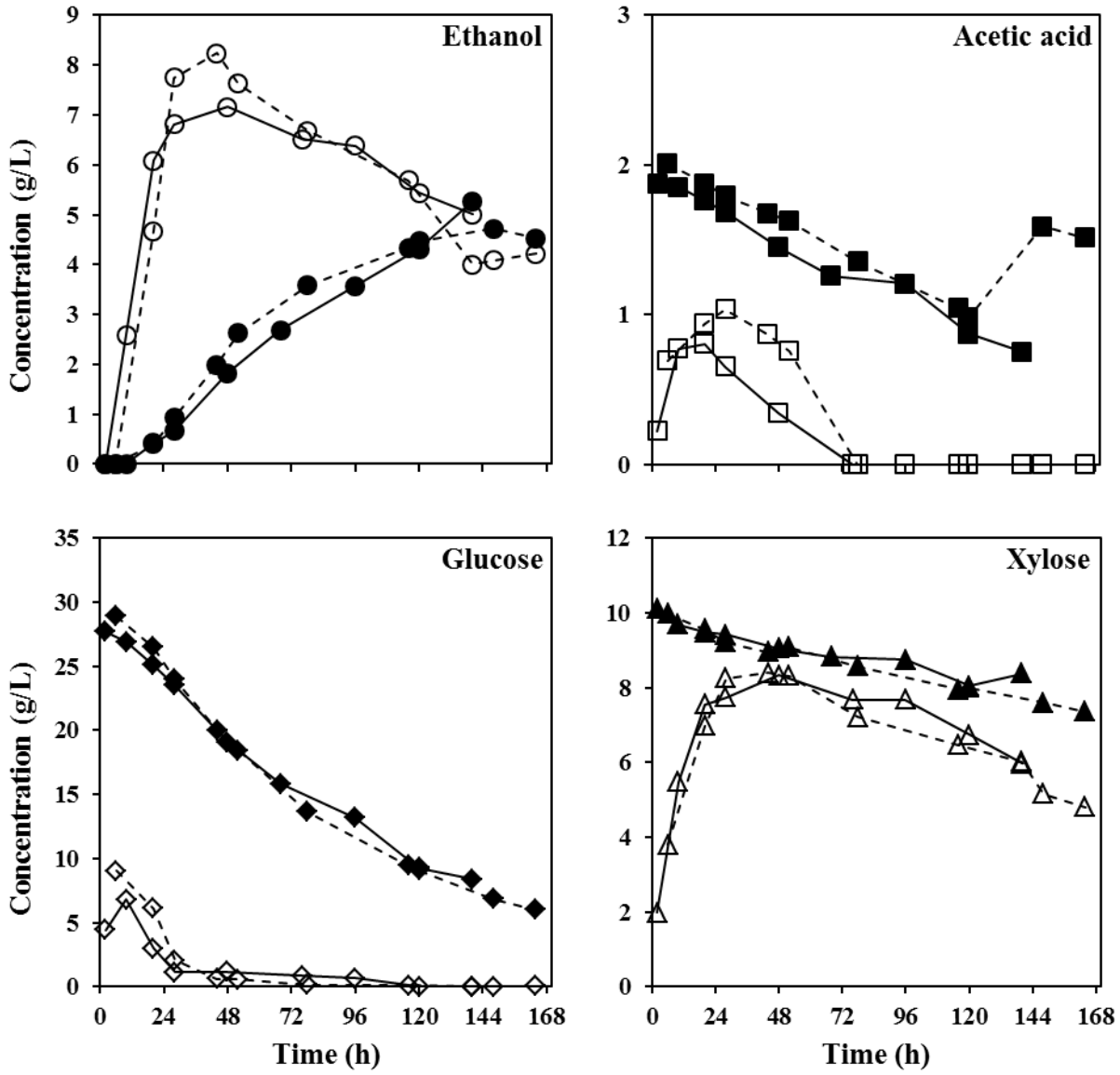

Figure 6.2: Concentrations of ethanol, acetic acid, glucose and xylose in SSFF for aerobic production of *Rhizopus* sp. biomass, following a 24 h of enzymatic hydrolysis before integration. Closed symbols denote concentrations in the ingoing feed; open symbols denote concentrations in the recirculation feed (**Paper VI**).

When no enzymes were added during the first 72 h of integration, no significant changes were observed in the concentration of glucose, acetic acid, ethanol or xylose. However, when enzymes were added and the oxygen was switched off, approximately 7 g/L of ethanol could be produced, but the performance of xylose and glucose assimilation was lower when compared to that when the cultivation was set under aerobic conditions (**Figure 6.3**). A yield of 0.034 gram of biomass per gram of consumed carbon sources was obtained.

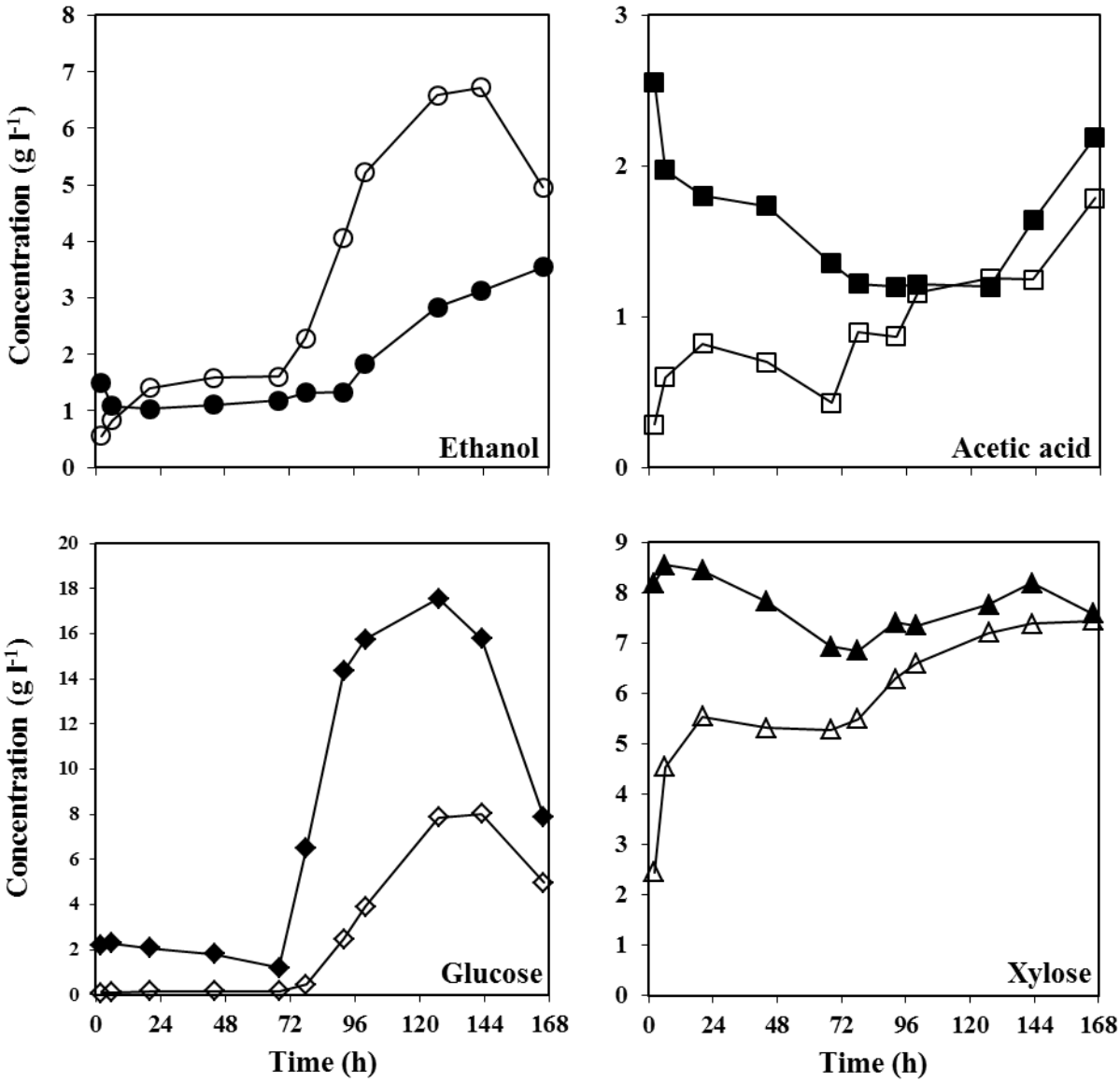

Figure 6.3: Concentrations of ethanol, acetic acid, glucose and xylose in SSFF for aerobic production of biomass followed by anaerobic fermentation by *Rhizopus* sp.. The air of the biomass vessel is switched off, and enzymes are added to the hydrolysis vessel at t = 72 h (**Paper VI**).

In another SSFF experiment, the integration was preceded by a 54 h enzymatic hydrolysis of the pretreated wheat slurry with added dry baker's yeast, leading to a residual amount of glucose. The medium was distilled and used for integration. A biomass yield of 0.34 g per gram of consumed xylose and acetic acid was achieved.

The addition of pretreated wheat straw to the 1st generation bioethanol process is of high importance and it could constitute an important step towards the realisation of commercial production of 2nd generation bioethanol. It is well known that such a facility cannot rely just on the production of one value-added product (i.e. ethanol) but on many. Therefore, the present strategy seems very promising regarding the production of two value-added products from pretreated wheat straw. However, using the present technology on large scale will mean scale-up challenges; thus, further research is needed.

6.3 Spent sulphite liquor

Spent sulphite liquor was 50% diluted and supplemented with nitrogen sources and used for production of *Rhizopus* biomass in a 26 L airlift bioreactor (**Figure 6.4**). The yield of biomass increased at gradually higher aeration rates indicating a high oxygen demand, where the highest effect was observed when changing the aeration rate from 0.15 to 0.5 vvm (**Figure 6.4**). Approximately, 7 g/L of biomass (0.34 g/g sugars) could be produced at 1 vvm. *Rhizopus* sp. grew as perfect small pellets at this aeration rate.

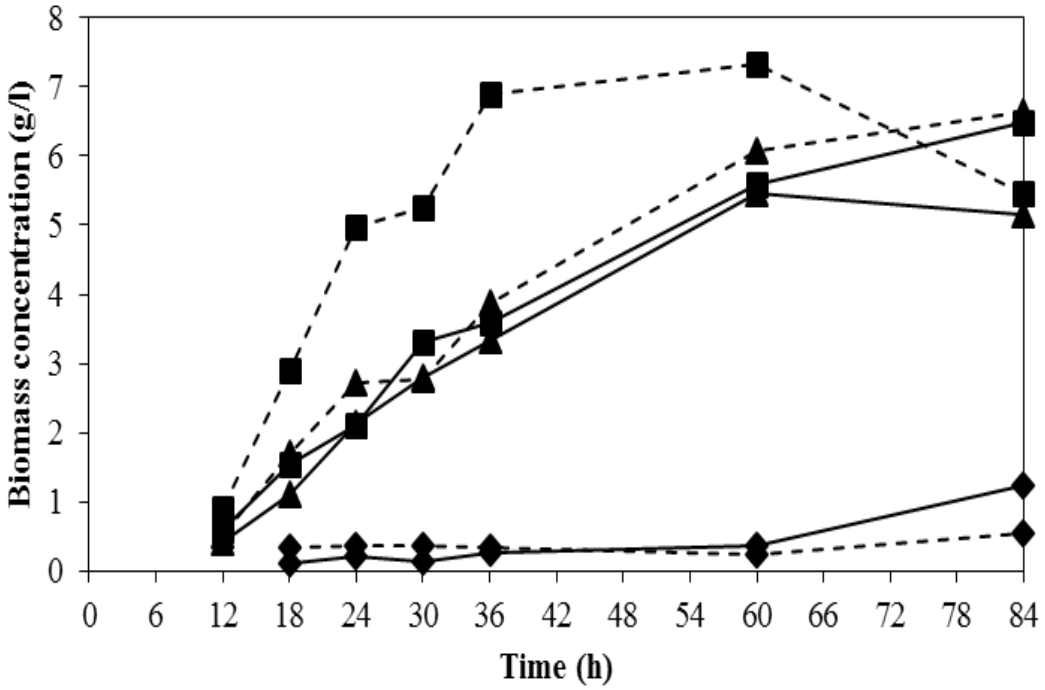

Figure 6.4: Biomass profiles during *Rhizopus* sp. cultivation in SSL50% using an airlift bioreactor, at 0.15 (diamonds), 0.5 (triangles) and 1 vvm (squares). Straight and dashed lines represent the replicates 1 and 2, respectively (**Paper I**).

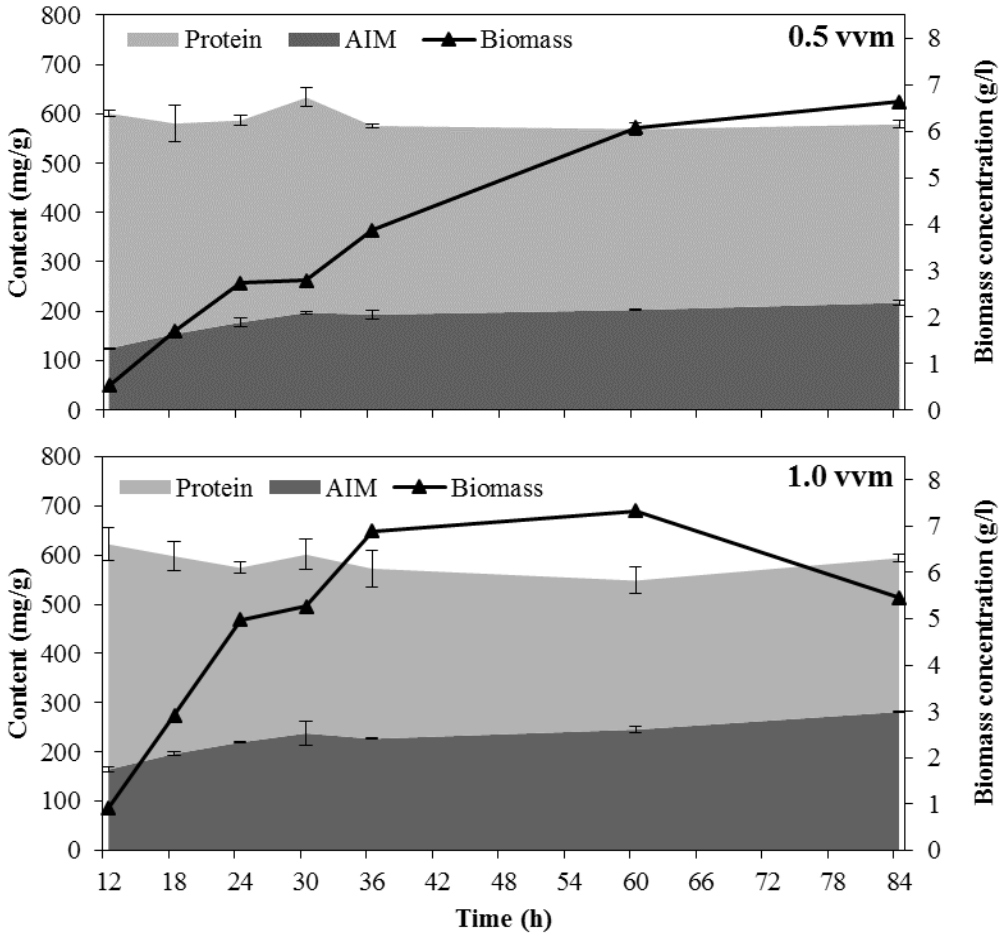

Figure 6.5: Profiles of AIM, protein and biomass for the replicate 2 of *Rhizopus* sp. cultivation in SSL50% using an airlift bioreactor. The error bars represent ± 2 s.d. (**Paper I**).

During cultivation, some trends were observed (**Figure 6.5**). The cultivation proceeded together with a thickening of the cell wall translated by an increase in the AIM content. Accordingly, previous studies point towards ageing cells with thicker cell walls [261]. A decrease in the protein content was also observed as the cultivation proceeded, being quite notorious difference between the fastest growth curve at 1 vvm, compared to that at 0.5 vvm. In the same way, the lipid content decreased at gradually higher aeration rates (**Table 6.1**). Higher initial fungal activity, higher rates of synthesis as well as nutrient limitation might explain those observations. Altogether, biomass protein and lipid contents accounted for about 0.40 to 0.50 g/g until 36 h of growth and for about 0.35 g/g after 84 h.

The contents of GLcN and GlcNAc, the main building blocks of chitosan and chitin, respectively, were followed during cultivation in SSL50% in the airlift bioreactor

(**Figure 6.6**). The GlcN content ranged from 220–270 and 220–320 mg/g, while its acetylated form, GlcNAc, ranged from 100–160 and 100–200 mg/g, at aeration rate of 0.5 and 1 vvm, respectively (**Figure 6.6**). Therefore, a GlcN/GlcNAc ratio of 1.6-2.2 was obtained throughout the cultivation. Per liquid volume, the maximum volumes corresponded to 0.33 and 0.53 g/L GlcN and 0.22 and 0.31 g/L GlcNAc, at 0.5 and 1 vvm, respectively. Previous studies have shown that the cationic chitosan in the cell wall of the zygomycetes is followed by polyphosphate and other polymeric anions [262]. Such an observation was not gotten in this study since the values of GlcN and phosphate could not be correlated (**Figure 6.6**). The presence of polyphosphates might be to serve as phosphate reserves, but it is still unclear and further studies are needed.

Table 6.1: Lipid fractions of *Rhizopus* biomass quantified after 84 h of cultivation in SSL50% in an airlift bioreactor. The error (± 2 s.d.) was estimated as 17% of the reported values (**Paper I**).

Aeration (vvm)	Replicate	Lipid fraction (mg/g)
0.15	1	90
0.15	2	87
0.5	1	74
0.5	2	42
1.0	1	46
1.0	2	25

SSL was found to be a good substrate for production of nutritionally-rich biomass using the zygomycete *Rhizopus* sp.. Actually, production of biomass with another zygomycete strain has already been tested using an ALB of a few m^3 in size and feeding trials replacing fishmeal have been also conducted [263-265] (**Paper II**). The achieved results looked promising: the protein content was high, the amino acid composition comparable to that of fishmeal and the proportion of C18 unsaturated fatty acids of approximately 60%. During feeding trials, with carnivorous fish species such as rainbow trout and Atlantic salmon, good growth, high palatability and minor difference from fish fed with a standard diet were observed. However, any added substrate or further valorisation of substrates of the ethanol plant must not affect

the final characteristics of the DDGS. Therefore, further studies are needed in order to ascertain the impact of the SSL lignosulphonates in the DDGS and their use for feed.

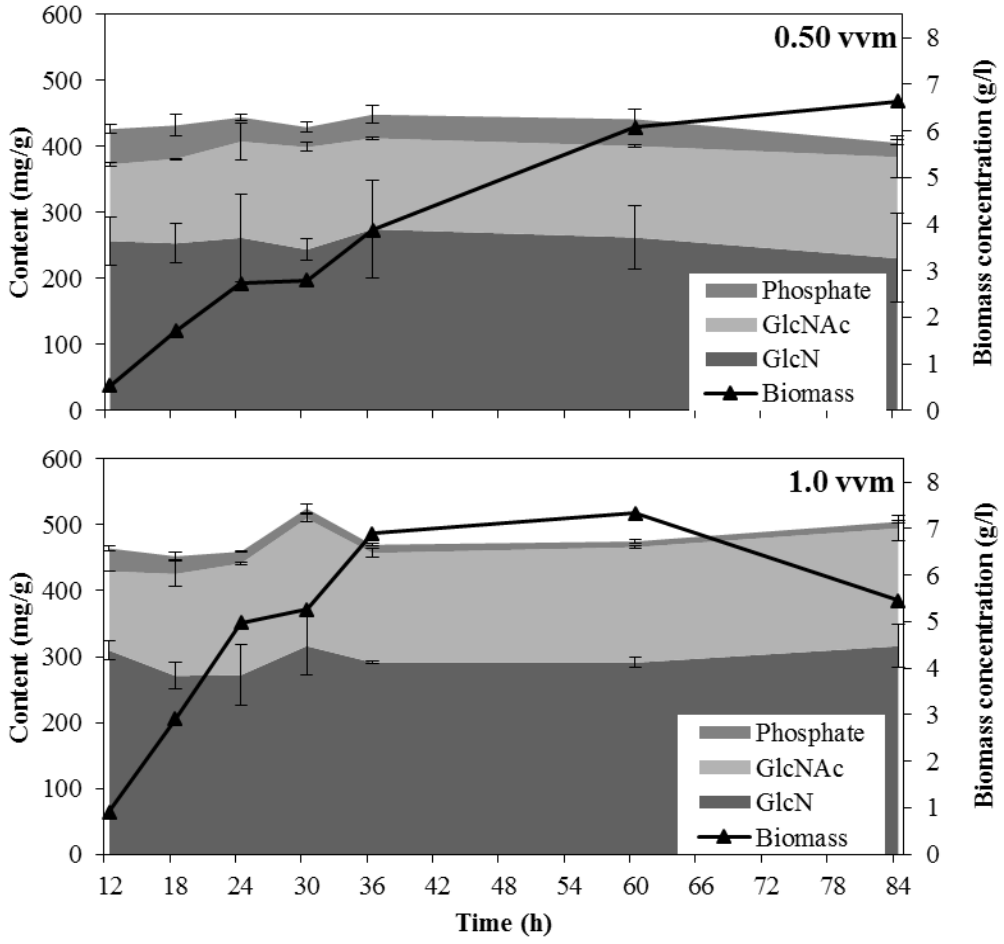

Figure 6.6: AIM profiles of GlcN, GlcNAc and phosphate compared to the biomass concentration from *Rhizopus* biomass during the replicate 2 in SSL50% at 0.5 and 1 vvm. The error bars represent ± 2 s.d. (**Paper I**).

6.4 Thin Stillage

A more detailed study was carried out on production of biomass and ethanol from thin stillage with *N. intermedia* at bioreactor scale. *N. intermedia* was first cultivated in thin stillage using the 26 L ALB where the aeration rate was varied between 0.5 and 2 vvm in order to study the upper and down limits regarding ethanol and biomass production (**Figure 6.7**). A clear trend was observed where higher the aeration rate applied, the lower ethanol and the higher biomass production achieved and vice versa. The highest amount of ethanol of around 3 g/L was achieved at 0.5 vvm while the highest amount of produced biomass of around 9 g/L was

achieved at 2 vvm. An aspect worth noting is that at aeration rates higher than 0.5 vvm, the ethanol concentration decreased after 24 h of cultivation. The crude protein of the biomass decreased slightly during cultivation at all tested aeration rates applied, where ranges of 48-53% w/w were obtained.

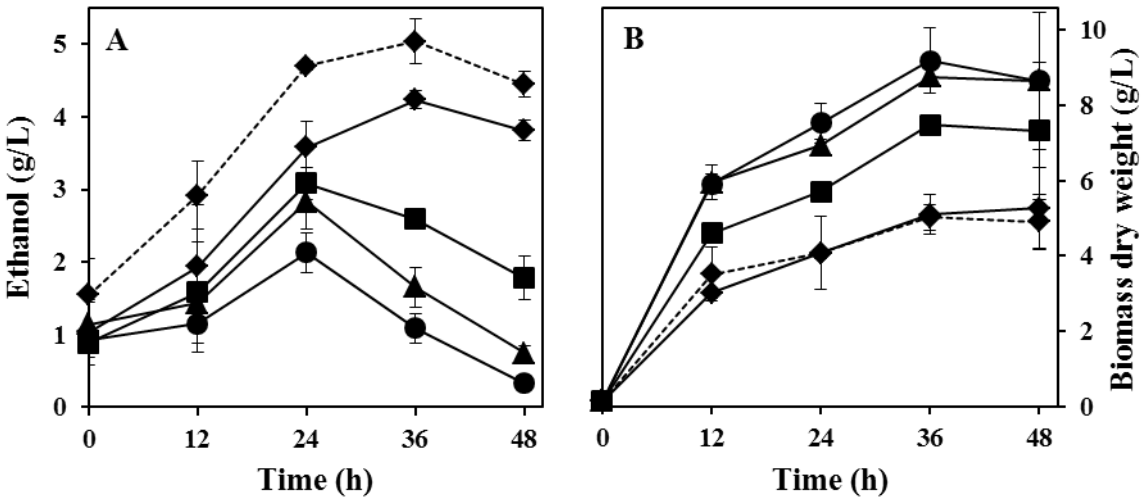

Figure 6.7: Profiles of (**A**) ethanol and (**B**) biomass dry weight during 48 h of cultivation of *N. intermedia* in ALB (straight lines) and BC (dashed line) at 0.5 (diamonds), 1 (squares), 1.5 (triangles) and 2 vvm (circles). All error bars represent ± 2 s.d. (**Paper IV**).

The valorisation of the thin stillage was also investigated in a BC. In practice, this was carried out by removing the internal loop tube from the ALB and applying aeration rate of 0.5 vvm. As shown in **figure 6.7** (dashed line), the ethanol and biomass production profiles between ALB and BC were similar. The maximum production of ethanol and biomass was 3.5 ± 0.2 and 5.0 ± 0.3 g/L, respectively. Therefore, there is the possibility to use a simpler (thus, cheaper) reactor for production of ethanol and biomass from thin stillage. Accordingly, the biomass harvested at the end of cultivation in the bubble column was further analysed for crude protein, lipids and respective amino acids and fatty acids profiles (**Table 6.2**). A comparison was tentatively made with the composition of the DDGS. All nine essential amino acids for humans were present in *N. intermedia* biomass and accounted for 40% of its amino acid composition. The most striking differences between DDGS and the fungal biomass were the amounts of glutamic acid, lysine and proline. The lipid content of the biomass was mainly

composed of linoleic acid (47.6 ± 0.7%), palmitic acid (20.5 ± 1.5%), oleic acid (15.3 ± 0.4%), α-linoleic acid (4.6 ± 0.1%) and stearic acid (3.8 ± 0.2%) (**Table 6.2**).

Table 6.2: Protein and lipid composition of industrial DDGS and *N. intermedia* biomass obtained from 48 h cultivation in BC bioreactor at 0.5 vvm (**Paper IV**).

Component (mg/g)	DDGS	*N. intermedia*	Component (mg/g)	DDGS	*N. intermedia*
Protein content and amino acid profile			**Lipid content and fatty acid profile**		
Crude protein	514 ± 13	529 ± 65	Lipid	77 ± 2	116 ± 11
Alanine	20.2 ± 0.5	32 ± 4	C 14:0	ND [a]	< 1
Ammonia	17.2 ± 0.4	13 ± 3	C 15:0	ND	< 1
Arginine	23.1 ± 0.6	29 ± 8	C 16:0	ND	24 ± 5
Aspartic	28.6 ± 0.7	39 ± 9	C 16:1 n-7	ND	< 1
Cysteine	11.1 ± 0.3	6 ± 1	C 17:0	ND	< 1
Glutamic acid	143.2 ± 3.6	52 ± 10	C 17:1 n-7	ND	< 1
Glycine	19.3 ± 0.5	22 ± 6	C 18:0	ND	4 ± 0
Histidine [b]	11.2 ± 0.3	12 ± 3	C 18:1 n-9	ND	18 ± 1
Isoleucine [b]	21.8 ± 0.6	21 ± 7	C 18:2 n-6	ND	55 ± 4
Leucine [b]	37.7 ± 1.0	32 ± 10	C 18:3 n-3	ND	5 ± 0
Lysine [b]	15.7 ± 0.4	33 ± 9	C 20:0	ND	< 1
Methionine [b]	8.4 ± 0.2	8 ± 3	C 20:1 n-9	ND	< 1
Ornitine	0.3 ± 0.0	6 ± 1	C 20:2 n-6	ND	< 1
Proline	46.0 ± 1.2	19 ± 3	C 22:0	ND	< 1
Phenylalaline [b]	25.2 ± 0.6	18 ± 6	C 24:0	ND	< 1
Serine	26.2 ± 0.7	21 ± 5	C 24:1 n-9	ND	< 1
Threonine [b]	16.8 ± 0.4	21 ± 5			
Tryptophan [b]	5.7 ± 0.1	7 ± 2			
Tyrosine	18.1 ± 0.5	15 ± 4			
Valine [b]	26.1 ± 0.7	27 ± 9			

[a] Not determined; [b] Essential amino acids for humans

The cell wall fraction increased by 60% during cultivation, accounting for up to 30.3 ± 1.6% w/w of the biomass dry weight; up to 1.5 ± 0.1 g/L of AIM was potentially available in the biomass. In the cell wall of the ascomycetes, the chitosan found in the zygomycetes was replaced by glucan [266]. Nevertheless, albeit chitin is the main component of the ascomycetes cell wall and native chitosan was not found, extraction of the latter has been reported from the ascomycetes cell walls [267]. The biomass of *N. intermedia* is thus a potential source of chitosan. The biomass ash content accounted for 5.1 ± 0.1% w/w at the end of the cultivation.

By setting up a continuous cultivation mode, the size of the needed reactor can be considerably reduced. Dilution rates up to 0.2 h^{-1} could be applied without *N. intermedia* cell wash out at 0.5 vvm in the bubble column. The maximum production of ethanol (5.1 ± 1.0 g/L) was obtained at the lowest dilution rate of 0.1 h^{-1}, while the highest production rate (778 ± 59 mg/L/h and 27% higher than that at 0.1 h^{-1}) was achieved at 0.2 h^{-1} (**Figure 6.8**). The trend for biomass production was similar to that of ethanol, where the highest biomass production of 3.6 g/L was obtained at dilution rate of 0.1 h^{-1}. The crude protein of the biomass harvested at the end of the stage at 0.1 h^{-1} was found to contain 50.1 ± 3.8% g/g on a dry weight basis.

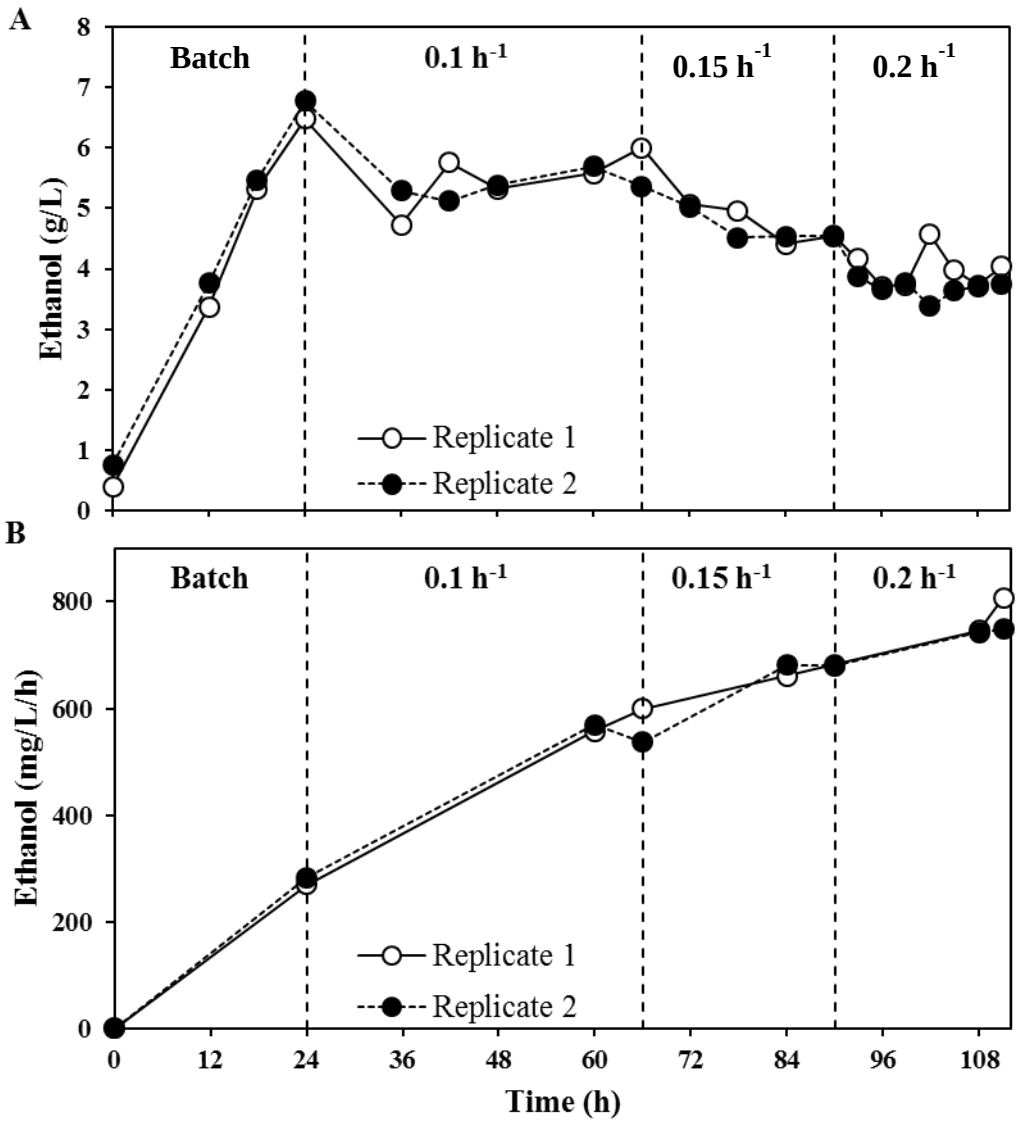

Figure 6.8: (A) Ethanol concentration and **(B)** ethanol production rate during continuous cultivation of *N. intermedia* in thin stillage at dilution rates of 0.1, 0.15 and 0.2 h^{-1}. The cultivation was shifted to continuous mode after 24 h of batch cultivation.

6.4.1 Ethanol and biomass from 1st generation bioethanol thin stillage - implications

The production of ethanol and biomass from thin stillage can take advantage from the equipment already available at the 1st generation bioethanol plant, meaning that so little investment is needed. The ethanol produced can be sent back to the process together with the condensate (as it is already done nowadays), which will increase the ethanol concentration in the mash that can be then recovered by distillation (**Figure 6.9**).

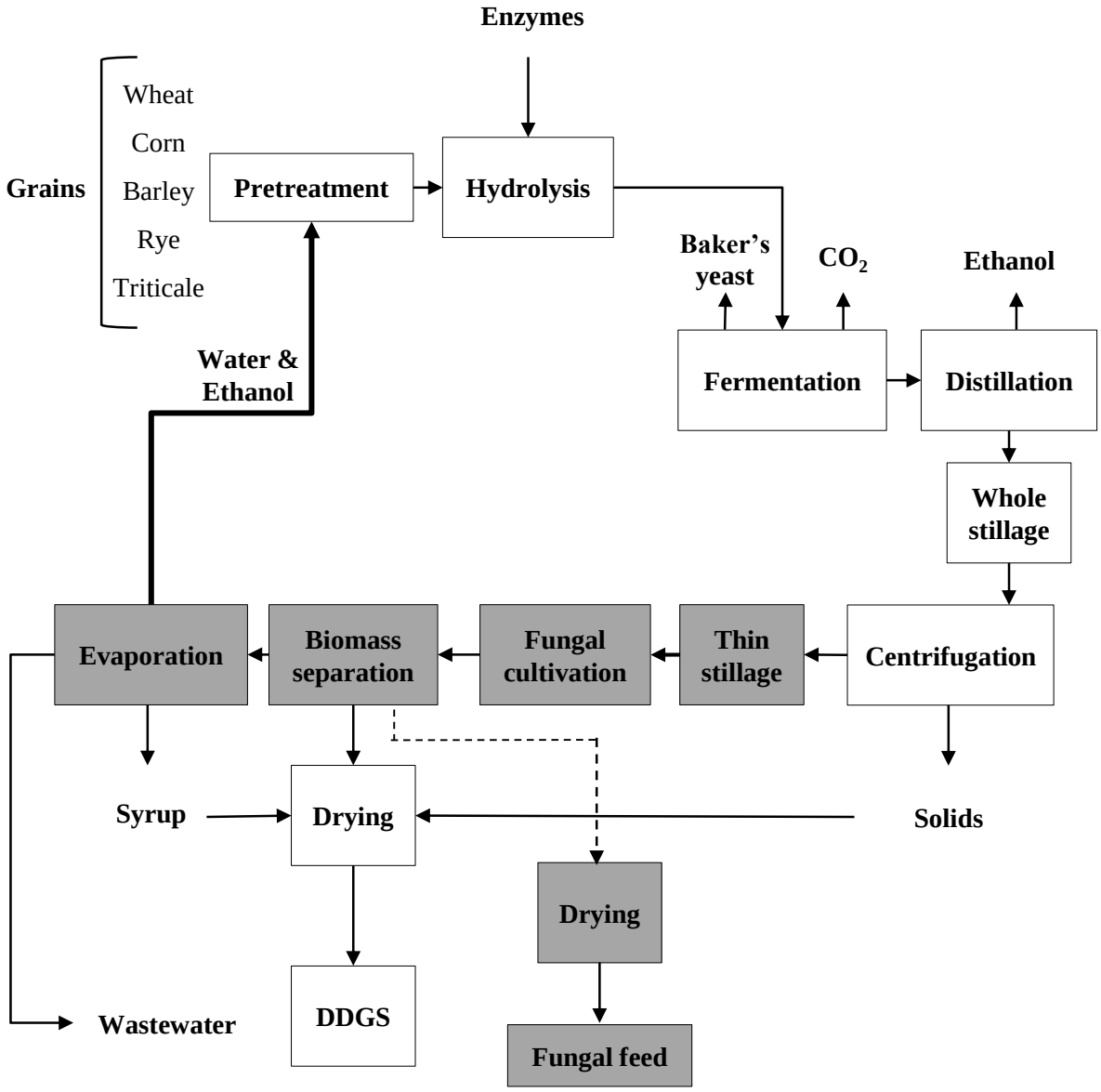

Figure 6.9: Schematic representation of the implications of the inclusion of the process of ethanol and fungal biomass production from thin stillage. Adapted from **Figure 2.1.**

The results presented in this thesis show that more than 11,000 m^3 of ethanol can be produced, representing a process improvement of 5.5%. Furthermore, the possibility of setting the cultivation in continuous mode using a dilution rate of 0.1 h^{-1} would mean that a reactor of 2,000 m^3, around four times smaller than that needed for a batch process of 36 h, could be used.

The produced biomass can be dried together or separate from the wet distiller's grains to produce animal feed products (**Figure 6.9**). Further studies are needed in order to evaluate if producing fungal biomass as a separate product would be more advantageous than drying it together with the wet distiller's grains. At the dilution rate of 0.1 h^{-1}, around 6,300 tonnes of high-quality biomass for animal feed can potentially be produced.

Another aspect to consider is the spent medium after fungal cultivation. In order to produce ethanol and biomass, the ascomycetes have to assimilate organic matter in the medium. Therefore, it is hypothesised that since the spent medium is less viscous and lower in solids, the process of evaporation and drying should become easier. The recycling of water back to the process might also be enhanced due to lower content of solids.

N. intermedia was found to assimilate dissolved saccharides, including glucose-, arabinose- and xylose-based saccharides where a reduction of 70% was obtained (**Figure 6.10**). A total of 16.0 ± 0.1 g/L of carbon sources were reduced in the liquid fraction. Under continuous cultivation, the reduction of dissolved glucose- and xylose-based saccharides decreased gradually at higher dilutions rates; at 0.1 h^{-1} their reduction was 14.7 ± 1.6 g/L. *N. intermedia* could also assimilate sugar polymers, where a reduction range of 57–91% (2.5 ± 0.2 g/L) was achieved during batch cultivation. Under continuous cultivation, the reduction of xylan in the suspended solids decreased gradually at higher dilution rates. The total reduction amount was 2.4 ± 0.2 g/L at 0.1 h^{-1}. During continuous cultivation at a dilution rate of 0.1 h^{-1}, the reduction of measured sugars both in liquid and solid fraction (18.1 ± 1.4 g/L) was comparable to that of total solids (16.9 ± 3.8 g/L). Thus, at this dilution rate, the yield of ethanol was 303 ± 9.5 mg/g of reduced solids. Preliminary studies pointed towards a reduction of around 60% in the viscosity of thin stillage after treatment with filamentous fungi and the biomass had been harvested.

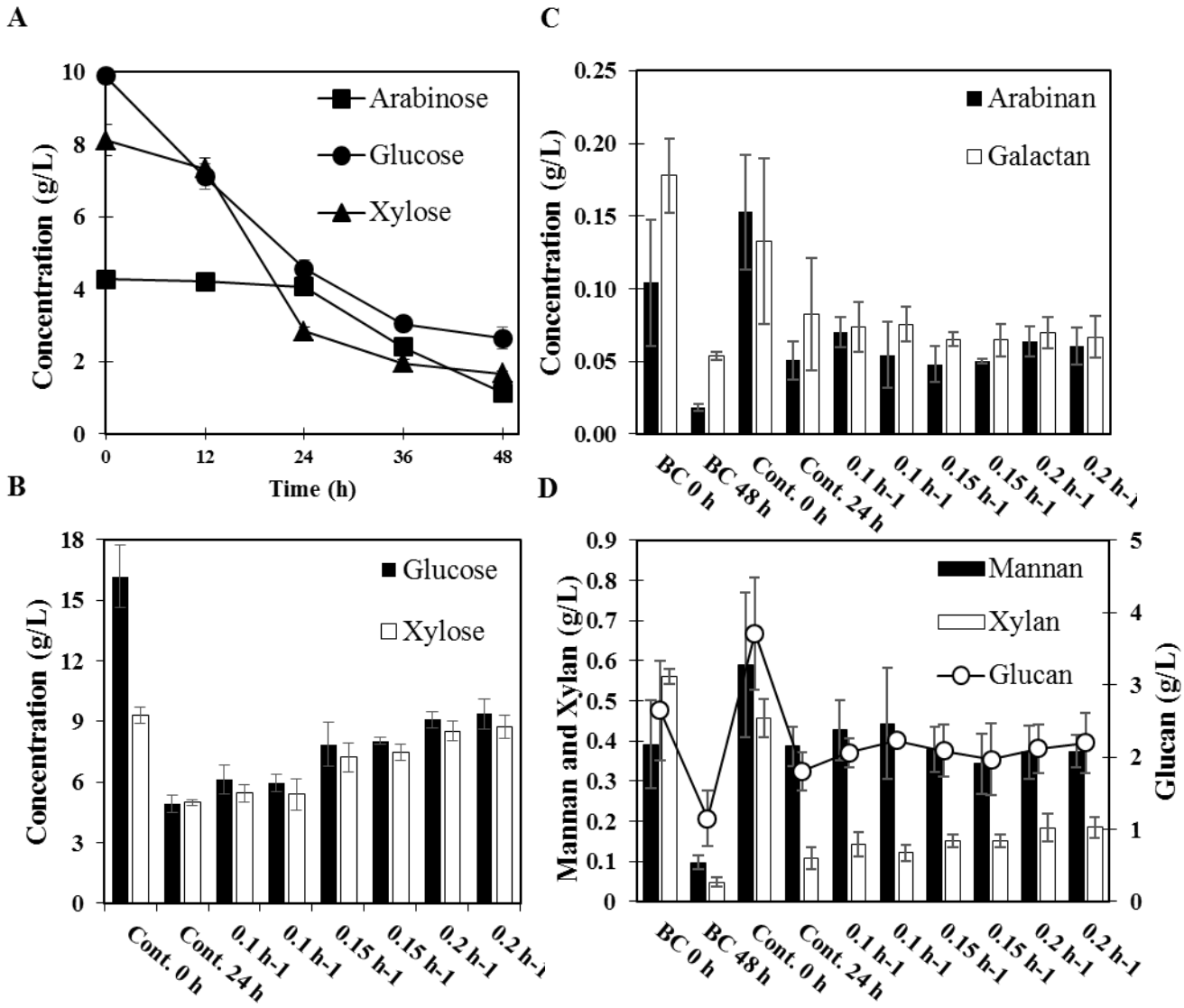

Figure 6.10: Profiles of (**A** and **B**) dissolved saccharides in the liquid fraction and (**C** and **D**) sugar polymers in the solid fraction of thin stillage during batch and continuous cultivation of *N. intermedia* in the bubble column. Error bars represent ± 2 s.d.. "**BC**" stands for the batch process in the bubble column, "**Cont.**" stands for the continuous cultivation, and **"Cont. 0 h**" and "**Cont. 24 h**" represent the 24 h of batch cultivation preceding the continuous cultivation. The graph **A** includes the sugar profiles during batch cultivation in the bubble column (**Paper IV**).

6.4.2 Process scale-up using 2.3 m^3 and 80 m^3 CSTRs

The process of production of ethanol and fungal biomass from thin stillage showed interesting and promising results when reactor-scale studies were performed in the laboratory. Therefore, a step forward was made and trial runs were carried out at Agroetanol on larger scales.

The process was tested using the same operating conditions as those used at the laboratory. The reactors used instead were two CSTRs of 2.3 and 80 m^3 capacity, where the agitation was 5% of the turbine speed. When using the 2.3 m^3 reactor, the working volume was 1.2 m^3 while in the 80 m^3 reactor, the volume was 20 m^3. As can be seen in the **Figure 6.11**, similar ethanol maxima could be reached at all reactor scales, with somewhat varied lag phases. Therefore, in the same way that changing from an ALB to a BC did not lead to significant changes in the final maximum of ethanol production, the use of CSTRs also did not give rise to significant changes, except the lag phases. Running the 80 m^3 reactor in fed-batch mode (F**igure 6.11**) was found to be successful, where a stable phase was reached after 54 h of cultivation. The reached medium volume was of 27.6 m^3.

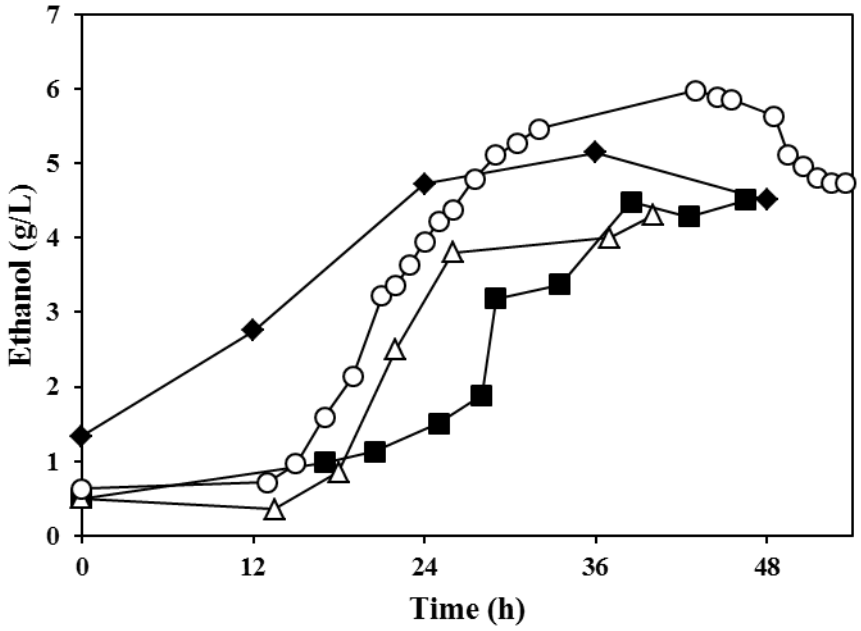

Figure 6.11: Production of ethanol with *N. intermedia* using a 26 L ALB (diamonds), a 2.3 m^3 CSTR with 1.2 m^3 working volume (squares), a 80 m^3 CSTR with 20 m^3 working volume (triangles), and the same 80 m^3 CSTR starting with 20 m^3 working volume and the volume was increased to 27.6 m^3 at 2 m^3 thin stillage per hour (circles). Single replicates are presented.

Transferring a process developed in the laboratory to an established industrial process has its challenges. The trial runs will always be dependent on the available equipment. A different reactor design was used (CSTR vs ALB or BC in the laboratory); however, it seems that such

difference, in the tested conditions, had a more striking influence just on the cultivation lag phase (**Figure 6.11**). The design of the facility will also have impact on the trial runs. The temperature of the thin stillage is quite high (around 80 °C), thus, it has to be cooled down to 35 °C at which temperature the cultivation is carried out. Furthermore, when working on larger scales, the need for a group of pipelines to transport the warm thin stillage to the used bioreactor represents higher probabilities of contamination. Contaminations were a hurdle during the many trial runs carried out at the facility. Here, the addition of antimicrobial agents played an important role for cultivation performance improvements. Future ambitions with the current process include feeding trials and the scale up of the process using 1,000 m^3 bioreactors.

CHAPTER 7

7 CONCLUDING REMARKS AND FUTURE WORK

7.1 Concluding remarks

The insights obtained in this thesis support a high potential for improving the 1st generation dry-mill biorefinery using filamentous fungi. This can be accomplished via valorisation of substrates, either intrinsic or extrinsic to the process of production of ethanol and biomass for feed.

Among the substrates studied in this thesis, the research milestone was accomplished when using thin stillage as cultivation substrate for production of ethanol and biomass for feed using the ascomycete *N. intermedia*. The process of ethanol and biomass production under continuous mode at dilution rate of 0.1 h^{-1} as well as the scale up to around 30 m^3 using a 80 m^3 CSTR were successfully carried out. The potential for feed was unveiled by the high contents in protein and lipids and respective profiles of essential amino acids and long-chain fatty acids found in the *N. intermedia* biomass. Furthermore, the spent medium after the biomass had been harvested is lower in solids and so less viscous, which will possibly simplify the process of water removal during evaporation and drying. Besides, it could be possible to send more thin stillage back to the process as back-set water, resulting in lower loads in the evaporators and driers.

The valorisation of another sidestream of the 1st generation bioethanol process, namely, whole stillage for production of ethanol and biomass was also studied. However, if a single biomass product is desired, the inclusion of the process using whole stillage as substrate will lead to higher investment costs. An additional step requiring separation of solids is needed prior to biomass production.

The inclusion of extrinsic materials of lignocellulosic origin to the industrial process is of special interest for 2nd generation bioethanol industrial realisation. Production of ethanol and biomass was successfully achieved from filtered monomers from pretreated wheat slurry using a cross-filter membrane. However, further studies are needed in order to evaluate the robustness of the process when scaling up the membrane size. Production of high-protein

biomass for feed was successfully achieved using the extrinsic to the process SSL. However, further studies are needed regarding the impact of the lignosulphonates present in the SSL on the characteristics of DDGS and further impact on feed.

7.2 Future Work

A proposal for future research includes:

- Conduct specific studies in order to understand how the type and amount of enzymes expressed by *N. intermedia* when grown in thin stillage are influenced by cultivation conditions.

- Study the effects of addition of different enzymes (e.g. lipases, phytases and cellulases) to the thin stillage on the production of ethanol and fungal biomass.

- Develop cell retention systems for the *N. intermedia* cells in order to be able to run the process at higher dilution rates.

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PAPER I

SPENT SULPHITE LIQUOR FOR CULTIVATION OF AN EDIBLE *RHIZOPUS* SP.

Jorge A. Ferreira,[a] Patrik R. Lennartsson,[a,b,]* Claes Niklasson,[b] Magnus Lundin,[a] Lars Edebo,[c] and Mohammad J. Taherzadeh[a]

Spent sulphite liquor, the major byproduct from the sulphite pulp production process, was diluted to 50% and used for production of an edible zygomycete *Rhizopus* sp. The focus was on production, yield, and composition of the fungal biomass composition. The fungus grew well at 20 to 40°C, but 32°C was found to be preferable compared to 20 and 40°C in terms of biomass production and yield (maximum of 0.16 g/g sugars), protein content (0.50-0.60 g/g), alkali-insoluble material (AIM) (ca 0.15 g/g), and glucosamine content (up to 0.30 g/g of AIM). During cultivation in a pilot airlift bioreactor, the yield increased as aeration was raised from 0.15 to 1.0 vvm, indicating a high demand for oxygen. After cultivation at 1.0 vvm for 84 h, high yield and production of biomass (up to 0.34 g/g sugars), protein (0.30-0.50 g/g), lipids (0.02-0.07 g/g), AIM (0.16-0.28 g/g), and glucosamine (0.22-0.32 g/g AIM) were obtained. The fungal biomass produced from spent sulphite liquor is presently being tested as a replacement for fishmeal in feed for fish aquaculture and seems to be a potential source of nutrients and for production of glucosamine.

Keywords: Spent sulphite liquor; Airlift bioreactor; Zygomycetes; Rhizopus; Chitosan; Lipids; Protein

*Contact information: a: School of Engineering, University of Borås, 501 90, Borås, Sweden; b: Department of Chemical and Biological Engineering, Chalmers University of Technology, 412 96, Gothenburg, Sweden; c: Department of Clinical Bacteriology, University of Gothenburg, 423 46 Gothenburg, Sweden; * Corresponding author: Patrik.Lennartsson@hb.se*

INTRODUCTION

Filamentous fungi have played a vital role in the progress of biotechnology, especially for production of the majority of primary and secondary metabolites, such as organic acids, enzymes, antibiotics (Yin et al. 1998), and several other drugs (Casas López et al. 2004). Interestingly, DNA-based phylogenetic studies have pointed out fungi as being more closely related to animals than to plants (Baldauf and Palmer 1993).

Edible moulds from the genus *Rhizopus* belonging to the phylum *Zygomycota* have been used for centuries by many countries, mainly in Southeast Asia and China, for the preparation of fermented foods, e.g. tempeh (Nout and Aidoo 2002), which increases both the digestibility and the protein value of the foods (Beuchat 1978). This also means that those species are classified as GRAS (Generally Regarded As Safe), which is a great advantage if the fungal biomass is produced for animal or human feed. Additionally, the cell wall of these fungi has been pursued as an alternative source for production of chitosan (White et al. 1979), a valuable polymer for agriculture, food, and pharmaceutical industries (Taherzadeh et al. 2003; Tharanathan and Kittur 2003). Another major

advantage of zygomycetes is their ability to assimilate both hexoses and pentoses (Millati et al. 2008; Sues et al. 2005). More recently, zygomycetes isolated from fermented foods have been investigated as an alternative for the large volumes of fish meal produced (Edebo 2009; Mydland et al. 2007; Bankefors et al. 2011; Sveälv and Edebo 2011). This could prove particularly advantageous, since zygomycetes are known producers of polyunsaturated fatty acids (Weete and Gandhi 1992), which would enhance the lipid content of the food.

Spent sulphite liquor (SSL), a by-product from sulphite pulp mills, has been considered as a raw material for production of valuable products for almost a century (Johnsen and Hovey 1919). SSL is produced in the process of delignification of wood chips in an aqueous solution of acid bisulphites with an excess of SO_2. As a consequence, the lignin is solubilized, and the cellulose remains largely undegraded, while hemicellulose is hydrolyzed to monosaccharides (Heikkila 1986; Mueller and Walden 1970). Discounting water, the main constituents are 50 to 70% lignosulphonates, 20 to 30% sugars, and 6 to 10% polysaccharides (Mckee and Quicke 1977; Mueller and Walden 1970). SSL has been used for the production of a variety of products such as yeast (Mueller and Walden 1970), filamentous fungi (Pretorious and Lempert 1993; Romantschuk and Lehtomäki 1978), ethanol (Taherzadeh et al. 2003), and xylanases (Chipeta et al. 2005). SSL has received the approval of the US Food and Drug Administration as a binding agent in animal feed (FDA 2002) indicating its non-harmful properties at low concentrations and lack of persistent toxic and/or accumulating chemicals. However, so far to the best of our knowledge, no reports exist that deal with the production and composition of *Rhizopus* sp. fungal biomass from SSL and the effect of the culture conditions, especially in airlift bioreactors.

In this work, the influence of process parameters on the growth and biomass composition of an edible strain of *Rhizopus* sp. grown in spent sulphite liquor was studied in bench-scale and 2 m high airlift. In the bench-scale assessments were made on the effect of temperature, cultivation time, and growth media; spent sulphite liquor was compared with a GYV media (Glucose, Yeast extract and Vitamins). In the airlift, the effect of aeration rate, which influences both the oxygen transfer and the total mixing, and the cultivation time were assessed.

EXPERIMENTAL

Fungal Strain

Rhizopus sp. isolated from Indonesian tempeh, named previously as Zygomycete IT (Millati et al. 2005), was used in all experiments. The strain was maintained by successive subcultures on potato dextrose agar (PDA) plates composed of (g/L): D-glucose 20, agar 15, and potato extract 4. The spores were produced on PDA plates by incubation for four days at 28°C and were then stored at 4°C for a maximum of 30 days until use. For cultivation, spore suspensions were obtained by flooding the plates with 20 mL of sterile distilled water.

Cultivation in Shake Flasks

Series of cultivations were performed in 250 mL cotton-plugged baffled Erlenmeyer flasks containing 100 mL of medium. Temperature was maintained at 20, 32, or 40°C using a water bath agitating at 125 rpm. The semi-synthetic (GYV) medium was composed of 30 g/L glucose, 5 g/L yeast extract, salts, trace metals, and vitamins (Sues et al. 2005). Spent sulphite liquor medium (SSL50%) composed of (g/L) mannose 19.1, galactose 6.5, glucose 6.2, xylose 8.0, and arabinose 3.0, was supplemented with 2 mL/L 1 M $NH_4H_2PO_4$ and 6.5 mL/L 25% NH_3 and diluted to 50% with distilled water. The pH was adjusted to 5.5 by addition of NaOH or HCl. The medium was inoculated with 20 mL/L spore suspension. The vitamin solution was sterilized by filtration, and the others were autoclaved at 121°C for 20 min.

Different harvesting times were chosen depending on cultivation medium and temperature in order to produce samples associated with the exponential phase, and the stationary and/or the declination phase. For GYV medium, the harvesting times were set as follows: (a) at 20°C, the biomass was harvested after 72, 96, 144, and 192 h; (b) at both 32 and 40°C, it was harvested after 18, 24, 72, and 144 h. For SSL50% medium, and growth at 20°C, the fungal biomass was harvested after 114, 120, 168, and 216 h; while at both 32 and 40°C, the biomass was harvested after 42, 48, 96, and 144 h. Harvesting was done with a sieve, and the biomass was washed three times with distilled water, frozen, freeze-dried, and stored at room temperature until used. For lipid and total nitrogen analysis drying was done overnight in an oven at 70°C.

Cultivation in Airlift Bioreactor

A 2 m high, 15 cm diameter airlift reactor (Bioengineering, Switzerland) with 26 L total volume of the internal-loop concentric tube reactor model was sterilized *in situ* with injection of steam (121°C, 20 min). SSL50% for a total working volume of 21 L was supplemented with 0.1 mL/L antifoam (VWR International, USA). Inoculum was grown in three baffled 1 L Erlenmeyer flasks, with liquid volumes of 50 mL diluted and supplemented SSL each, in a 125 rpm agitating water bath at 32°C for a total of 72 h. After 48 h, an additional 50 mL diluted and supplemented SSL was added to each flask. The cultivations in the airlift were performed at 32°C. Different aeration rates were tested namely 0.15, 0.5, and 1.0 vvm (air volume per culture volume per minute). The pH was maintained at 5.50±0.07 by addition of 2 M NaOH and 2 M H_2SO_4. Dissolved oxygen was monitored with an oxygen probe. The growth was followed by sampling 0.5 L volumes after 12, 18, 24, 30, 36, 60, and 84 h.

Determination of Protein Composition

The protein content of dried biomass was measured according to a Biuret method previously used for cell wall materials (Zamani et al. 2007).

Total Nitrogen

Crude protein (N×6.25) was determined by Kjeldahl digestion using a 2020 Kjeltec Digestor and a 2400 Kjeltec Analyser unit (FOSS Analytical A/S Hilleröd, Denmark).

Determination of Gross Lipids

Determination of gross lipids was performed according to the EU standard method (Official Journal of the European Communities 1984) using a 1047 Hydrolyzing Unit and a Soxtec System HT 1043 Extraction Unit (FOSS Analytical A/S).

Cell Wall Composition

The preparation of alkali-insoluble material (AIM) from fungal cells followed a method previously described (Zamani et al. 2008; Zamani and Taherzadeh 2010) based on treatment with 0.5 M NaOH.

The content of N-acetylglucosamine (GlcNAc) and glucosamine (GlcN) in AIM was determined according to a previous publication (Zamani et al. 2008) with one modification. Prior to addition of MTBH, the pH was adjusted to approximately 3 by the addition of 1 mL 0.5 M NaAc (Plassard et al. 1982). The acetic acid concentration for measuring GlcNAc from cold sulfuric acid hydrolysates was quantified by HPLC.

The phosphate content of the AIM was quantified from the cold acid soluble phase after the second step of sulphuric acid hydrolysis with the ammonium molybdate spectrometric method according to European Standard ISO 6878 (SIS 2005).

Chromatography

The sugars, inhibitors, and metabolites from SSL fermentation and acetic acid from hydrolysates for determination of GlcNAc were analysed by HPLC (Waters 2695, Waters Corporation, Milford, USA). An Aminex HPX-87H column (Bio-Rad, Hercules, USA) at 60°C and 0.6 mL/min 5 mM H_2SO_4 as eluent was used for most analyses of the medium composition and the metabolites. Additionally, an Aminex HPX-87P (Bio-Rad) at 85°C and 0.6 mL/min ultrapure water was used for separation of mannose, galactose, xylose, and arabinose. A UV absorbance detector (Waters 2487), operating at 210 nm wavelength was used in series with a refractive index (RI) detector (Waters 2414).

Statistical Analysis

All experiments and analyses were carried out in duplicate and analyzed with the software package MINITAB®. All error bars and intervals reported represent two standard deviations. One way ANOVA tables were used to evaluate the results after the data was reduced to single factors. Differences between single treatments were evaluated via pairwise comparisons according to the Bonferroni method.

RESULTS AND DISCUSSION

In this work, spent sulphite liquor, the major byproduct in the sulphite pulp process, was successfully used for cultivation of a *Rhizopus* sp. isolate from tempeh. The high biomass production after scale-up to airlift makes the fungus a potentially valuable new product with two potential applications. The high protein and lipid contents make it useful for production of nutrients, and the high concentrations of GlcN found in the cell walls make it a potential source of chitosan.

Cultivation in Shake Flasks

Fungal growth

Cultivations of *Rhizopus sp.* both on GYV and SSL50% media were performed at 20, 32, and 40°C. In GYV medium, maximum growth was reached after 144 h of cultivation (Fig. 1a-c) with a biomass concentration of ca 5 g/L (0.17 g/g glucose) regardless of temperature. The major difference between various temperatures applied was the lag phase (not shown in detail). While 72 h cultivation was necessary at 20°C for enough biomass to be harvested, at 32 and 40°C, just 18 h was sufficient. In contrast, in SSL50%, the growth reached maxima of 7.1 g/L (0.33 g/g sugars) at 20°C, along with 3.3 and 3.1 g/L (0.16 and 0.15 g/g sugars) at 32 and 40°C, respectively, after very different cultivation times (Fig. 1d-f). Furthermore, when the start of growth in the two series of flasks was compared, it showed great differences at 20°C, indicating that initiation of growth is irregular at this temperature.

The high, albeit slow, biomass production at 20°C can probably be explained by the high oxygen access. Since the growth was very slow, relatively more oxygen was available, which led to more biomass. This is also supported by the lower ethanol production, viz. 1.7, 4.3 and 3.8 g/L at 20, 32 and 40°C respectively, as it is produced mainly under oxygen-limited conditions. Growth at 20°C was also the only SSL50% cultivation, which initially grew as perfectly spherical pellets, similar to the more favourable GYV cultivations. The lack of growth after extended cultivation at the higher temperatures could be explained by the tendency of fungi to remain in the same growth mode during the whole cultivation (unpublished data). The slow utilisation of pentoses and corresponding lower biomass yields is well known for fungi of this type (Millati et al. 2005; Vially et al. 2010).

Protein content

The potential of *Rhizopus* sp. biomass as a source of protein was evaluated by protein content measurement during cultivation. When grown on GYV medium, the biomass initially contained ca 0.50 g/g protein. However, at 32 and 40°C the protein content decreased during the cultivations (Fig. 1a-c). In contrast, during growth in the SSL50%, the protein content increased with maximal values at 32 and 40°C (Fig. 1d-f). This maintenance of high protein values when *Rhizopus* sp. was grown in SSL50% might be related to the longer presence of sugar throughout the cultivation, which would allow the fungal cells to remain metabolically active.

AIM concentration

The fungal AIM fraction was also analysed with respect to GlcN, GlcNAc, and cell wall phosphate. In general, the AIM content of fungal biomass increased during cultivation with the highest values in GYV at 32 and 40°C (Fig. 1). It has been previously reported that the AIM content can be influenced by different factors. Generally, cell aging and environmental stress are accompanied by increase of the cell wall AIM, particularly its GlcNAc portion (Zamani, et al., 2008). However, this change in AIM content was only obvious in *Rhizopus* sp. cultivation in GYV but not so prominent when the zygomycete was cultivated in SSL50% (Fig. 1).

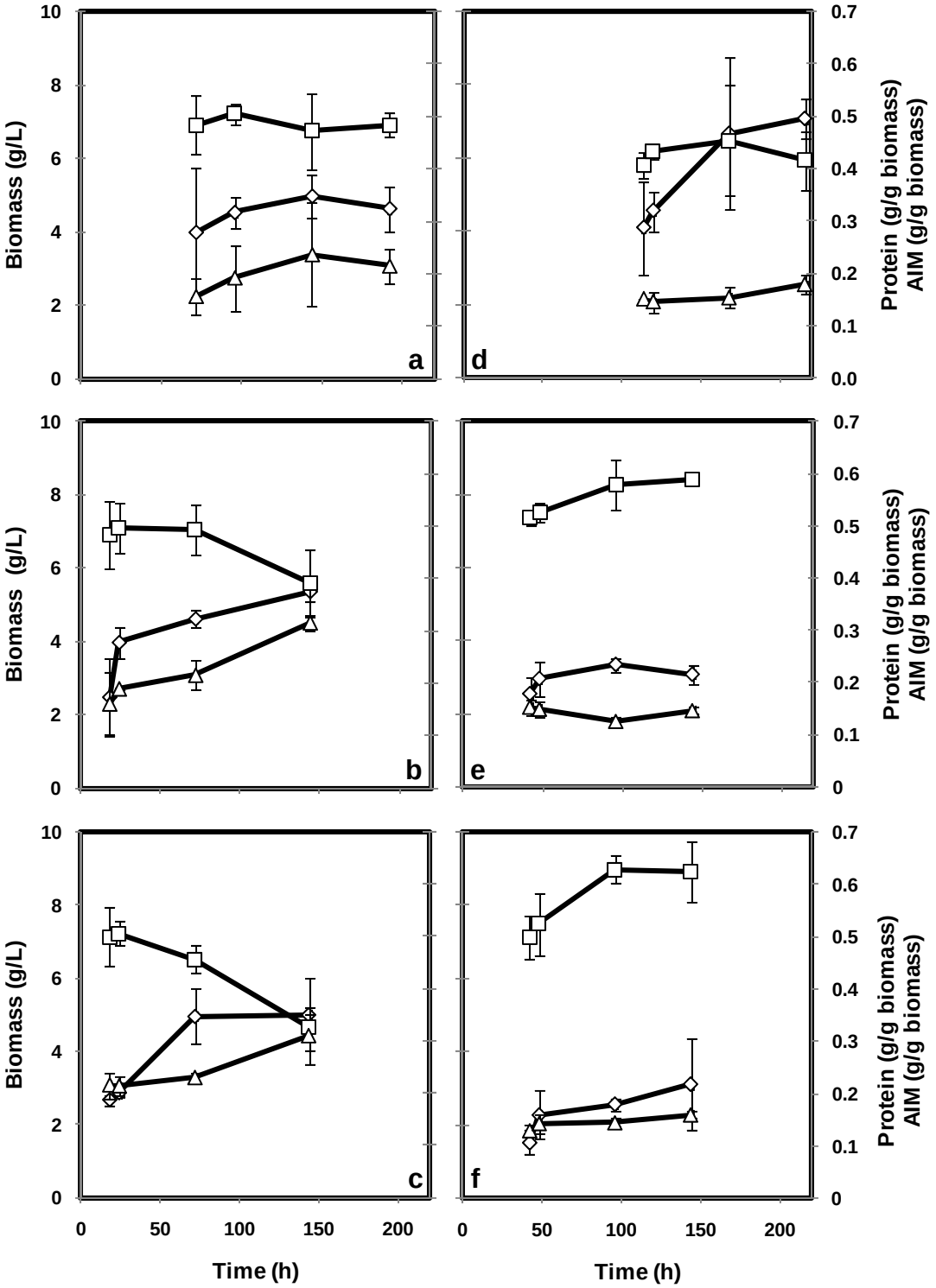

Figure 1. Biomass concentration (◊), protein (□) and AIM (Δ) profiles obtained from *Rhizopus* sp. cultivation in GYV (a – c) and SSL50% (d – f) at 20 (a, d), 32 (b, e) and 40°C (c, f)

Cell wall composition

Zygomycetes have gained increasing interest partly due to the valuable content of chitosan in the cell walls. Thus, in addition to their potential as a protein source, the production of GlcN and GlcNAc were also evaluated in this context. Phosphate, which has been reported to be associated with chitosan (Zamani et al. 2007), was also measured.

The GlcN content of AIM behaved quite differently at various conditions applied. During growth in GYV at 32 and 40°C it generally decreased, while levels were generally increasing in the other conditions (Fig. 2). Concurrently, the GlcNAc generally increased during cultivation, regardless of temperature or cultivation media (Fig. 2). The sum of GlcN and GlcNAc was generally more than 70% of the fungal AIM. Furthermore, the phosphate of AIM content decreased during cultivation, except at 20°C in GYV, in which the trend was on the opposite (Fig. 2).

In general, increase of AIM seems to be mainly due to production of GlcNAc, as shown for other zygomycetes strains (Zamani et al. 2008), indicating that it is not strain specific. However, the amount of GlcN per biomass was constant at 32 and 40°C in GYV, while it increased under all other conditions. Thus, growth in the harsher SSL50% media, and growth at a generally more unfavourable temperature, might cause the cells to produce more GlcN than under more favourable conditions. It was reported (Bartnicki-Garcia and Davis 1984) that the degree of (de-)acetylation of fungal cell walls is coordinated by tandem action of two enzymes, chitin synthase and chitin deacetylase. The first enzyme is responsible for elongation of chitin chains, while the second enzyme can cut this chain when it reaches a critical length. For aging cells, the degree of deacetylation is lower, since chitin deacetylase cannot get access to the acetylated growing chains due to higher crystallization. It results in higher levels of GlcNAc in the cell walls.

The phosphate content of fungi ranges from 0.001 to 0.02 g/g of the cell wall dry weight up to 0.23 g/g in *Mucorales* (Ruiz-Herrera 1992). However, comparing the data found in this study, the used microorganism and cultivation conditions might strongly influence the phosphate content in the cell walls. The remaining fraction of the fungal cell walls is considered to contain sugars, proteins, glucuronic acid, and ash (Zamani et al. 2007).

Altogether, cultivation at 32°C seems to be the most advantageous, taking into account biomass yield, protein content, and GlcN in fungal biomass. Thus, 32°C was the chosen temperature for the airlift cultivations.

Growth in Airlift Bioreactor

The culture of filamentous fungi using bioreactors has been a challenge for researchers partly due to their morphological diversity. Particularly, the dispersed mycelial form of fungi increases the viscosity of the medium, wraps around internal mechanical parts of fermentors, such as baffles and impellers, and causes blockages of sampling and overflow lines. As a consequence, reduction of oxygen and mass transfer rates may occur, which greatly influences the reactor performance and the production yield and rate (Yin et al. 1998). On the other hand, airlift bioreactors have been considered as a suitable choice for fungal cultivation as well as for industrial scale up.

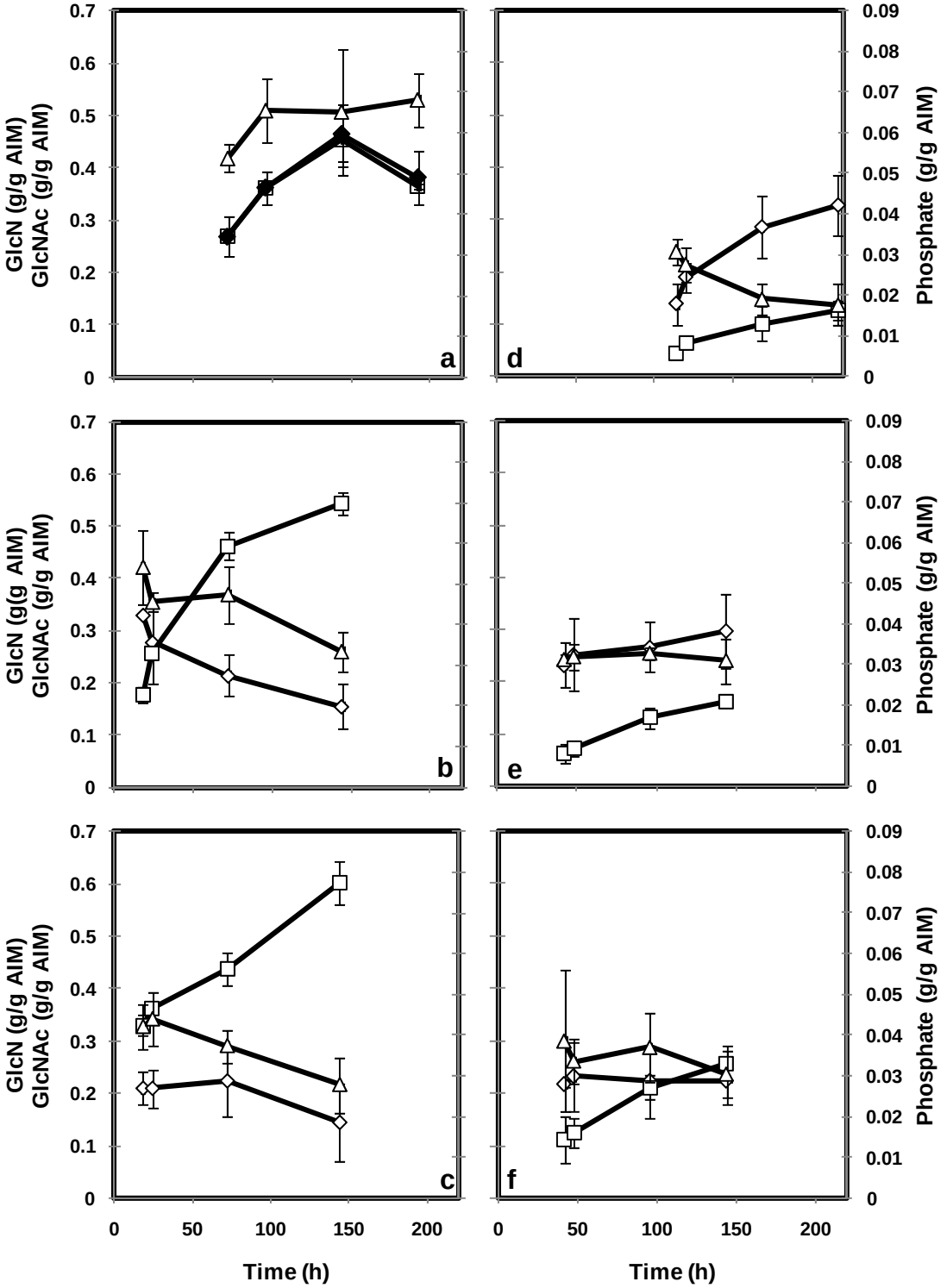

Figure 2. GlcN (◊), GlcNAc (□) and phosphate (Δ) content in acid-hydrolysed AIM derived from *Rhizopus* sp. cultivation in GYV (a–c) and SSL50% (d–f) at 20 (a, d), 32 (b, e) and 40°C (c, f)

The simple reactor design of airlift decreases both the risk of contamination and the overall process costs. In addition, the oxygen and mass transfer rates in airlifts are enhanced compared to traditional stirred-tank reactors (Merchuk and Siegel 1988). Accordingly, a 2 m airlift reactor was used for the scale up of *Rhizopus* sp. cultivation in SSL50%. The growth and biomass composition at different aeration rates (0.15, 0.5 and 1.0 vvm) were studied, and the results are summarized in Figs. 3-5 and Tables 1-2.

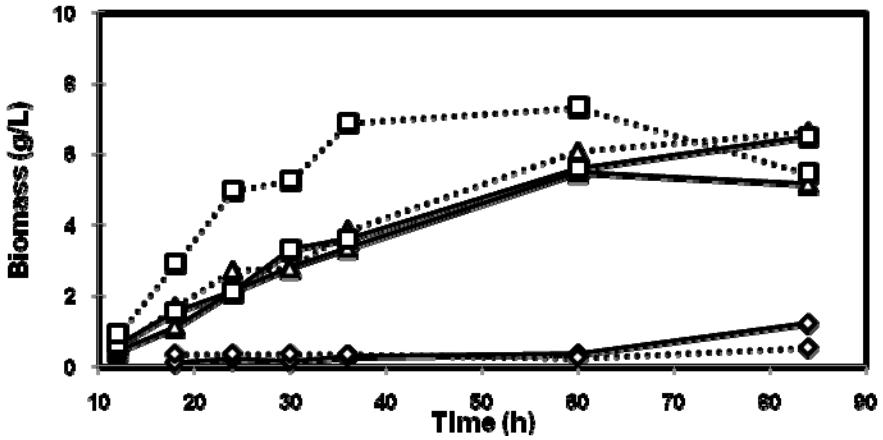

Figure 3. Biomass production of *Rhizopus* sp. in airlift at 0.15 (◊), 0.5 (Δ) and 1.0 vvm (□) on SSL50%. All experiments were done in duplicate, experiment 1 (—) and 2 (····), and their individual growth performance curves are presented

Fungal growth

Maximum growth of 1.23, 6.64, and 7.33 g/L (0.06, 0.31, and 0.34 g/g sugars) were reached at 0.15, 0.5, and 1.0 vvm, respectively (Fig. 3). Furthermore, the growth profiles at 0.5 and 1.0 vvm were similar, although the exact growth rates differed at 1.0 vvm (Fig. 3). Additionally, at 1.0 vvm the zygomycete grew as perfectly spherical pellets with increasing size throughout the cultivation, while it grew as dispersed filaments otherwise. The growth was also reflected in the sugar consumption; 0.15 vvm required 60 h for complete hexose utilisation, while 0.5 and 1.0 vvm required only 30 to 36 h and had consumed all of the xylose in 60 h. In addition to the biomass, some metabolites were also produced, including 4.0, 1.7, and 1.0 g/L ethanol and 1.0, 0.9, and 0.4 g/L lactic acid at 0.15, 0.5, and 1.0 vvm cultivations, respectively. Low quantities of glycerol (about 0.5 g/L) were also produced in all cultivations. However for the 0.5 and 1.0 vvm experiments, all of these metabolites and the initially present acetic acid had been consumed before the end of cultivation.

Accordingly, the oxygen availability for *Rhizopus* sp. played a striking role for the growth performance. The largest effect was seen by the increase from 0.15 to 0.5 vvm, while the change from 0.5 to 1.0 vvm had a smaller and irregular impact. Furthermore, the impact of oxygen limitation hypothesized for the bench-scale cultivations in SSL50% was clearly supported by the cultivations in the airlift bioreactor.

Protein content

The protein content of the fungal biomass in the airlift was measured during cultivation at 0.5 and 1.0 vvm (Fig. 4). The highest concentrations of proteins were

recorded in the beginning, and then decreased as the cultivation continued (Fig 4). The strongest effect was observed with the fastest growing culture at 1.0 vvm (experiment 2 in Fig. 3), which ended with the lowest protein content. Nevertheless, a common maximum protein yield of ca 2.40 g/L was reached when *Rhizopus* sp. was cultivated at both 0.5 and 1.0 vvm. The 0.15 vvm cultivations had protein contents of 0.41 and 0.49 after 84 h, which was the only time it could be detected and measured.

There was most likely a link between fungal growth and its protein content. Higher biomass yields resulted in lower protein fractions. Higher initial fungal cell activity and consequently higher protein synthesis might explain this observed inverse proportionality. Thus, *Rhizopus* sp. biomass was shown to contain a reasonably high protein fraction in the airlift as well.

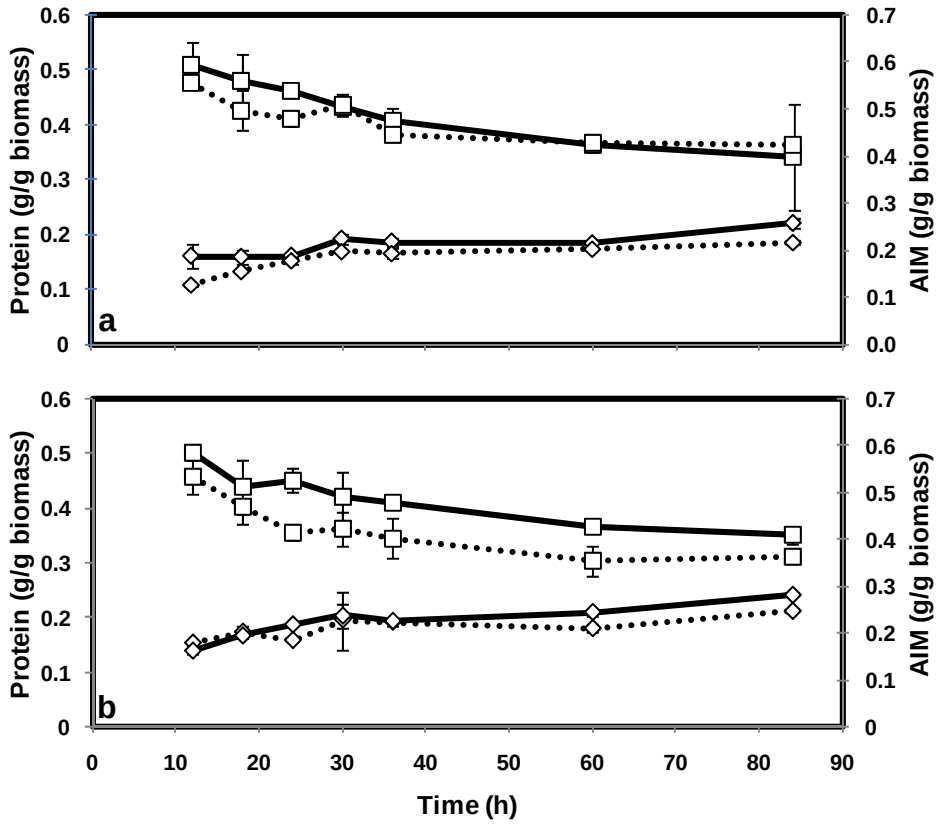

Figure 4. Protein (□) and AIM (◊) profiles during *Rhizopus* sp. cultivation in SSL50% using an airlift reactor at 0.5 (a) and 1.0 vvm (b). The individual performance of experiment 1 (—) and 2 (····) are presented.

AIM content

The AIM content of *Rhizopus* sp. biomass was found to be similar during cultivation at 0.5 and 1.0 vvm. Generally, the AIM content of the fungal biomass increased slowly during growth at 0.5 vvm and 1.0 vvm (Fig. 4). This AIM increase is in accordance with the shake-flask cultivations in sulphite liquor. AIM maxima of 1.44 g/L after 84 h and 1.80 g/L after 60 h were reached during cultivation at 0.5 and 1.0 vvm, respectively. The AIM fraction from *Rhizopus* sp. biomass cultivated at 0.15 vvm was shown to be around 20% at the end of cultivation.

Lipid content

The lipid fraction of *Rhizopus* biomass was measured after 84 h at the end of cultivation (Table 1). The largest lipid fractions were obtained after cultivation at 0.15 vvm, which was associated with the lowest biomass yields. With greater aeration and growth, the lipid fraction decreased to the lowest point (Table 1).

Table 1. Lipid Fraction (g/g) of *Rhizopus* sp. Biomass from Airlift Cultivation at 0.15, 0.5, and 1.0 vvm after 84 h growth on SSL50% *

Aeration (vvm)	Replicate	Lipid fraction (g/g)
0.15	1	0.090
0.15	2	0.087
0.5	1	0.074
0.5	2	0.042
1.0	1	0.046
1.0	2	0.025

* Measurement error (±2s) was estimated to 17% of the reported values.

The lipid content was also measured for the fastest growing culture (2; Fig. 3) at 1.0 vvm during cultivation. The analysis revealed lipid fractions of 0.065, 0.059, 0.062, 0.067, 0.042, 0.029, and 0.025 g/g after 12, 18, 24, 30, 36, 60, and 84 h, respectively. Thus, for the first 30 h, the fungal biomass contained about 0.06 to 0.07 g/g lipids, which decreased at the end to a level of about 0.02 g/g. The decline in lipid and protein (Fig. 4) contents might be related to reduction of sugar concentration in the cultivation medium. However, compounds other than sugars in SSL can also play a role in the protein and lipid profiles obtained. Taken together, the protein and lipid fractions were found to compose about 0.40 to 0.50 g/g of the fungal biomass until 36 h of growth and about 0.35 g/g after 84 h.

Cell wall composition

The content of the AIM components, GlcN, GlcNAc, and phosphate, were studied and results are presented in Fig. 5. When *Rhizopus* was grown at 0.5 vvm, the GlcNAc content of *Rhizopus* increased at the beginning of the cultivation, and then it remained somewhat constant until the end of cultivation (Fig. 5). On the other hand, during *Rhizopus* sp. growth at 1.0 vvm, a more pronounced increase in GlcNAc content occurred throughout cultivation. Thus, both a quantitative increase of AIM (Fig. 4) and a proportional increase of its GlcNAc component (Fig. 5) are taking place as the growth of biomass is proceeding. Nevertheless, an approximate GlcN/GlcNAc ratio of 2 was maintained throughout cultivation at 0.5 and 1.0 vvm. Greater increases of GlcNAc were observed in the flask cultures with synthetic GYV medium (Fig. 2). The phosphate content of the AIM during cultivation was generally decreased during cultivation at 0.5 and 1.0 vvm (Fig. 5), similar to the effects obtained in shake-flasks. The generally low phosphate content in the AIM might be an indication of phosphate limiting conditions.

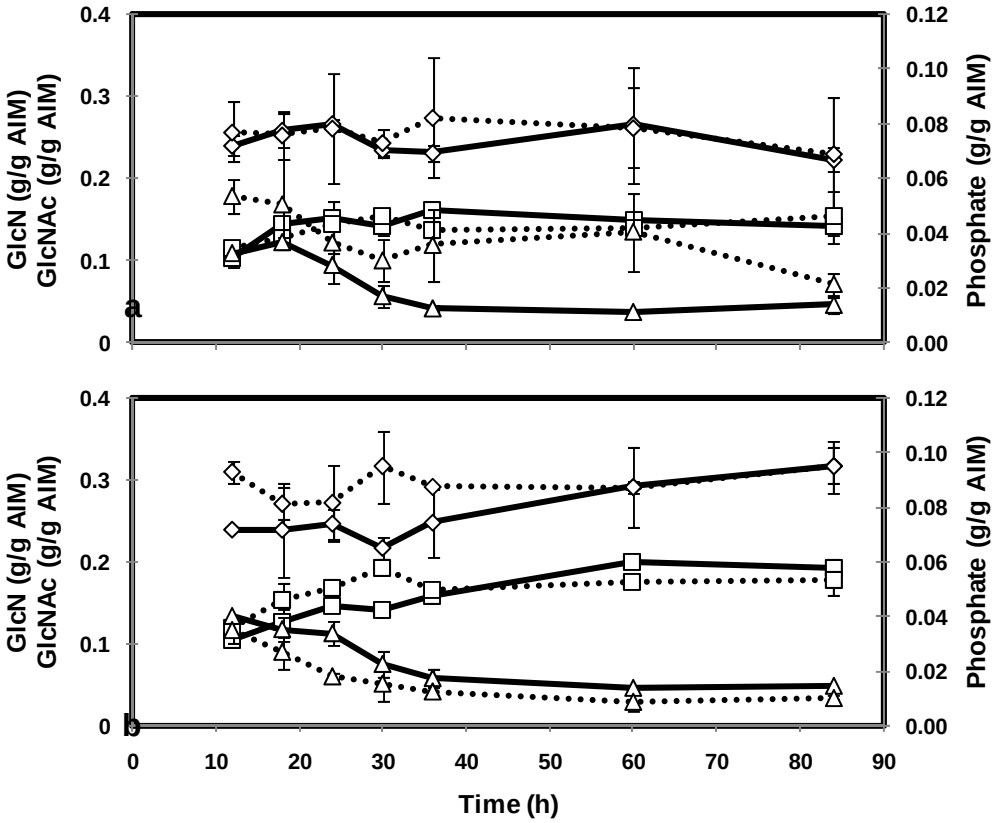

Figure 5. GlcN (◊), GlcNAc (□) and phosphate (Δ) content of AIM derived from *Rhizopus* sp. cultivation in SSL using an airlift at 0.5 (a) and 1.0 vvm (b). The individual performance of experiment 1 (—) and 2 (····) are presented.

Comparison of Protein Content with Crude Protein

The nutritional value of feed is often based on its content of nitrogen (Kjeldahl method), which is used as an indicator of protein. In general, a conversion factor of 6.25 of protein to nitrogen is used. A few fungal biomass samples were also analysed for Kjeldahl nitrogen.

As can be observed in Table 2, the results from biomass grown in flasks on GYV medium and in the airlift on SSL50% differed. In the GYV medium, the nitrogen-based protein contents were 0.15 g/g higher than the Biuret-based values, while they were approximately equal for biomass grown in the SSL50%. The high nitrogen contents in the GYV can be explained by adsorption of nitrogenous compounds to the cell wall from the rich media, such as the added yeast extract. The similarity of the different methods from the biomass grown on SSL50% also have some methodological implications, as the standard crude protein calculation is based on the total nitrogen content including e.g. chitosan and nucleic acids. The GlcN and GlcNAc of AIM alone can be calculated to contribute ca 0.05 g/g of the calculated crude protein. Thus the Biuret method may have overestimated the protein content. This might have been a consequence of different amino acid composition of *Rhizopus* biomass and bovine serum albumin used for calibration of Biuret. It should also be considered that the nitrogen content of *Rhizopus* protein may not be 16% and the multiplication factor not 6.25. However, both methods

followed the same trends and the results can thus be assumed to be used for comparisons. Nevertheless, care should be taken if comparisons are to be made based on results from different methods.

Table 2. Protein Content Calculated from Kjeldahl Nitrogen and Corresponding Biuret Values from Previous Sections

	GYV in shake-flasks at 32°C			
Time (h)	Crude (g/g)*		Biuret protein (g/g)§	
18	0.65	±0.025	0.48	±0.065
24	0.64	±0.021	0.50	±0.048
72	0.63	±0.025	0.49	±0.047
144	0.55	±0.057	0.39	±0.064
	SSL50% in airlift, 84 h of growth			
Aeration and replicate	Crude (g/g)*		Biuret protein (g/g)§	
0.15 vvm rep1	0.36		0.41	±0.009
0.15 vvm rep2	0.41		0.49	±0.026
0.5 vvm rep1	0.34		0.34	±0.097
0.5 vvm rep2	0.37		0.36	±0.008
1 vvm rep 1	0.35		0.35	±0.014
1 vvm rep 2	0.34		0.31	±0.009

*Crude protein was calculated from N×6.25
§ Method used for the values reported in this article

General Discussion

The scale-up of *Rhizopus* sp. biomass production using an airlift reactor reproduced the data obtained at bench-scale with improvement of biomass yields, pointing out the important role played by oxygen availability for fungal growth. Furthermore, the biomass yield and its composition of protein, AIM, GlcN, GlcNAc, and phosphate evaluated during this study were similar at 0.5 and 1.0 vvm. However, considering the lower production of lactic acid and ethanol during cultivation at 1.0 vvm compared to 0.5 vvm (0.5 and 1.13 g/L against 2.03 and 1.77 g/L, respectively), 1.0 vvm might better satisfy the oxygen demands. The increase of the fungal cell wall skeleton component AIM, and its components GlcN and GlcNAc during cultivation in airlift were in accordance with those obtained from shake-flasks. Besides, even during the scale up of the process, *Rhizopus* sp. was shown to be a valuable source of single cell protein, given the valuable protein and lipid contents obtained. The edible characteristics of *Rhizopus* sp. biomass may serve several purposes in the supply of nutrients. Moreover, the fat content found in *Rhizopus* biomass could further improve the lipid amount of reared fish. Additionally, *Rhizopus* sp. also proved to be a suitable source of GlcN residues and thus, a good alternative for chitosan production as another valuable product from the process of the zygomycetes biomass production.

CONCLUSIONS

1. Spent sulphite liquor diluted to 50% was successfully used for production of the edible zygomycetes fungus *Rhizopus* sp. biomass both in shake-flask and in a pilot airlift bioreactor.
2. Cultivation in airlift bioreactor at 1.0 vvm resulted in high biomass production (up to 0.34 g/g sugar), with high levels of protein (0.30-0.50 g/g) and lipids (0.02-0.07 g/g). The fungal biomass may thus make an excellent animal feed ingredient *e.g.* for fish.
3. The high contents of AIM (0.18-0.28 g/g biomass) and GlcN (0.22-0.32 g/g AIM) obtained during cultivation in airlift bioreactor at 1.0 vvm means that the produced biomass could also be used as a source of chitosan.
4. At bench-scale, 32°C was a more advantageous temperature in comparison to 20 and 40°C. A maximum biomass production and yield (0.16 g/g sugar) as well as fractions of 0.50-0.60 g protein/g biomass and up to 0.30 g GlcN/g AIM were obtained during cultivation at this temperature in spent sulphite liquor diluted to 50%.
5. Aeration played a crucial role for successful production of fungal biomass on diluted spent sulphite liquor.
6. Given the cultivation conditions used in the airlift bioreactor, 1.0 vvm seemed to be the best aeration rate tested for *Rhizopus* sp. biomass production accounting for biomass yield, protein and lipid contents, GlcN content of fungal AIM, and fulfilment of oxygen demand of the fungal cells.

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PAPER II

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Zygomycetes-based biorefinery: Present status and future prospects

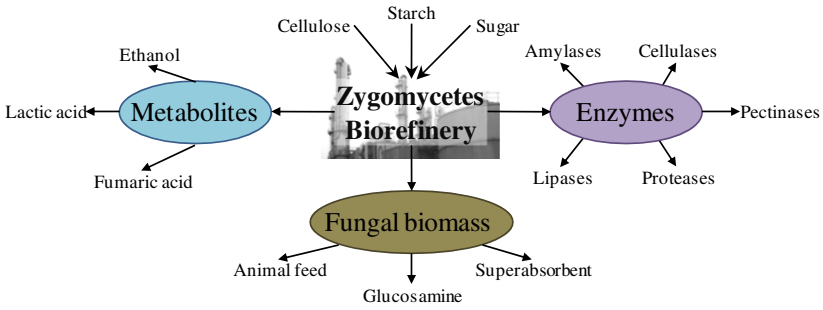
Jorge A. Ferreira [a], Patrik R. Lennartsson [a], Lars Edebo [b], Mohammad J. Taherzadeh [a,*]

[a] School of Engineering, University of Borås, SE 501 90 Borås, Sweden
[b] Department of Clinical Microbiology, University of Gothenburg, SE 413 46 Gothenburg, Sweden

HIGHLIGHTS

- Zygomycetes meet all requirements for being utilized as catalysts in biorefineries.
- Metabolites such as lactic acid, fumaric acid, and ethanol can be produced.
- Starch, cellulose, phytic acid, and proteins can be assimilated.
- Zygomycetes fungi are the source of a great diversity of enzymes.
- Potential source of single-cell protein with high nutritional value.

GRAPHICAL ABSTRACT



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ABSTRACT

Fungi of the phylum Zygomycetes fulfil all requirements for being utilized as core catalysts in biorefineries, and would be useful in creating new sustainable products. Apart from the extended use of Zygomycetes in preparing fermented foods, industrial metabolites such as lactic acid, fumaric acid, and ethanol are produced from a vast array of feedstocks with the aid of Zygomycetes. These fungi produce enzymes that facilitate their assimilation of various complex substrates, e.g., starch, cellulose, phytic acid, and proteins, which is relevant from an industrial point of view. The enzymes produced are capable of catalyzing various reactions involved in biodiesel production, preparation of corticosteroid drugs, etc. Biomass produced with the aid of Zygomycetes consists of proteins with superior amino acid composition, but also lipids and chitosan. The biomass is presently being tested for animal feed purposes, such as fish feed, as well as for lipid extraction and chitosan production. Complete or partial employment of Zygomycetes in biorefining procedures is consequently attractive, and is expected to be implemented within a near future.

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1. Introduction

Microbial fermentation has been used since ancient times. During the last century, however, petroleum-based refineries have prevailed over microbial routes for production of fine chemicals, due to the abundance and low cost of crude oil. Due to oil depletion forecasts, global warming, and the steadily increasing amount of waste products, the situation is now changed. Sustainable development is presently on the agenda, and great efforts have been made by seeking to replace oil-based refineries with biorefineries based on renewable feedstocks. Using filamentous fungi, with diverse and expanding products, constitute an important contribution to this development. Fermentation processes for the production of organic acids, antibiotics, enzymes, food components, and other miscellaneous products have already been applied (Gibbs et al., 2000).

Given their structural and physiological properties, Zygomycetes are receiving increased attention within the biotechnological field. They are already well known due to their extended use in China and Southeast Asia, for the production of fermented foods such as tempe and tofu (Lennartsson et al., 2012). The fungi have in recent times been investigated and used for production of a wide range of metabolic products. Examples include organic acids,

* Corresponding author. Address: University of Boras, School of Engineering, Allegatan 1, 501 90 Boras, Sweden. Tel.: +46 33 4355908; fax: +46 33 4354008.
E-mail address: Mohammad.Taherzadeh@hb.se (M.J. Taherzadeh).

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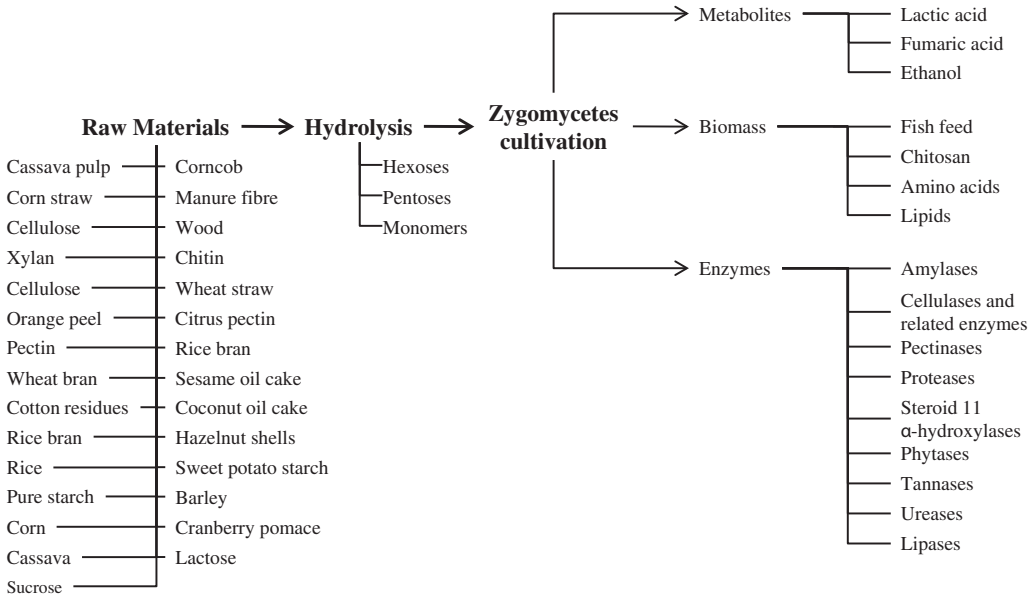

Fig. 1. Overall scheme of a Zygomycetes-based biorefinery, illustrating the alternative feedstock inputs as well as the product outputs, including metabolites, enzymes, and potential cell mass applications.

enzymes, and biofuels such as bioethanol and biodiesel. In addition, the Zygomycetes biomass contains beneficial quantities of proteins, lipids, amino acids, chitosan, and chitin. Thus, the biomass is envisaged for production of animal feed, human food, and chitosan. Zygomycetes are able to grow on a large variety of carbon sources at different temperatures, oxygenation rates, and pH-values. They are amylolytic, and able to consume pentose sugars. They are also able to perform simultaneous saccharification and fermentation (SSF) of starch materials. Contemplating the minimal requirements of growth, the easy recovery of fungal mycelium, and the variety of feasible co-products, Zygomycetes biomass holds the capacity of playing an important role in the future establishment of economically attractive Zygomycetes-based biorefineries, and may well be exploited in already established industrial processes to enhance profitability.

The aim of the present review is to gather the different biotechnological applications of Zygomycetes holding the highest potential for a biorefinery (Fig. 1), which mainly refers to flexibility in terms of the raw materials to be used, and their capability of producing metabolites, enzymes, and biomass.

2. Zygomycetes as core catalysts in biorefineries

The kingdom *Fungi* is considered to comprise four phyla; *Chytridiomycota*, *Zygomycota, Ascomycota*, and *Basidiomycota. Zygomycota* is divided into two classes, Trichomycetes and Zygomycetes, which are found worldwide as saprophytes growing on dead organic matter. Zygomycetes have long been used in food production. However, they have also been observed to be pathogens of plants, animals, and other fungi, and suitable strains must hence be selected (Lennartsson et al., 2012).

Zygomycetes are versatile filamentous fungi with well demonstrated growth ability in a vast array of culture media. The medium requirements are generally characterized as inexpensive and unspecific, and include, apart from some salts, a variety of nitrogen and carbon sources (Zhang et al., 2007), the range of the latter being wide and expanding, mostly due to the variety of enzymes carbon sources produce (Fig. 1). Glucose has been the first choice as carbon and energy source. However, some strains are able to consume more complex sugars, such as the disaccharides sucrose and lactose (Guo et al., 2010; Vamvakaki et al., 2010). Valorization of cheese whey, a lactose-containing dairy waste, as a renewable substrate for Zygomycetes has also been investigated (Vamvakaki et al., 2010). Furthermore, these fungi have, as amylase producers, the prerequisites for simultaneous saccharification and fermentation (SSF) of starchy materials. SSF has been widely examined due to its capacity to enhance process productivity, reduce the reactor volume required, and hence also capital costs. Starchy crops, such as barley, cassava, corn, oats, and rice, have been used as carbon sources for Zygomycetes in the production of lactic acid (Zhang et al., 2007). Koutinas et al. (2007) designed an oat-based biorefinery for production of lactic acid and various value-added co-products, including β-glucan and an anti-irritant solution. Due to their renewable character, abundance, and cheap price, lignocellulosic materials are also promising feedstocks for biotechnological processes (Zhang et al., 2007). A wide variety of lignocellulosic materials, providing cellulosic and hemicellulosic feedstocks, have been evaluated for production of value-added products, using Zygomycetes (Fig. 1). Corn straw and wood hydrolysates have for instance been examined for production of fumaric acid and ethanol, respectively, while wheat bran, cellulose, and xylan have been analyzed for production of cellulases and xylanases (Lennartsson et al., 2012; Xu et al., 2010). Valorization of spent sulphite liquor, a waste hemicellulosic product from paper pulp mills, for producing high-value Zygomycetes biomass for fish feed as well as chitosan, has also been carried out (Bankefors et al., 2011; Lennartsson et al., 2012; Mydland et al., 2007). Although lignocellulosic materials have been scrutinized for ethanol production for decades, developing a feasible commercial process has not yet been accomplished. In a facility using lignocellulosic materials, the total value might be raised with Zygomycetes producing biomass as a second product (Lennartsson et al., 2012). At present, agricultural crop-based facilities are being used for ethanol production. The process results in large volumes of low value by-products, thin stillage being a major one. Thin stillage may however be utilized by Zygomycetes for cell mass production. The process results in a product enriched with protein (including amino acid composition), lipids, and chitosan (van Leeuwen et al., 2012). Zygomycetes are also capable of utilizing lipids and fatty acids. Being lipase producers,

Zygomycetes have been examined extensively for biodiesel production, using plant oil as well as animal fat as substrates (Ghosh and Ray, 2011).

In addition to carbon sources, presence of nitrogen in the culture medium is crucial for optimal Zygomycetes growth and final product yields. Various inorganic and organic nitrogen sources have been employed. Typically, inorganic nitrogen such as ammonium sulfate and ammonium nitrate has been used, but the organic nitrogen of peptone, urea, yeast extract, corn steep liquor, or even fish protein hydrolysate, may also be used (Vattem and Shetty, 2002; Zhang et al., 2007). Furthermore, inorganic salts, including KH_2PO_4, $MgSO_4$, $ZnSO_4$, $CaCl_2$, and $Fe_2(SO_4)_3$, may also play a significant role for securing optimal fermentation (Lennartsson et al., 2012; Zhang et al., 2007). Non-expensive feedstocks containing the aforementioned nutrients might reduce the cost and enhance the sustainability of the process. Dairy manure has been used as carbon as well as nitrogen source for fumaric acid production (Liao et al., 2008). In exploiting the ability of Zygomycetes to hydrolyse complex macromolecules (Koutinas et al., 2007), oat flour as sole nutrient source for lactic acid production was tested.

Being filamentous fungi, Zygomycetes can adopt diverse morphological forms when cultivated in submerged culture. Various factors such as medium composition, inoculum size, pH, temperature, aeration rate, and agitation speed, affect the fungal morphology (Gibbs et al., 2000). They may grow as uniform filaments, evenly distributed throughout the cultivation medium, but also as entangled filaments, in clumps and pellets. The growth mode affects the rheological properties of the cultivation medium, and consequently, the overall process performance and final product yields (Gibbs et al., 2000).

The pellet-mode of growth has been dominating in the Zygomycetes research, since it results in lower broth viscosity. The possibility of performing high-density cultivation yielding significantly higher productivity has also been a significant factor. However, pellets need to be kept below a critical size to prevent oxygen limitation (Roa Engel et al., 2011), and strategies for controlling the Zygomycetes morphology has consequently been developed. After optimizing the medium, Liao et al. (2007) succeeded to cultivate *Rhizopus oryzae* in pellet-form. This form enhanced the production of lactic as well as fumaric acid in comparison with clump morphology. Zhang et al. (2008) controlled the morphology of *Rhizopus arrhizus* in a continuous stirred-tank reactor (CSTR) by using an acid-adapted preculture approach. Loose small pellets achieved the highest lactic acid production and yield from waste potato starch. Fu et al. (2009) developed a multi-stage preculture strategy in order to control the morphology of *R. oryzae* in a CSTR. Small fluffy pellets, produced in the third preculture at pH 3.0, resulted in the highest fumaric acid production. Zhou et al. (2011) controlled the pellet size by varying the initial glucose and peptone concentrations. They observed that cell mass and fumaric acid yield tended to increase with decreasing pellet diameter.

To summarize, the ability to utilize a variety of non-expensive feedstocks for the production of valuable and diverse products (Fig. 1) warrants a high plausibility of Zygomycetes being used as a core catalyst in a biorefinery. The capability of simultaneously generating more than one valuable product, e.g., a metabolite and biomass, might in particular have a significant impact on biorefining as a whole.

3. Metabolites of Zygomycetes

The metabolic versatility of Zygomycetes makes them well suited for biorefining, and is central to the great research interest. They are reported to be valuable producers of metabolites, mainly lactic and fumaric acid, but also ethanol, from many different substrates, and using various fermentation designs. So far, research has focused primarily on two genera, *Rhizopus* for production of organic acids, and *Mucor* for ethanol production. Focus has then been on production of lactic acid, rather than on production of the other metabolites.

In the metabolism of Zygomycetes, pyruvate, the final glycolysis product, can be directed into different pathways. The shortest pathway leads directly to the production of lactic acid via lactate dehydrogenase. Another pathway is via the cytosolic reductive TCA, after which fumaric acid is produced, and in which cytosolic fumarase plays an important role. Pyruvate may also be diverted via pyruvate decarboxylase for acetaldehyde production, and be further converted into ethanol by alcohol dehydrogenase (Bai et al., 2004). Consequently, activity and substrate affinity of these enzymes differ, depending on the individual strain, and will thus decide the amounts of metabolites produced (Oda et al., 2002). Stress has been shown to be a requirement for production of organic acids and ethanol. Nitrogen-limiting conditions trigger production of organic acids, whilst oxygen-limiting or anaerobic conditions are necessary for production of ethanol (Lennartsson et al., 2012). *Rhizopus* species are divided into lactic and fumaric acid producers, based on the yields of these organic acids (and other metabolites). Interestingly, the *Rhizopus* species mainly producing lactic acid possess two lactate dehydrogenase genes, ldhA and ldhB, while the fumaric acid producers only had the ldhB gene (Saito et al., 2004). The metabolic differences have been confirmed by a phylogenetic analysis of DNA sequences (Abe et al., 2007). Since the production of organic acids is associated with non-growth, a two-stage strategy comprising a cell-growth stage and a production stage needs to be applied. In the cell growth stage, the Zygomycetes are kept in a relatively rich medium. In the production stage, the produced biomass is cultivated in a nitrogen-limited medium to promote production of organic acids (Wu et al., 2011). Additional requirements for the organic acid production are oxygen-rich conditions and a neutralizing agent to maintain the pH-value in the medium (Zhang et al., 2007).

3.1. Lactic acid

Lactic acid ($CH_3CHOHCOOH$) is the most widely occurring organic acid in nature, and has two known isomers, L(+)-lactic acid and D(−)-lactic acid. Food applications require L-lactic acid, and this isomer is also the preferred monomer for polylactide manufacturing. Other applications for lactic acid are found within the pharmaceutical, leather, cosmetic, chemical, and textile industries. The global market for L-lactic acid has steadily increased, and has been projected to reach 500,000 (metric) tons per year by 2012 (John et al., 2007).

L-lactic acid production by *Rhizopus* strains has been extensively investigated, seeking to replace the chemical route of fermentation with lactic acid bacteria. *Rhizopus* species hold an important advantage over bacteria in their ability to produce L-lactic acid in a less expensive medium. Zhang et al. (2007) reviewed the intense research on the development of L-lactic acid production, using free and immobilized *Rhizopus* cells. The research on this subject is still continuing, and the findings are summarized in Table 1. Glucose is the most studied substrate for L-lactic acid production. However, other production routes, using starchy and lignocellulosic materials as more cost-effective feedstocks, have also been explored (Zhang et al., 2007). In addition to glucose, sucrose is being examined for L-lactic acid production; the yield of 0.81 g/g is however difficult to obtain on a regular basis (Guo et al., 2010). In a recent study concerning the use of starch-containing substrates, a yield of 0.72 g L-lactic acid per gram potato starch was attained, when using *Rhizopus* in pellet form (Yen and Lee, 2010).

Table 1
Recent insights on L-lactic acid production by *Rhizopus* cells.

Free cells						
Microorganism/ morphology[c]	Medium	Mode of operation	L-LA (g/L)	P[a] (g/L/h)	Y[b] (g/g)	References
R. oryzae CGMCC 2681	Sucrose (120 g/L)	EF	98	1.02	0.81	Guo et al. (2010)
	Glucose (160 g/L)		115	1.60	0.81	
	Xylose (60 g/L)		54	0.45	0.90	
	Glucose + xylose 1:1 (80 g/L)		66	0.91	0.81	
R. oryzae BCRC 33071 pellet	Sweet potato starch (60 g/L)	5 L CSTR batch	43	1.23	0.72	Yen and Lee (2010)
Cotton-like floc			25	0.82	0.42	
Filamentous			41	0.48	0.69	
Pellet			36	1.14	0.59	
R. oryzae adapted HM11	Xylose (80 g/L)	5 L CSTR batch	61	0.68	0.76	Bai et al. (2008)
	Corncob hydrolysate (97 g/L)		77	0.99	0.80	
R. oryzae NRRL 395	Glucose (~70 g/L)	EF	22	0.43	0.31	Thongchul et al. (2010)
	HCl treated cassava pulp hydrolysate (70 g/L sugars)		7	0.16	0.09	
	Enzyme treated cassava pulp hydrolysate (70 g/L sugars)		17	0.29	0.24	
R. oryzae UMIP 4.77 pellet	Glucose (<20 g/L residual)	4 L CSTR fed-batch	73	2.37	0.86	Vially et al. (2010)
	Xylose (120 g/L)	4 L CSTR batch	8	0.41	0.64	
Filamentous	Hemicelluloses (50 g/L; 71% xylose)		10	0.27	0.26	
Not reported	Cellulosic material (100 g/L)		24	0.50	0.28	
R. oryzae NRRL 395 pellet	Glucose (105 g/L)	EF	57	1.87	0.56	Yao et al. (2010)
Pellet and cotton-like	+30% crude protein		50	0.77	0.43	
Clump	+1.68 g/L crude protein		58	0.91	0.55	
Clump	+0.42 g/L N protein hydrolysates		56–59	–	0.53–0.56	
R. oryzae AS3.819 pellet	Glucose (120 g/l + 100 g/L feeding medium)	EF 20 cycle batch	64–95	–	0.79–0.9	Wu et al. (2011)
		7 L CSTR 25 cycle batch	71–104	2.20–3.90	–	
R. oryzae NBRC 5378	Wheat straw (50 g/L–12% cellulose; 41% hemicellulose)	1 L CSTR	6	0.06	0.23	Saito et al. (2012)

Nomenclature: EF, Erlenmeyer flasks; CSTR, continuous-stirred tank reactor.
[a] L-lactic acid productivity.
[b] L-lactic acid yield.
[c] when available.

Glucose, however, has been reported to yield up to 0.90 g/g L-lactic acid (Wu et al., 2011), which also surpasses the yield and productivity, using xylose as substrate. Some improvements have nevertheless been made towards xylose conversion into L-lactic acid. Guo et al. (2010) used a new soil-isolated *R. oryzae* strain, obtaining a lactic acid yield of 0.90 g/g at a C/N ratio of 30. However, the lactic acid productivity only reached 0.45 g/L/h. Further development at laboratory scale is required before valorization of xylose-rich lignocellulosic materials is to be realized for L-lactic acid production. More complex substrates have also been used for lactic acid production. Corncob acid hydrolysate was for instance tested, gaining a lactic acid yield of 0.80 g/g sugars (Bai et al., 2008), while Saito et al. (2012) reported a lactic acid yield of only 0.23 g/g sugars from simultaneous saccharification and fermentation of wheat straw. Dairy manure appears more promising, probably due to its carbon and nitrogen contents. The crude protein was used as nitrogen source, resulting in approximately 0.60 g/g lactic acid (Yao et al., 2010).

Maintaining high yields in the L-lactic acid production processes during long-term fermentations has been hindered by unbalanced fungal growth, relating to nitrogen sources. However, progress has been made. Wu et al. (2011) successfully performed 20 repeated cycles in shake flasks in a continuous stirred-tank reactor. High L-lactic acid yields in the range 0.79–0.90 g/g were procured, which was achieved by optimizing the feeding of medium, and by using uniform *Rhizopus* pellets.

The intense research towards L-lactic acid production, using *Rhizopus* species, resulted in a scale-up of the process to 5 m^3 airlift bioreactors, using glucose as carbon source. Prospects of designing airlift bioreactors larger than 3000 m^3 have previously been provided (Liu et al., 2006). The use of starchy materials for L-lactic acid production in a biorefinery also appears very promising since yields comparable to those from glucose have been obtained. Lignocellulosic materials, however, require further analyses prior to economical evaluations.

3.2. Fumaric acid

Fumaric acid ($HO_2CCH{=}CHCO_2H$) is a naturally occurring four-carbon dicarboxylic acid, and has been identified as one of the top ten chemicals to be produced via fermentation on an industrial scale. Due to its non-toxic and non-hygroscopic properties, it is frequently used in food and pharmaceutical industries. Fumaric acid was recently considered for treatment of psoriasis, and as feed additive for cattle (Roa Engel et al., 2008). Its structure with a double bond and two carboxylic groups, makes it furthermore suitable for production of biodegradable polymers, synthetic resins, and intermediates for chemical syntheses (Roa Engel et al., 2008).

Using *Rhizopus* strains for fumaric acid production has been appraised with the purpose of changing the chemical route from maleic anhydride produced from butane (Roa Engel et al., 2008). Glucose is the major carbon source used in fumaric acid fermentation, yielding up to 0.70 g/g fumaric acid, which is lower than the L-lactic acid yields (Huang et al., 2010). However, a two-stage dissolved oxygen control strategy has been applied in an attempt to attain high fumaric acid yield as well as high productivity from glucose. The dissolved oxygen concentration was kept controlled at 80% during the first 18 h of fermentation, and then switched to 30%. Although the fumaric acid yield of 0.54 g/g was low, it was nevertheless the highest yield acquired when using continuous

stirred-tank reactors (Fu et al., 2010). It has previously been claimed (Roa Engel et al., 2008) that the low pKa value of fumaric acid (3.03 at 25 °C) is a property that can be exploited for product recovery. Indeed, when Roa Engel et al. (2011) late in the batch phase switched off the pH control, cell-specific productivity, fumaric acid yield, and fumaric acid titre were not affected, and a pH of 3.6 was achieved. The pH control required less inorganic base, and acid recovery should be relatively easy at that pH value. Ding et al. (2011) demonstrated the relationship between carbon:nitrogen ratio, cytosolic fumarase, and fumaric acid titre. In urea of low concentration, the cytosolic fumarase activity increased 300% and the production of fumaric acid increased up to 40 g/L.

Studies on lignocellulosic materials for fumaric acid production are scarce (Table 2). Liao et al. (2008) reached a maximum fumaric acid yield of 0.31 g/g, using acid hydrolysate of manure fibers supplemented with glucose. Xu et al. (2010) applied a two-stage process of corn straw and reported a fumaric acid yield of 0.35 g/g. The highest yield reported, 0.44 g/g, was accomplished when *Eucalyptus globulus* wood hydrolysate was used (Rodríguez-López et al., 2011).

Research on fumaric acid production on a larger scale is missing in literature; Exploring high-performance *Rhizopus* strains, is still at want, and so is a broader perspective in terms of utilizing alternative substrates. Even so, given its potentials, the production of fumaric acid will most likely trigger intense experimentation in coming years, and its conceivable suitability for application in a biorefinery concept should therefore not be ignored.

3.3. Ethanol

Several Zygomycetes species are known to produce ethanol (CH_3CH_2OH) when the oxygen supply is limited. The *Mucor* genus, and in particular *M. indicus*, is the most explored group. Taherzadeh et al. (2003) were the first to have fuel ethanol in mind, and reported ethanol yields ranging between 0.20 and 0.37 g/g glucose, when using *R. oryzae*. However, aerobic conditions were used at the time and lactic acid was produced as well, limiting the ethanol yield. Millati et al. (2005) compared the Zygomycetes strains *R. oryzae, Mucor corticolous, M. hiemalis,* and *M. indicus*, reporting yields of 0.37–0.43 g ethanol/g glucose under aerobic conditions. The same strains produced ethanol from xylose, yielding 0.15–0.28 g/g. No ethanol production was reported when using *Rhizomucor pusillus* or *Rhizomucor miehei*. Optimization of the growth conditions for *M. indicus* resulted in ethanol yields of up to 0.46 g/g glucose in anaerobic cultivations (Sues et al., 2005). Most recently, edible Zygomycetes were isolated from tempe, a traditional fermented dish in Indonesia, which resulted in 32 isolates with ethanol yields of 0.26–0.41 g/g glucose (Wikandari et al., 2012). Two of the isolates in this study, tentatively identified as belonging to the *Rhizomucor* genus, were grown under microaerobic conditions, producing ethanol from glucose with yields of 0.46–0.47 g/g.

Zygomycetes have also been used for fermentation of lignocellulosic hydrolysates into ethanol. Taherzadeh et al. (2003) pioneered the current research by using *R. oryzae* for ethanol production from spent sulphite liquor. The yield, however, left room for improvement. Only 0.16 g ethanol/g sugar was produced. Significantly higher yields were achieved by *M. indicus* and *M. hiemalis* from dilute-acid hydrolysate, mainly from spruce; both species reached an ethanol yield of 0.44 g/g sugar (Millati et al., 2005). Furthermore, *M. indicus* produced ethanol from orange peel hydrolysate (based on 12% solids) under limited aerobic conditions and without detoxification, with a yield of 0.40 g/g hexoses (Lennartsson et al., 2012). No other organism has been reported to be able to grow, or to remain metabolically active, under those conditions. The dimorphic behavior of *M. indicus*, i.e., its ability to grow in either a yeast-like or a filamentous mode, depending on growth conditions, and whether the mode affects the ethanol production, has also been investigated. No measurable differences were however detected (Lennartsson et al., 2012).

Although Zygomycetes have been explored for fuel ethanol production for a decade with promising results, no scale-up is as yet reported. The reason is probably the focus towards 2nd generation biofuels, whose realization has met with difficulties. The process would probably stand a better chance if a starch or sugar based substrate were used. However, this would require a replacement of the core of an already functioning and well-known industrial process, with a novel biorefinery process. If the achievable economical benefits are sufficiently large to overcome the risks involved, remains to be seen.

4. Enzymes

Zygomycetes fungi are the source of a great diversity of enzymes (Table 3). Ghosh and Ray (2011) reviewed the enzymatic characteristics of *R. oryzae*, and their commercial potential. Reasonably, behind the high ability of Zygomycetes fungi to assimilate a large variety of substrates, lies the capacity to produce different hydrolytic enzymes, such as those needed for the degradation of plant carbohydrates, including amylases, cellulases, and xylanases, but also proteases and lipases, and the isolation, purification, and application of these fungal enzymes in different relevant industrial processes, have been extensively pursued.

Table 2
Recent insights on fumaric acid production by *Rhizopus* strains, using glucose and lignocellulosic materials as substrates.

Microorganism/morphology[c]	Medium	Bioreactor/ operation mode	FA (g/L)	P[a] (g/L/h)	Y[b] (g/g)	References
R. oryzae ATCC 20344/Pellet form	Glucose + Acid hydrolysate of manure fiber (100 g/L)	1 L CSTR/batch	31	0.32	0.31	Liao et al. (2008)
Mutant *R. oryzae* ZJU11 from ZD-35	Glucose (85 g/L)	EF 5 L CSTR/batch	57 41	0.48 0.37	0.68 0.48	Huang et al. (2010)
Mutant *R. oryzae* ME-F12 from ATCC 20344	Corn straw hydrolysate (Glucose (80 g/L)	EF	28	0.33	0.35	Xu et al. (2010)
Mutant *R. oryzae* ME-F12 from ATCC 20344	Glucose (100 g/L)	7 L CSTR/batch	56	0.70	0.54	Fu et al. (2010)
R. arrhizus DSM 5772/pellet form	*Eucalyptus globulus* wood hydrolysate (glucose (23.39 g/L))	2 L CSTR/batch	10	0.43	0.44	Rodríguez-López et al. (2011)

Nomenclature: EF, Erlenmeyer flasks; CSTR, continuous-stirred tank reactor.
[a] Fumaric acid productivity.
[b] Fumaric acid yield.
[c] when available.

4.1. Amylases

The production of amylases and glucoamylases enables the Zygomycetes to carry out simultaneous saccharification and fermentation (SSF) of starch-based materials, without a need for adding extra enzyme. Amylases are intensively applied within food industries to obtain glucose, and to manufacture glucose syrup for the pharmaceutical, beer, and beverage industries (Peixoto-Nogueira et al., 2008). Actually, in industrial utilization, the conversion of starch into glucose syrups by glucoamylase from *Rhizopus* and *Aspergillus* is well established (Chou et al., 2006). Clearly, amylases also play crucial roles in the starch-based ethanol production field. *Rhizopus* strains produce amylases when cultivated in agricultural commodities, including barley, corn, oats, rice, and cassava, or in starch (Ghosh and Ray, 2011). Peixoto-Nogueira et al. (2008) observed amylase production by *Rhizopus microsporus* var. *rhizopodiformis* in solid-state fermentation, using a mixture of wheat bran, corncob, starch, and saline solution. *R. oryzae* isoamylase was capable of using starch-containing waste food-stuffs. These included rice extract and bred dust, as well as soluble potato starch and various domestic starchy effluents, including arrowroot, tamarind kernel, tapioca, and oat (Ghosh and Ray, 2011).

In conclusion, with reference to Zygomycetes being very potent amylase producers, already involved in industrial processes, it is practically certain that they will continue to be commercially exploited. The potentiality of amylase production playing an important role in biorefining may thus be deemed high.

4.2. Cellulases and related enzymes

Zygomycetes fungi have also been screened for production of enzymes exhibiting cellulolytic activities. Saha (2004) demonstrated the presence of a complete cellulase system in *Mucor circinelloides*, comprising endoglucanase, exoglucanase, and β-glucosidase. Endoglucanases produced by Zygomycetes in liquid medium containing chitin or wheat bran, have been investigated and compared to commercial endoglucanases. Potential applications for endoglucanases appear in the brewery industry, where these enzymes lead to lower viscosity (Celestino et al., 2006), and in the textile industry, where they induce higher defibrillation activities on lyocell fabric as well as lower resistance to anionic surfactants and oxidizing agents (Shimonaka et al., 2006). In the animal feed industry, the enzymes showed higher thermostability and activity at pH 2.6–6.5 than current commercialized β-glucanases (Boyce and Walsh, 2007). *R. oryzae* has also been shown to produce glucanases when grown on a variety of agro wastes, such as dried flower, dried grass, water hyacinth, sugarcane bagasse, or fruit peel (Ghosh and Ray, 2011). β-glucosidase activity of species belonging to various Zygomycetes genera, e.g., *Gilbertella*, *Mucor*, *Rhizomucor*, and *Rhizopus*, has been described (Krisch et al., 2010). Takii et al. (2005) tested 12 *Rhizopus* strains for β-glucosidase activity in solid-state fermentation, using wheat bran. In one of these strains, *R. oryzae*, the fermentation resulted in a significantly higher enzymatic activity (0.308 U/mg of protein) in comparison with the other 11 strains (<0.05 U/mg). The β-glucosidase activity of a food grade fungus, *Rhizopus oligosporus*, grown on cranberry pomace in solid-state fermentation, was enhanced by NH_4NO_3 and fish protein hydrolysate, increasing the amounts of extractable phenolics as well as the antioxidant activity. Ellagic acid, a cranberry compound with anti-carcinogenic properties, was enriched to a level of 375 mg/g dry weight of pomace extract (Vattem and Shetty, 2002). Shuvaeva and Sysoeva (2010) optimized the production of xylanase from *Rhizopus microsporus* by employing solid-state fermentation, based on wheat bran, straw, and grain sprouts, and also submerged fermentation, based on xylose, maize extract, and salts. Endo-xylanase production by a *R. oryzae* strain during cultivation in xylan-containing agricultural by-products, including wheat straw, cotton residues, hazelnut shells, corn cobs, and oak sawdust, has furthermore been reported (Ghosh and Ray, 2011).

The production of a variety of cellulases and related enzymes by different Zygomycetes has been well demonstrated. However, acknowledging the competition from fungi specialized in cellulose degradation, Zygomycetes-based enzymes will probably reach only limited markets. The most feasible enzyme solutions are those with various types of activities and niche markets. Whether there is sufficient incentive for commercial production of Zygomycetes-based enzymes, remains to be seen.

4.3. Pectinases

Zygomycetes are promising sources of pectinases. Pectin is a polymer of mainly α-(1,4)-linked D-galacturonic acid, some rhamnose, and with side chains rich in arabinose and galactose (Zhang et al., 2005). Maleki et al. (2011) screened 22 Zygomycetes isolates from soil for pectinase production, all with positive results. *Rhizopus* pectinases have been produced in solid culture, using citrus peel of orange, and the crude extract was used for clarifying orange juice (Kareem and Adebowale, 2007). *Rhizopus* pectinases have been studied for flax retting, the initial microbiological step in the process of making linen. The retting abilities of different enzyme mixtures are strongly correlated to polygalacturonase activity, and a pure polygalacturonase is able to fulfil efficient retting. Hence, endopolygalacturonases have been isolated from *Rhizopus* strains and characterized (Zhang et al., 2005). Using oxalic acid or EDTA as chelators, enhanced retting efficiency. Furthermore, the retting efficiency of *Rhizopus* endopolygalacturonase was comparable to that of the commercial Flaxzyme, a retting mixture comprising xylanase, cellulase, polygalacturonase, and with pectin lyase activities (Akin et al., 2002). The authors scaled up the endopolygalacturonase production to a 100 L bioreactor. Polygalacturonase is also believed to play an essential role in the maceration of mulberry roots by *Rhizopus oryzae* (Ghosh and Ray, 2011). Damásio et al. (2011) analyzed the production of polygalacturonase and pectin lyase by *R. microsporus* var. *rhizopodiformis*, grown in more than 20 different carbon substrates, and the highest activity of the two enzymes was detected in medium of lemon peel.

To summarize, Zygomycetes fungi appear to be very well suited for production of pectinases, as is confirmed by the presence of these enzymes on the market today, which makes pectinases one of the top products to be derived from a Zygomycetes-based biorefinery.

4.4. Proteases

Zygomycetes are since long acknowledged as potential sources of proteases. The high protein content and digestibility of fermented foods such as tempe, is related to the proteolytic activity of *Rhizopus* sp. on soybean seed proteins (Heskamp and Barz, 1998). Proteases are categorized as alkaline, neutral, or acidic, based on their catalytic mechanism and consequent pH optimum for activity. Alkaline proteases have extensive applications in washing-materials, whereas acidic proteases are used in food processing. Fungal acidic proteases are considered feasible substitutes for three important proteases involved in food processing, namely, pepsin, rennin, and papain (Ikasari and Mitchell, 1996). Several aspartic proteases (including pepsins and rennins) have been isolated, purified, and characterized from Zygomycetes. *Rhizomucor* species are good producers of rennins with milk-clotting properties, and have been used in cheese production for a long time. Several *Mucor* strains are also confirmed to be a promising source of milk-clotting enzymes, with unique technological properties. Their

Table 3
Overview of the diversity of enzymes produced by Zygomycetes fungi in submerged (SbmF) as well as solid-state fermentation (SSF), using a wide range of substrates.

Microorganism	Medium	Culture mode/ bioreactor	Enzyme	References
R. microsporus var. *rhizopodiformis*	Wheat bran + corn cobs + starch + saline solution	SSF	Amylase	Peixoto-Nogueira et al. (2008)
Mucor circinelloides NRRL 26519	Lactose, cellulose, Sigmacell 50	SbmF/EF	Cellulase system	Saha (2004)
R. microsporus var. *microsporus*	Chitin, xylan, cellulose	SbmF/EF	Endoglucanase	Celestino et al. (2006)
R. oryzae MIBA348	Wheat bran	SSF/EF	β-glucosidase	Takii et al. (2005)
R. oligosporus	Cranberry pomace	SSF/EF	β-glucosidase	Vattem and Shetty (2002)
Rhizopus var. *microsporus* 595	Wheat bran xylose	SSF/thermostat SbmF/ EF	Xylanase	Shuvaeva and Sysoeva (2010)
R. oryzae NRRL 29086	Citrus pectin	SbmF/14 L CSTR	Polygalacturonase	Zhang et al. (2005)
R. oryzae NRRL 29086	Pectin	SbmF/100 L fermenter	Endopolygalacturonase	Akin et al. (2002)
R. oligosporus ACM 145F	Rice bran	SSF/EF	Protease	Ikasari and Mitchell (1996)
R. oryzae CH4	Wheat gluten, starch	SbmF/CSTR	Protease	M'hir et al. (2012)
R. nigricans ATTC 6227b	Progesterone	SbmF/EF	11α-Hydroxylase	Žnidaršič et al. (1998)
R. nigricans Tj 108	16,17α-Epoxyprogesterone	SbmF/EF	11α-Hydroxylase	Zhou et al. (2009)
Mucor racemosus NRRL 1994	Wheat bran + sesame oil cake	SSF/EF	Phytase	Roopesh et al. (2006)
R. oryzae NRRL 1891	Coconut oil cake + sesame oil cake	SSF/EF	Phytase	Ramachandran et al. (2005)
R. microsporus var. oligosporus	Rice flour	SbmF/EF	Phytase	Azeke et al. (2011)

Nomenclature: EF, Erlenmeyer flasks; CSTR, continuous-stirred tank reactor.

production in submerged culture has been analyzed, using casein, gelatine, glucose, and fructose, or more complex substrates such as soybean meal, wheat bran, and cheese whey, as carbon substrates (Yegin et al., 2011). In solid-state fermentation studies, the typical source was wheat bran. The production of aspartic proteinases from *Mucor* sp. has already been scaled up in a continuous stirred-tank reactor (Yegin et al., 2011) of 1.5 m^3 capacity. Also *R. oligosporus* has been reported to hold promise for protease production; it produces a satisfactorily calf rennet substitute at laboratory scale, and does not produce toxins (Ikasari and Mitchell, 1996). M'hir et al. (2012) produced *R. oryzae* protease in a 20 L continuous stirred-tank reactor after bench-scale optimization, using a wheat gluten and starch-containing medium.

Protease production is widespread among Zygomycetes fungi. In view of this and the valuable protease applications within the food industry, the processes for producing these enzymes by using Zygomycetes will most certainly continue their straightforward development towards biorefining.

4.5. Steroid 11 α-hydroxylases

The 11α-hydroxylation of progesterone by *Rhizopus nigricans*, an important reaction in the production of therapeutic steroid drugs and hormones, has been utilized in the industry for over half a century (Zhou et al., 2009). Nonetheless, probing cost-effective routes, and economically viable industrial processes for this biohydroxylation, is continuing. The operational activity and stability of the 11α-hydroxylation system has been tested with immobilized *Rhizopus* cells. However, repeated batch processes failed to maintain the hydrolysing activity for long periods (Žnidaršič et al., 1998), prompting Žnidaršič et al. (1998) to perform a detailed analysis of the morphology and physiology of *R. nigricans* pellets, in consecutive cycles. Conversion was optimally achieved in the second cycle, by pellets from the cultivation with the highest agitation and aeration. Zhou et al. (2009) optimized the bioconversion of 16,17α-epoxyprogesterone by means of a uniform design, where the medium contained (in g/L): glucose (25), corn steep liquor (22), $(NH_4)_2SO_4$ (0.1,) and yeast extract (0.86).

Although the use of steroid 11α-hydroxylases at industrial scale is well established today, there is indeed room for improving the versatility of the process. Studies on different types of substrates are limited, and so is the knowledge relating to morphological control during continuous cultures. Regardless, considering the value of the process, its potential within biorefining is granted.

4.6. Phytases

Phytases are acidic phosphohydrolases that catalyze the release of phosphate from phytate (myco-inositol hexaphosphate), the main form of phosphorous occurring predominantly in cereal grains, legumes, and oilseeds. Interest in phytase production has arisen from its viable application as animal feed supplement; phytase increases the phosphorous availability in phytate-rich feed. This not only aids proper skeleton growth of fed animals, but reduces phosphate pollution as well. Although phytases are produced by a large number of plants, animals, and microorganisms, fungal phytases are preferred in the industry (Pandey et al., 2001).

Phytase production by Zygomycetes has mostly been examined in solid-state fermentation. Sesame and groundnut oil cakes, and a mixture of wheat bran and sesame oil cake, were good substrates for phytase production by *Mucor racemosus* (Roopesh et al., 2006). A mixture of coconut and sesame oil cakes, supplemented with glucose and ammonium nitrate, was also a good solid medium for phytase production by *Rhizopus* sp. (Ramachandran et al., 2005). Two intracellular phytases have recently been isolated from *R. oligosporus* grown in rice flour suspension, and were shown to have broad affinity for various phosphorylated compounds (Azeke et al., 2011).

Research on phytase production has been somewhat limited to solid-state fermentation, where scalability is a problem. Studies addressing phytase production in submerged culture are probably needed to fully evaluate the potential of commercializing the process. However, contemplating its economic potential, if the scalability problems are solved, phytase production could play a central role in a future Zygomycetes-based biorefinery.

4.7. Other enzymes and enzymatic co-production

Tannase, urease, and lipases are other enzymes produced by Zygomycetes (Ghosh and Ray, 2011). Lipases, in particular, are among the most used biocatalysts in organic reactions. Beyond being able to catalyze the hydrolysis of fats and oils, releasing free fatty acids, diacylglycerols, monoacylglycerols, and glycerol, lipases can also catalyze synthetic reactions in non-aqueous media. A relevant practical application of *Rhizopus* lipases has been the transesterification of plant oils and animal fats, with methanol. The reaction produces fatty acid methyl esters (biodiesel), which has gathered increased interest due to its promise as a green replacement of petroleum based fuels (Ghosh and Ray, 2011). An-

other aspect that might be worthy of consideration is the expression of multiple enzymes, occurring in correlation with adequate induction by specific substrates. Oda et al. (2002) claimed for instance that *R. oryzae* grown in potato pulp produced cellulase, pectinase, and amylase, which made it suitable for L-lactic acid production. Moreover, the capability of *R. oryzae* to use pearled oat flour has been utilized for production of glucoamylase, protease, and phytase (Koutinas et al., 2007).

Undoubtedly, Zygomycetes fungi are sources of a wide range of enzymes, and the potentiality of these fungi in various industrial areas is far from being fully realized. A full-scale process for biodiesel production based on lipase-producing Zygomycetes might well be conceivable in a near future. The process would be particularly interesting if integrated with a biorefinery concept.

5. Zygomycetes biomass

5.1. Composition

Zygomycetes have attracted great interest within science concerning their biomass composition. The recognized value of these fungi as sources of single-cell protein with high nutritional value can be tracked back for centuries, on account of their use in fermented food production. Indeed, *Rhizopus* strains, including those isolated from fermented foods, and *Rhizopus oligosporus*, a food-grade fungus, were revealed to be composed of up to 50% protein when cultivated in spent sulphite liquor, thin stillage, or starch processing wastewater (Bankefors et al., 2011; Edebo, 2008; Lennartsson et al., 2012; Mydland et al., 2007). The biomass protein contents are depending on harvesting, dewatering, and drying methods. Nitrogen concentration in the medium is also believed to affect fungal protein contents (van Leeuwen et al., 2012).

Zygomycetes have long been recognized as valuable sources of lipids, including the unsaturated omega-6 (linoleic and γ-linolenic acids), omega-7 (palmitoleic acid) and omega-9 (oleic acid), and the saturated C16 (palmitic) and C18 (stearic) fatty acids (Sajbidor et al., 1988). Long-chain omega-3 polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have also been found in Zygomycetes biomass. Maximally, 0.46 g/L of EPA and 0.34 g/L of DHA were extracted from a cultivation of *Mucor* sp. in sugarcane molasses and urea, using a C/N ratio of 35 (Li et al., 2008). The lipid content was approximately 13% in *Rhizopus* sp. grown in spent sulphite liquor, while the oleaginous fungus *M. circinelloides*, grown in thin stillage with addition of extra glycerol, contained 61% (Bankefors et al., 2011; Mitra et al., 2012). Due to its nutritional and pharmaceutical importance, γ-linolenic acid (GLA – C18:3) has sparked a great interest in the science world (Vamvakaki et al., 2010). Many *Mucor* strains have been observed to produce GLA in their biomass. By using normal conditions supporting lipid production, Mamatha et al. (2010) measured GLA contents of approximately 14% in the *M. indicus (rouxii)* biomass. Moreover, when cultivated in nitrogen-limited cheese whey, a maximum of 177 mg/L GLA was produced by a *Mucor* strain (Vamvakaki et al., 2010), while a maximum of 1.35 g/L of GLA was previously reported by Li et al. (2008). Interestingly, Oda et al. (2003) found that lactic acid-producing *R. oryzae* strains contained more γ-linolenic acid than strains producing fumaric and malic acid. Addition of $CaCO_3$ raised the GLA content to 30% of the total lipid amount. Single-cell oil production by *Mucor circinelloides*, rich in γ-linolenic acid, has already been scaled up to commercial size. In 1985, a process based on 220 m^3 continuous stirred-tank reactors was launched, although they are not in use today (Ratledge, 2006). This established a realistic practicability of commercial production of fungal oil, with a potentiality of replacing plant oils as substrate for biodiesel production, or as food.

Lysine, methionine, tryptophan, and threonine are of high relevance for non-ruminants, lysine and methionine usually being the foremost limiting factors in corn-soybean meal diets for swine and poultry, respectively (van Leeuwen et al., 2012). Zygomycetes have been reported to produce amino acids, and for instance biomass of *R. oligosporus* grown in thin stillage contained lysine (1.8%), tryptophan (0.3%), threonine (1.5%), methionine (1.8%), calculated as percentage of protein. The amounts of the first three amino acids were comparable to those in soybean meal, but fungal methionine content was significantly higher (van Leeuwen et al., 2012). Other amino acids, including arginine, aspartic acid, cysteine, phenylalanine, glutamic acid, histidine, isoleucine, leucine, lysine, proline, serine, tyrosine, and valine, were also present in relevant amounts in *Rhizopus* biomass (Edebo, 2008; van Leeuwen et al., 2012).

Another remarkable characteristic of the composition of Zygomycetes is the high concentration of chitosan in their cell walls. In *R. oryzae*, it amounts to 42% of the total cell mass (Hjorth et al., 2005; Lennartsson et al., 2012), and deriving chitosan from these fungi might thus offer an alternative to the chitosan production involving deacetylation of chitin from marine crustacean shells, a by-product of food industries. Deacetylation of crustacean shells is normally conducted through harsh alkaline hydrolysis at high concentration and temperature, which entails long processing time, environmental pollution, and inconsistent physicochemical properties of the produced chitosan (Aranaz et al., 2009). Production of chitosan from Zygomycetes under milder controlled conditions could yield a readily available and much more consistent product.

*Muc*or dimorphism decides the cell wall chitosan content. *M. indicus* is known to contain more chitosan when grown in filamentous form than in yeast-like form (Lennartsson et al., 2012). The amount of glucosamine has been extensively used to evaluate the feasibility of chitosan production from Zygomycetes fungi. In a *Rhizopus* strain isolated from Indonesian tempe, cultivated on spent sulphite liquor in an air-lift bioreactor, up to 42% of its cell envelope consisted of glucosamine monomers (Hjorth et al., 2005; Lennartsson et al., 2012). Glucosamine levels have furthermore been estimated during co-production of secondary metabolites. Liu et al. (2008) reported that *R. oryzae* biomass cultured in glucose for L-lactic acid production comprised 25% glucosamine. Ammonia was added to maintain the glucosamine levels in the *R. oryzae* cell wall, without influencing L-lactic acid production. Liao et al. (2008) used manure as carbon and nitrogen source for co-production of fumaric acid and glucosamine, and observed the glucosamine fraction to be 21% of the *R. oryzae* biomass.

In a world facing forecasts of food-grade protein depletion, Zygomycetes will undoubtedly provide a high value alternative to plant proteins and vegetable oils. Its high-value composition of protein, nutritionally relevant lipids, amino acids, and chitosan will certainly promote committed research towards Zygomycetes biomass production, which appears particularly promising within a biorefinery concept. The commercial production of single-cell oil from Zygomycetes in biorefineries has long been a reality.

5.2. Applications of Zygomycetes cell mass

In addition to their valuable biomass composition, Zygomycetes possess advantages concerning application within feed production. The strains already employed in preparing fermented foods for human consumption are deemed to be generally regarded as safe (GRAS), which significantly reduces the amount of testing required prior to a full-scale process (Lennartsson et al., 2012). Further, Zygomycetes generally contain relatively small amounts of nucleic acids that, due to the uric acid produced when catabolized, are a limiting factor for human or animal consumption (Solomons and Litchfield, 1983). The pleasant taste and smell of the produced biomass, and its easy separation from the medium, are additional

advantages of using these fungi in the feed industry (Lennartsson et al., 2012).

Biomass of a *Rhizopus* isolate from tempe, cultivated in spent sulphite liquor, is currently tested as an ingredient replacing fishmeal in fish feed (Bankefors et al., 2011; Edebo, 2008; Mydland et al., 2007). The aquaculture sector is at present responsible for the consumption of ca. 70% of the world fishmeal and 90% of the fish oil production, hence respectively and contributing to being instrumental in over-fishing and subsequent lack of fish for food, particularly in developing countries (Edebo, 2008; Lennartsson et al., 2012). Zygomycetes biomass might thus represent an alternative to the vast quantities of fishmeal consumed within fish aquaculture. The results so far are promising. The protein concentration is high, the amino acid composition comparable to that of fishmeal, and the proportion of C18 unsaturated fatty acids, is approximately 60%. Most importantly, carnivorous fish species, such as rainbow trout (Mydland et al., 2007) and Atlantic salmon (Bankefors et al., 2011), fed a diet containing *Rhizopus* biomass, grew well and had a high palatability, showing only minor differences from fish fed a standard diet. Production of Zygomycetes biomass from spent sulphite liquor has already been tested in an airlift bioreactor, a few m^3 in size, and production at larger scales are expected in a near future.

The potentiality of chitosan production reinforces the already high value of Zygomycetes biomass. The cationic behavior of chitosan provides this polymer with numerous and unique features, e.g., being nontoxic, biocompatible, biodegradable, haemostatic, analgesic, tumour inhibiting, mucoadhesive, permeation enhancing, anticholesterolemic, antimicrobial, and antioxidative, granting great possibilities within many different industries (Aranaz et al., 2009). Chitosan has held great importance within various fields, e.g., the biomedical field, including wound healing and drug delivery systems, the food industry as a dietary ingredient and preservative, waste water treatment, removing water pollutants, and as immobilization support for cells and enzymes in biocatalysis processes (Aranaz et al., 2009).

The first steps towards a commercial scale of Zygomycetes biomass production have been taken, using spent sulphite liquor as substrate. The acceptance of the biomass as fish feed has already been confirmed. It is therefore highly conceivable that Zygomycetes biomass will be a part of biorefining, as a foundation for feed production.

6. Conclusions

The establishment of biorefineries with Zygomycetes as central catalysts is undoubtedly promising. Their ability to grow on starch-based and lignocellulose-based substrates opens the possibility of using an array of cost-effective waste materials. Altogether, Zygomycetes hold great potential for the establishment of a platform for production of fine chemicals, enzymes, fungal biomass for food purposes, and lipids. Given their versatility regarding fermentation designs, an intensification of Zygomycetes research to valorise organic waste materials, and an establishment of biorefineries, using these filamentous fungi in the main core, is expected in a near future.

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PAPER III

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Article

Production of Ethanol and Biomass from Thin Stillage Using Food-Grade *Zygomycetes* and *Ascomycetes* Filamentous Fungi

Jorge A. Ferreira *, Patrik R. Lennartsson and Mohammad J. Taherzadeh

Swedish Centre for Resource Recovery, University of Borås, Borås SE-50190, Sweden;
E-Mails: Patrik.Lennartsson@hb.se (P.R.L.); Mohammad.Taherzadeh@hb.se (M.J.T.)

* Author to whom correspondence should be addressed; E-Mail: Jorge.Ferreira@hb.se;
Tel.: +46-33-435-4638; Fax: +46-33-435-4008.

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Abstract: A starch-based ethanol facility producing 200,000 m^3 ethanol/year also produces *ca.* 2 million m^3 thin stillage, which can be used to improve the entire process. In this work, five food-grade filamentous fungi, including a *Zygomycete* and four *Ascomycetes* were successfully grown in thin stillage containing 9% solids. Cultivation with *Neurospora intermedia* led to the production of *ca.* 16 $g \cdot L^{-1}$ biomass containing 56% (w/w) crude protein, a reduction of 34% of the total solids, and 5 $g \cdot L^{-1}$ additional ethanol. In an industrial ethanol production process (200,000 m^3 ethanol/year), this can potentially lead to the production of 11,000 m^3 extra ethanol per year. Cultivation with *Aspergillus oryzae* resulted in 19 $g \cdot L^{-1}$ biomass containing 48% (w/w) crude protein and the highest reduction of the thin stillage glycerol (54%) among the *Ascomycetes*. Cultivation with *Rhizopus* sp. produced up to 15 $g \cdot L^{-1}$ biomass containing 55% (w/w) crude protein. The spent thin stillage had been reduced up to 85%, 68% and 21% regarding lactic acid, glycerol and total solids, respectively. Therefore, *N. intermedia*, in particular, has a high potential to improve the ethanol process via production of additional ethanol and high-quality biomass, which can be considered for animal feed applications such as for fish feed.

Keywords: *Ascomycetes*; biomass; ethanol; protein; thin stillage; *Zygomycetes*

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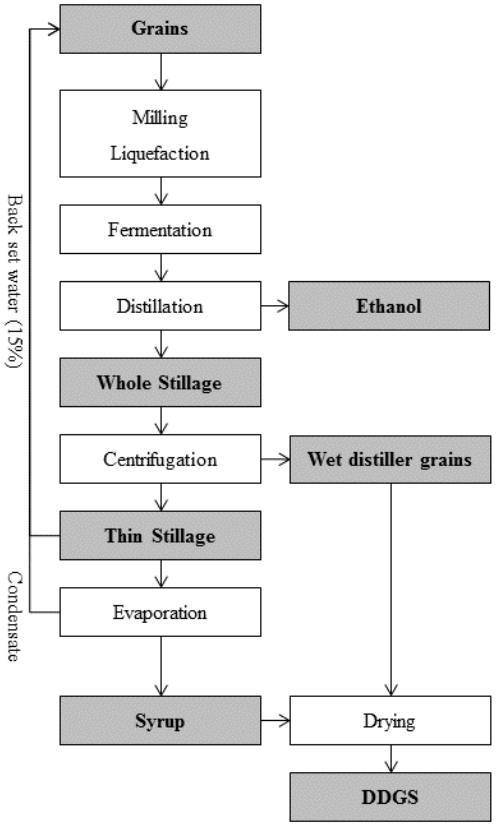
1. Introduction

Production of renewable fuels with comparable costs to fossil fuels is a hot topic. In particular, ethanol has triggered intense commercial interest due to its use in the transport sector as a viable alternative to petroleum fuel. Global production of ethanol was 86.1 billion liters in 2011, which corresponded to a contribution of around 0.6% to the worldwide energy [1]. Forecasts state that future ethanol production will reach 100 billion liters in 2015 [2]. In Sweden, the commercial production of ethanol is based on starch, mostly obtained from wheat [3]. Generally, starch-based ethanol processes give rise to two products: ethanol and an animal feed known as dry distiller grains with soluble (DDGS, Figure 1). Considering that the starch-based process gives rise to approximately the same amount of DDGS as ethanol, *ca.* 68 million tons DDGS was produced in 2011 [4]. Recently, research on the overall process leading to the production of feed products has been triggered. Thin stillage, in particular, has been considered to be a potential source of further improvement of the overall ethanol process via production of other products such as protein- and lipid-rich biomass [5,6].

Figure 1. General overview of the main starch-based ethanol process pathways leading to the production of ethanol and dry distiller grains with solubles (DDGS).



Filamentous fungi have been prime catalysts in biotechnological processes towards valorisation of a wide range of by-products. Alcohols, organic acids, or enzymes are a few examples of fungal products. Moreover, their biomass has been a target of intense research and considered to be a potential source of single cell protein. Particular interest has been paid to filamentous fungi with known ancient use for the production of human food products [7,8]. For instance, *Zygomycetes* fungi, mainly those belonging to the genus *Rhizopus* have been well known for hundreds of years for their use in the preparation of

fermented foods such as tempe and tofu [8]. On the other hand, the *Ascomycetes* include *Fusarium venenatum*, which under the trade name Quorn® might be the most studied microorganism for the production of human food [9]; *Aspergillus oryzae*, which is one of the most studied fungal species at the industrial scale for production of various fungal products [10]; *Monascus purpureus*, which has been used for production of red fermented rice for over a thousand years in Asian countries [11] and *Neurospora intermedia*, which is used for the preparation of oncom, an indigenous Indonesian food [12].

Thin stillage from corn-based ethanol industries has already been researched for production of several products, such as high-value biomass made using *Rhizopus oligosporus* [5,13], butanol using *Clostridium pasteurianum* [14], single-cell oil using *Mucor circinelloides* [6], eicosapentaenoic acid (EPA) using *Pythium irregulare* [15], and biogas [16] and ethanol using metabolically engineered *Escherichia coli* [17]. The production of ethanol from thin stillage is greatly interesting from a process economics standpoint since it could be recovered without needing additional steps: the produced ethanol left after the series of evaporations can be sent back into the process and follows the general stream towards the distillation column (Figure 1). Additionally, the produced biomass after a harvesting step could be simply directed to the installed dryers as proposed by Lennartsson, *et al.* [18] and the resulting effluent should be easier to treat due to prior removal of organic matter by the fungus.

In this work, a study on ethanol and high-quality fungal biomass production from mostly wheat-based thin stillage was carried out via submerged cultivation of food-grade microorganisms, namely a *Zygomycete Rhizopus* sp. and the *Ascomycetes A. oryzae*, *F. venenatum*, *M. purpureus* and *N. intermedia*. To the best of our knowledge this is the first research work on thin stillage valorisation using these *Ascomycetes* species. Special focus was placed on the influence of strain type on the production and composition of the fungal biomass as well as on the composition of the resulting thin stillage. The temperature effect upon *Rhizopus* sp. cultivation in thin stillage was also studied in this work.

2. Experimental Section

2.1. Thin Stillage

Thin stillage was provided by Lantmännen Agroetanol (Norrköping, Sweden), an ethanol production facility mostly based on wheat. The thin stillage used in this work originated from one single industrial batch. It was autoclaved in 5 L plastic bottles for 30 min at 121 °C and stored at 4 °C prior to use.

2.2. Microorganisms

Five different microorganisms belonging to the *Zygomycetes* or *Ascomycetes* fungal groups were used. The *Zygomycete* was a *Rhizopus* sp. previously identified as R15 isolated from starting cultures for tempe preparation [19]. The four *Ascomycetes* strains used were *Aspergillus oryzae* var. *oryzae* CBS 819.72 (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands), *Fusarium venenatum* ATCC 20334 (American Type Culture Collection, Manassas, VA, USA), *Monascus purpureus* CBS 109.07 and *Neurospora intermedia* CBS 131.92. All fungi were maintained on potato dextrose agar (PDA) slants containing (in $g \cdot L^{-1}$): glucose 20, agar 15 and potato extract 4. The slants were renewed every six months. New PDA plates were prepared via incubation for 3–5 days at 30 °C followed by storage at 4 °C. For spore solution preparation, *Zygomycetes p*lates were flooded with 20 mL of

distilled water, while *Ascomycetes* plates, except those with *M. purpureus* (5 mL), were flooded with 10 mL. A disposable plastic spreader was used to extract the spores.

2.3. Cultivation in Shake Flasks

Rhizopus sp. was first examined for consumption of pentose sugars in a semi-synthetic medium containing (in $g \cdot L^{-1}$): arabinose or xylose 20, yeast extract 5, $(NH_4)_2SO_4$ 7.5, KH_2PO_4 3.5, $CaCl_2 \cdot 2H_2O$ 1.0 and $MgSO_4 \cdot 7H_2O$ 0.75 and supplemented with trace metals ($10\ mL \cdot L^{-1}$) and vitamin ($1\ mL \cdot L^{-1}$) according to Sues *et al.* [20]. The cultivations were performed in 250 mL cotton-plugged Erlenmeyer flasks containing 50 mL medium at pH 5.5 at 30 °C. Spore concentration was 6×10^4 spores$\cdot mL^{-1}$. Cultivations were carried out for 12 and 5 days in arabinose-containing and xylose-containing medium, respectively. *Rhizopus* cultivations in undiluted thin stillage were performed in 1 L cotton-plugged Erlenmeyer flasks containing 0.2 L thin stillage adjusted to pH 4.5 ± 0.2 with 10 M NaOH. The flasks were maintained either at 25 °C, 30 °C, 35 °C, 40 °C or 45 °C. Spore concentration was 2×10^5 spores$\cdot mL^{-1}$ and the cultivation time was 96 h. Cultivations with *Ascomycetes* were performed using 250 mL cotton-plugged Erlenmeyer flasks containing 50 mL of undiluted thin stillage adjusted to pH 5.5 with 10 M NaOH. The flasks were maintained at 30 °C. Inoculum concentration was 2×10^6, 6×10^5, 7×10^5 spores$\cdot mL^{-1}$ and 9×10^4 colony-forming units (CFU)$\cdot mL^{-1}$ of *A. oryzae*, *N. intermedia*, *M. purpureus* and *F. venenatum*, respectively. The cultivation time was three days. All cultivations were carried out in water baths shaking at 125 rpm under aerobic conditions. The biomass was harvested either at the end or during cultivation using a sieve and extensively washed with distilled water until a clear effluent was obtained. All solutions except thin stillage were sterilised in an autoclave at 121 °C for 20 min.

2.4. Analytical Methods

Harvested biomass was dried to constant weight in an oven for 24 h at 70 °C and reported as biomass production in $g \cdot L^{-1}$. The biomass crude protein was determined according to the Kjeldahl method using block digestion and steam distillation (Application note 300, Rev. 8.0, FOSS, Eden Prairie, MN, USA) by Eurofins (Lidköping, Sweden). A Kjeltec™ 8400 analyser unit and a 2400/2460 Kjeltec™ autosampler system were used. Crude protein was determined as Nitrogen × Protein Factor (6.25). Total nitrogen in the thin stillage was determined using a Nanocolor® 500 D Universal Photometer (Macherey-Nagel, Düren, Germany). A Nanocolor total nitrogen kit within the range 5–220 $mg \cdot L^{-1} \cdot N$ was used.

The total solids and suspended solids in the thin stillage was determined according to the National Renewable Energy Laboratory (NREL) method for determination of total solids in biomass and total dissolved solids in liquid process samples [21]. The cell wall material as alkali-insoluble material (AIM) was prepared by dried biomass treatment with 0.5 M NaOH ($30\ mL \cdot g^{-1}$). The AIM was separated via centrifugation (5000 × g, 5 min), washed until neutral pH and dried using a freeze-dryer (Labconco, Kansas City, MO, USA). The cell wall contents of glucosamine and *N*-acetylglucosamine were determined according to a previous method [22] with some modifications; specifically, the acid hydrolysate was diluted to a glucosamine range of 0.01–0.1 $g \cdot mL^{-1}$ and the pH was adjusted to 3 with 0.5 M NaAc before addition of 3-metyl-2-benzothiozolone-hydrazone-hydrochloride (MTBH) [23].

The liquid fractions from the thin stillage and acid-treated *Rhizopus* cell wall were analysed using high-performance liquid chromatography (HPLC). A hydrogen-ion based ion-exchange column (Aminex HPX-87H, Bio-Rad, Hercules, CA, USA) at 60 °C and 0.6 mL·min^{-1}. 5 mM H_2SO_4 as eluent was used for analyses of acetic acid, ethanol, glycerol and lactic acid. Additionally, a lead (II)-based column (Aminex HPX-87P, Bio-Rad) at 85 °C and 0.6 mL·min^{-1} ultrapure water was used for separation of xylose and arabinose. An ultraviolet (UV) absorbance detector (Waters 2487, Waters Corporation, Milford, MA, USA), operating at 210 nm wavelength was used in series with a refractive index (RI) detector (Waters 2414). All samples for HPLC analysis were centrifuged for 5 min at 10,000 × g, and the supernatant was frozen at −20 °C.

2.5. Statistical Analysis

All experiments and analyses were carried out in duplicate and analysed with the software package MINITAB 15 (Minitab Inc., State College, PA, USA). All error bars and intervals reported represent two standard deviations. Results were analysed with ANOVA (analysis of variance) tables, using one-way models and factors were considered significant when their *p*-value was less than 0.05. When applied, pairwise comparisons were performed according to the Tukey test.

3. Results and Discussion

3.1. Characteristics of Thin Stillage and Rhizopus sp. Cultivation in Pentose Sugars

The thin stillage from corn-based ethanol production has previously been shown to be a good growth medium for production of nutritionally rich biomass using filamentous fungi [5]. However, research performed on thin stillage derived from ethanol production using other cereals such as wheat is scarce in literature. In this study wheat-based thin stillage from a Swedish ethanol facility was mostly used and some of its characteristics are presented in Table 1. Altogether, organic acids, glycerol, ethanol and pentose sugars represented around 13% of the *ca.* 92 g·L^{-1} of total solids. The thin stillage also contained around 5 g·L^{-1} of nitrogen reinforcing its high potential to be used as the sole cultivation medium. The remaining fraction of the thin stillage most likely consists of fiber, oil, other cereal-unfermented components, salts and dead yeast cells, which are also potential nutrient sources.

Reasonably, microorganisms able to consume pentose sugars would be preferable for cultivation in thin stillage. Contrary to *Saccharomyces cerevisiae*, *Zygomycetes* fungi are well known for their capability to consume pentose sugars. However, their ability to assimilate xylose is far better investigated than for arabinose [8]. *Rhizopus* sp. was first examined for assimilation of arabinose and xylose in semi-synthetic medium. It consumed 83% ± 1% of the initial arabinose after 12 days of cultivation. The fastest consumption rate of (2.0 ± 0.1) g·L^{-1}·d^{-1} was reached after six days. The produced biomass was (4.1 ± 1.0) g·L^{-1}, corresponding to a yield of (285 ± 78) mg·biomass·g^{-1} consumed arabinose. No metabolites were produced upon arabinose consumption. The *Rhizopus* sp. consumed all xylose within 60 h of cultivation. The highest xylose consumption rate of about (570 ± 90) mg·L^{-1}·h^{-1} was reached after 36 h. The produced biomass was 5.1 ± 0.0 g·L^{-1}, corresponding to a yield of (306 ± 2) mg·biomass·g^{-1} consumed xylose. Other than biomass,

Rhizopus sp. produced ethanol, glycerol, lactic acid and xylitol, with yields of (128 ± 12) $mg \cdot g^{-1}$, (88 ± 1) $mg \cdot g^{-1}$, (74 ± 11) $mg \cdot g^{-1}$ and (47 ± 15) $mg \cdot g^{-1}$ consumed xylose, respectively. The production of the intermediate xylitol indicates that xylose conversion occurs through the general fungal pathway [24]. The *Rhizopus* strain used in this study is evidently a potential candidate to be used as a catalyst in fermentations of pentose-containing substrates such as thin stillage.

Table 1. Characterisation of the industrial thin stillage derived from wheat-based ethanol production process used in the present work.

Parameter	Value	Parameter	Value
pH	3.5	Arabinose ($g \cdot L^{-1}$)	1.5 ± 0.1
Total nitrogen ($g \cdot L^{-1}$)	5.0 ± 0.4	Glycerol ($g \cdot L^{-1}$)	7.0 ± 0.1
Soluble total nitrogen ($g \cdot L^{-1}$)	2.1 ± 0.4	Lactic acid ($g \cdot L^{-1}$)	1.8 ± 0.1
Total solids (%, w/v)	9.2 ± 0.9	Acetic acid ($g \cdot L^{-1}$)	0.21 ± 0.01
Suspended solids (%, w/v)	3.8 ± 0.3	Ethanol ($g \cdot L^{-1}$)	1.2 ± 0.2
Xylose ($g \cdot L^{-1}$)	0.8 ± 0.1		

3.2. Cultivation in Thin Stillage with a Rhizopus sp.

The growth performance of filamentous fungi has been studied in corn-based thin stillage. Mitra *et al.* [6] and Liang *et al.* [15] have reported growth of *Mucor circinelloides* and *Pythium irregulare* in 6% total solids thin stillage, respectively. The latter was further shown to perform better in 50% diluted thin stillage. The need for the dilution of thin stillage containing 8% or more total solids has been reported when using *Rhizopus oligosporus* [5]. In this work, *Rhizopus* sp. was successfully grown in mostly wheat-based thin stillage containing *ca.* 9% total solids, and its growth profiles within the range 25–45 °C are depicted in Figure 2. Similar produced biomass values (around 6.5 $g \cdot L^{-1}$) were achieved at the beginning of cultivation within the range 30–40 °C; a lower value was achieved at 45 °C (*ca.* 4 $g \cdot L^{-1}$); and a longer lag phase was observed at 25 °C. The highest maximum produced biomass value (*ca.* 15 $g \cdot L^{-1}$) was obtained at 30 °C, while the lowest one was obtained at 45 °C (<10 $g \cdot L^{-1}$) (Table 2). Significant statistical differences were found on biomass production within examined temperatures ($p = 0.000$); 45 °C was found to be statistically different from the other tested temperatures. Maximum biomass productivities of (366 ± 26) $mg \cdot L^{-1} \cdot h^{-1}$, (364 ± 52) $mg \cdot L^{-1} \cdot h^{-1}$, (358 ± 19) $mg \cdot L^{-1} \cdot h^{-1}$ and (234 ± 40) $mg \cdot L^{-1} \cdot h^{-1}$ were obtained after 18 h of cultivation at 30 °C, 35 °C, 40 °C and 45 °C, respectively; a maximum of (282 ± 2) $mg \cdot L^{-1} \cdot h^{-1}$ was obtained after 24 h cultivation at 25 °C. The *Rhizopus* sp. also exhibited different growth morphologies during cultivation at different temperatures. It grew as small mycelial clumps at 30 °C, 35 °C and 40 °C, while it grew as small mycelial pellets at 25 °C and 45 °C. The capacity of *Rhizopus* sp. to grow well up to 40° C is very relevant, since this could potentially lead to energy savings during cooling of the thin stillage after distillation.

When evaluating the potential to use fungal biomass as a nutrient source for feed applications, a few compositional aspects are of special interest such as protein contents. The final crude protein of the *Rhizopus* sp. biomass was found to be within the range 49%–55% of biomass dry weight (Table 2), which was found to be similar to that at the beginning of cultivation (52%–54%). *Zygomycetes* fungi are also well known for the presence of chitosan in their cell walls. This polymer has been gathering

increasing interest due to its wide applications in e.g., biomedical and environmental fields [25]. Its acetylated form chitin can also be found in the *Zygomycetes* cell walls. Chitosan and chitin are polymers of glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc) in which GlcN is dominant (60%–100%) in chitosan, while GlcNAc is dominant (60%–100%) in chitin [26]. The cell wall fraction was measured as alkali-insoluble material (AIM), which presented an increasing trend during cultivation at all tested temperatures. Overall, the cell wall fraction of *Rhizopus* sp. biomass was in the range 10%–15% of biomass dry weight. Maximum concentration of (2.2 ± 0.4) $g \cdot L^{-1}$ and (1.5 ± 0.0) $g \cdot L^{-1}$ of the AIM were obtained at the end of cultivation at both 30 °C and 40 °C and (1.6 ± 0.1) $g \cdot L^{-1}$ after 72 h when cultivating *Rhizopus* sp. at 35 °C. Both glucosamine and *N*-acetylglucosamine contents were found to increase by 45%–50% of the AIM dry weight during cultivation in the thin stillage. Final glucosamine and *N*-acetylglucosamine contents were not found to be statistically different at the temperatures examined ($p = 0.953$ and $p = 0.199$, respectively). These monomers were present at about the same fractions (10%–25% each), making up to 40%–50% of the *Rhizopus* sp. cell wall (Table 2). Together, glucosamine and *N*-acetylglucosamine made up to 6% of the *Rhizopus* sp. biomass; up to (960 ± 77) $mg \cdot L^{-1}$, (657 ± 127) $mg \cdot L^{-1}$ and (778 ± 92) $mg \cdot L^{-1}$ of glucosamine and *N*-acetylglucosamine together could be produced during cultivation at 30 °C, 35 °C and 40 °C, respectively. Similar glucosamine trends were found by Ferreira *et al*. [23] when cultivating another *Rhizopus* strain in spent sulphite liquor. Higher production of glucosamine was observed either when changing from semi-synthetic medium to spent sulphite liquor or during cultivation under more unfavourable temperatures. *N*-acetylglucosamine content was found to increase at all tested conditions.

Figure 2. *Rhizopus* sp. biomass concentration during cultivation in thin stillage at different temperatures.

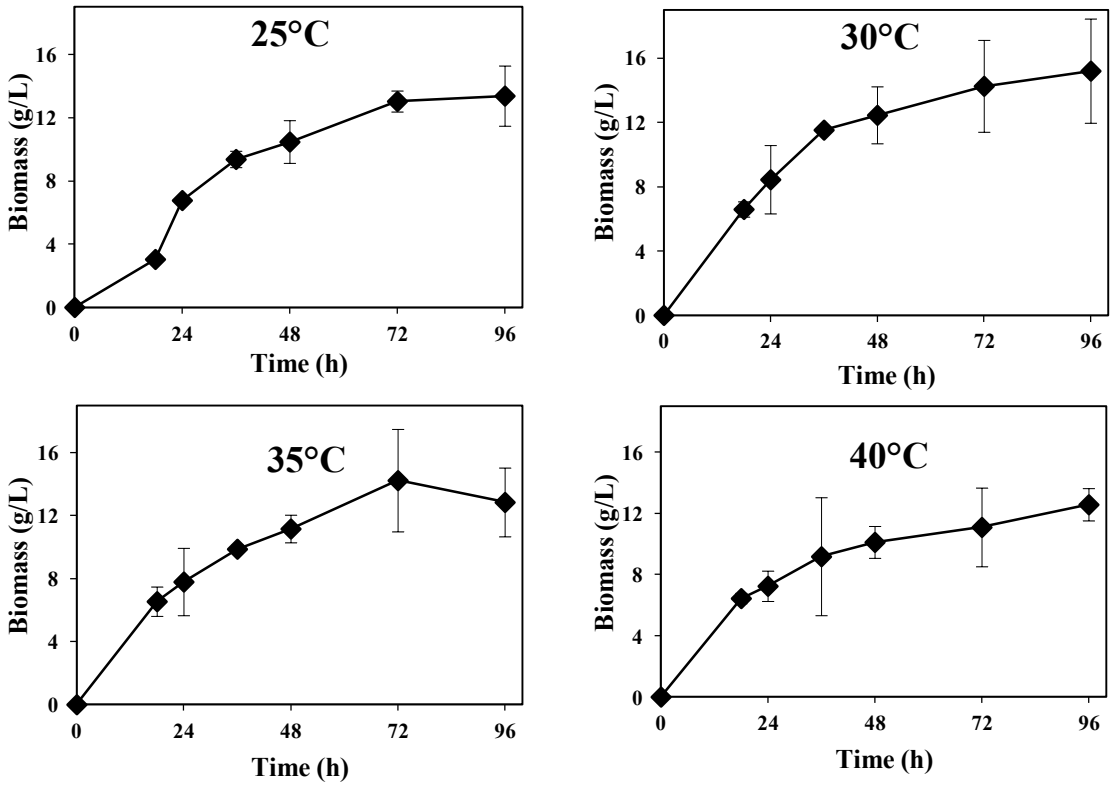
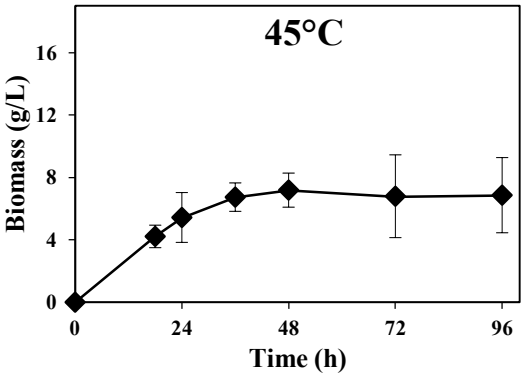
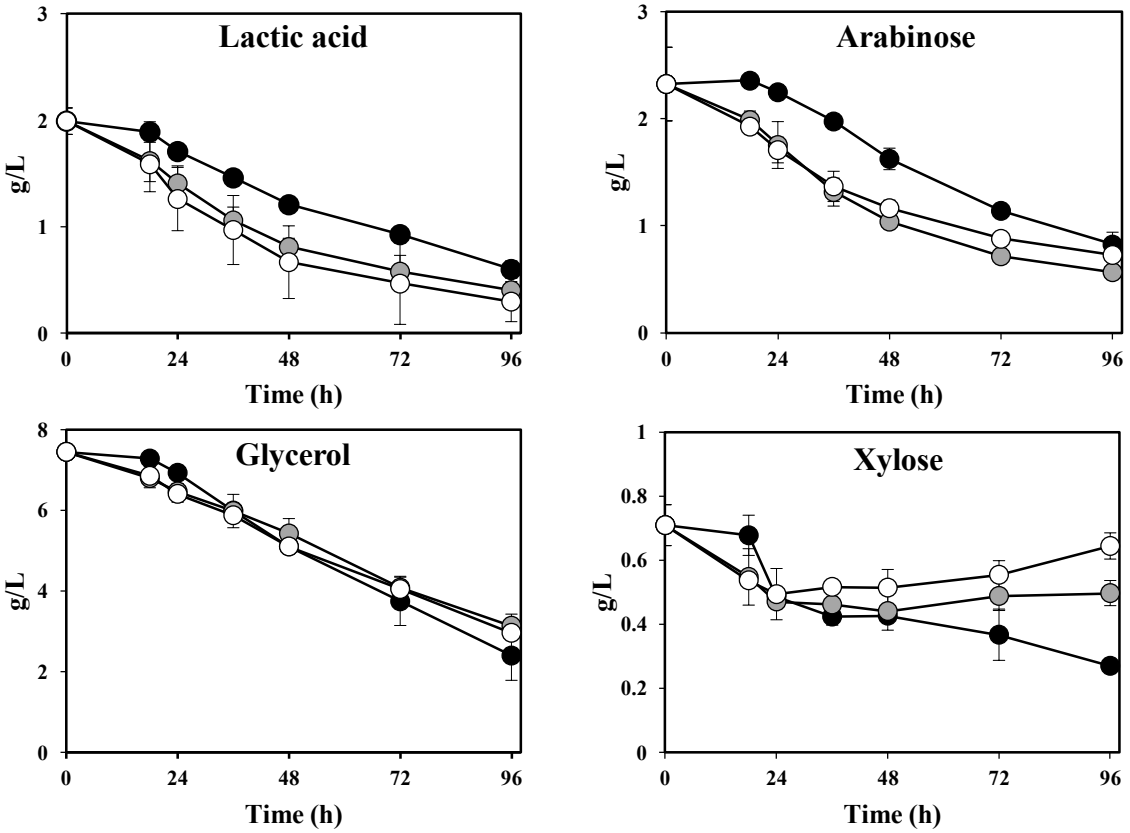


Figure 2. *Cont.*



In addition to the production of high-quality fungal biomass, a post-cultivation thin stillage with a lower level of organic load is of interest. The *Rhizopus* sp. was able to consume 70%–85% of the initial lactic acid and 58%–68% of the initial glycerol within the applied temperature ranges (Figure 3; Table 2). Final lactic acid and glycerol reduction values were not found to be statistically different between temperatures (p = 0.065 and p = 0.068, respectively). Acetic acid was completely consumed after 18 h of cultivation. In addition, a net output of 1 g·L^{-1} of ethanol was produced during cultivation at all tested temperatures (Figure 3; Table 2). The concentration of the main sugars in the thin stillage (arabinose and xylose) showed different patterns during cultivation (Figure 3). Arabinose decreased continuously during cultivation; reduction maxima of 64% ± 0%, 76% ± 0% and 69% ± 0% were achieved during cultivation at 30 °C, 35 °C and 40 °C, respectively. On the other hand, xylose presented a decreasing trend at 30 °C (maximum reduction of 61.9% ± 0.4%), whereas it presented a constant or slightly increasing trend after 24 h of cultivation at 35 °C and 40 °C (Figure 3). Overall, total solids and suspended solids in the thin stillage were comparably reduced by 16%–21% and 37%–54%, respectively, after cultivation with *Rhizopus* sp. No statistical differences were found between the tested temperatures (p = 0.188 and p = 0.072, respectively). However, if the sum of the consumed glycerol, lactic acid, arabinose and xylose is considered (around 8 g·L^{-1} at all tested temperatures), it is considerably lower than the reduction in total solids and suspended solids (15–20 g·L^{-1} and 14–20 g·L^{-1}, respectively). Clearly, other components of the thin stillage were assimilated by the *Rhizopus* strain. This might explain the constant high level of protein during cultivation. It is well known that *Zygomycetes* fungi can produce a wide range of enzymes depending upon the substrate they grow on being able to assimilate different carbon and nitrogen sources [8]. In absolute terms, the biomass production might be overestimated due to entanglement with solids in the fungal mycelium.

Figure 3. Concentration of lactic acid, glycerol, ethanol, arabinose and xylose during cultivation of *Rhizopus* sp. in thin stillage at 30 °C (black), 35 °C (grey) and 40 °C (white).



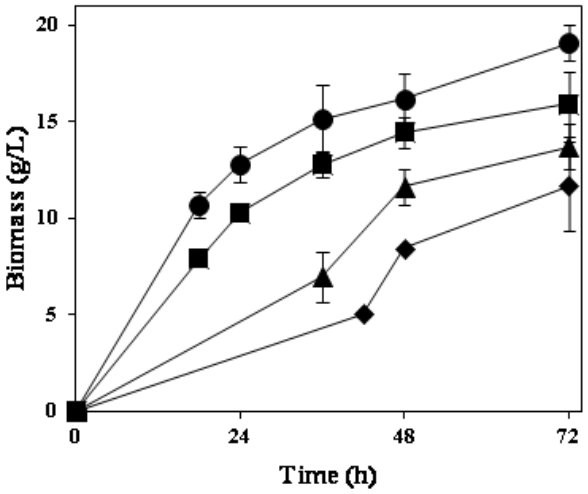
Altogether, cultivation in the thin stillage with *Rhizopus* sp. resulted in the production of biomass containing 49%–55% protein and 6% glucosamine-based polymers as well as a spent medium with a reduced amount of total solids (up to 21%). No addition of nutrients was required; pH adjustment with 25% NH_3 instead of NaOH did not lead to any further improvement in the biomass production (data not shown).

3.3. Cultivation in Thin Stillage Using Ascomycetes Fungi

A preliminary study on thin stillage valorisation was performed using four *Ascomycetes* fungi; their biomass production profiles are depicted in Figure 4. Common aspects for all of them are either their long tradition in the production of food products or their extensive use at industrial scale that make them well-known/studied microorganisms. Similar to the *Rhizopus* strain used, all four strains examined were able to grow extensively in the thin stillage containing 9% total solids. Cultivation with *A. oryzae* resulted in the highest amount of produced fungal biomass (19 $g \cdot L^{-1}$); *N. intermedia* gave rise up to 16 $g \cdot L^{-1}$of biomass while *F. venenatum* and *M. purpureus* presented a longer lag phase and *ca*. 14 $g \cdot L^{-1}$ and 12 $g \cdot L^{-1}$ of biomass were reached at the end of cultivation, respectively (Figure 4; Table 2). The final biomass concentration produced was found to be statistically different among tested strains (p = 0.003); final produced biomass with *A. oryzae* was statistically different from that of *M. purpureus* and *F. venenatum* but not from that of *N. intermedia*. Maximum biomass productivities of (595 ± 36) $mg \cdot L^{-1} \cdot h^{-1}$ and (439 ± 13) $mg \cdot L^{-1} \cdot h^{-1}$ were achieved after 18 h of cultivation with

A. oryzae and *N. intermedia*, respectively, while (242 ± 19) mg·L^{-1}·h^{-1} and (176 ± 2) mg·L^{-1}·h^{-1} were achieved after 48 h of cultivation with *F. venenatum* and *M. purpureus*, respectively. *A. oryzae* together with *F. venenatum* grew in a well dispersed mycelial form, whereas *N. intermedia* and *M. purpureus* grew as dense mycelial suspensions. All fungi examined were also found to contain high crude protein content since ranges of 44%–56% of biomass dry weight were obtained (Table 2). *A. oryzae* and *M. purpureus* exhibited a slight decrease in their crude protein during cultivation (52%–48% and 49%–44%, respectively). In contrast, the crude protein of *F. venenatum* increased slightly during cultivation (53%–56%), while that of *N. intermedia* remained somewhat constant. Final crude protein levels were statistically different (p = 0.001) among tested strains. No statistically significant differences were found between *A. oryzae* and *M. purpureus* and between *F. venenatum* and *N. intermedia*.

Figure 4. Produced biomass profiles during cultivation of *Ascomycetes* filamentous fungi in thin stillage at 30 °C. *A. oryzae* (circles), *N. intermedia* (squares), *F. venenatum* (triangles), *M. purpureus* (diamonds).

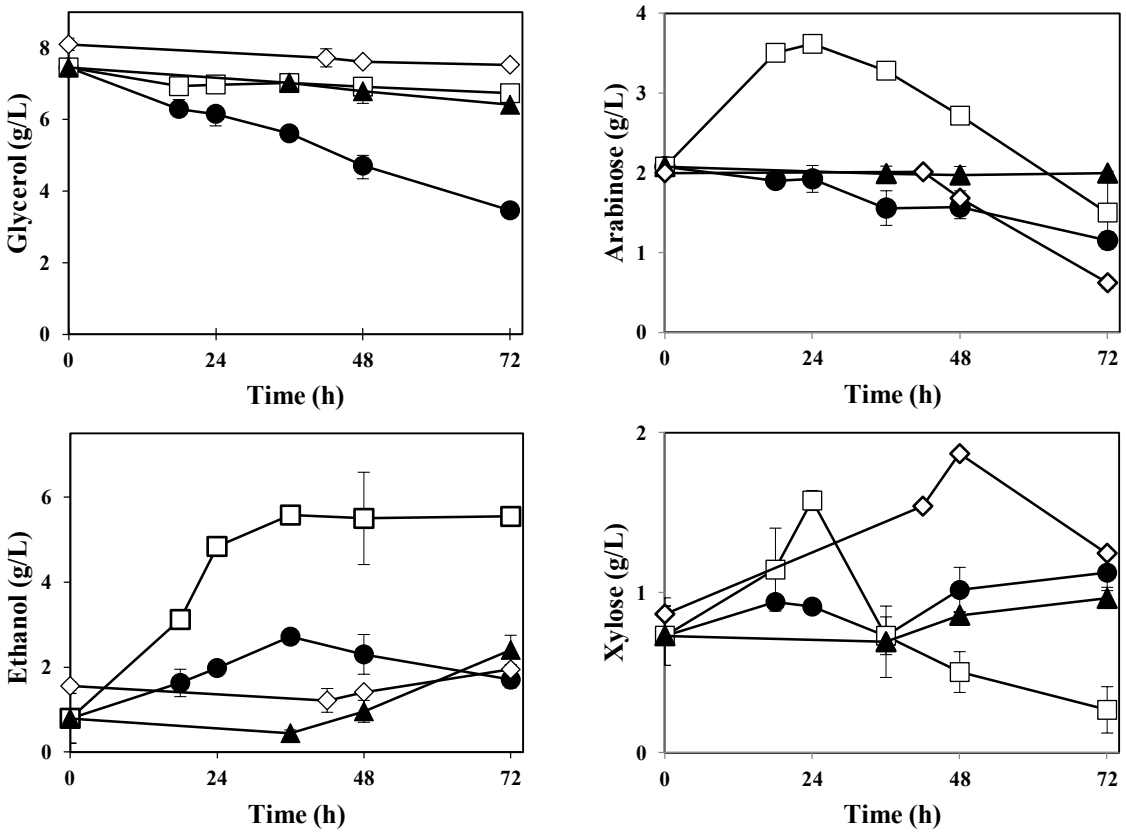


Interesting differences were found in the thin stillage after cultivation with different *Ascomycetes*. *M. purpureus*, *F. venenatum* and *N. intermedia* reduced the glycerol concentration by 7%–14%, while cultivation with *A. oryzae* resulted in a 54% reduction (Figure 5; Table 2).

Final glycerol reduction percentages were found to be statistically different (p = 0.000); total glycerol reductions by *A. oryzae* and *F. venenatum* were statistically different among each other as well as statistically different from those obtained by the remaining strains, while no statistical difference was found between glycerol reduction values by *M. purpureus* and *N. intermedia*. The initial acetic acid present in the stillage had been completely consumed after 18 h, while the concentration of lactic acid remained constant during cultivation with all applied *Ascomycetes* fungi (Table 2). The main sugars present in the thin stillage (arabinose and xylose) were also followed during cultivation with *Ascomycetes* (Figure 5). Both *A. oryzae* and *M. purpureus* assumed a consumption trend of arabinose reaching a maximum reduction of 45% ± 5% and 69% ± 3%, respectively. On the other hand, arabinose concentration remained constant during cultivation with *F. venenatum*, and increased during cultivation with *N. intermedia*; a maximum of (3.6 ± 0.0) g·L^{-1} was recorded at 24 h of cultivation (Figure 5). In contrast, an increase in xylose concentration was

observed for all strains examined. The highest concentration of (1.9 ± 0.0) g·L^{-1} was recorded during cultivation with *M. purpureus*. Cultivation with *N. intermedia* resulted in the lowest final amount of xylose (Table 2).

Figure 5. Concentration profiles of glycerol, ethanol, arabinose and xylose during cultivation of Ascomycetes in thin stillage at 30 °C. *A. oryzae* (circles), *N. intermedia* (squares), *F. venenatum* (triangles), *M. purpureus* (diamonds).



Additionally, *ca*. 5 g·L^{-1} extra ethanol were produced by *N. intermedia*. Cultivation with *A. oryzae* and *F. venenatum* gave rise to *ca*. 2 g·L^{-1} additional ethanol, while *M. purpureus* produced *ca*. 0.5 g·L^{-1} of extra ethanol (Figure 5). Maximum amounts of ethanol were found to be statistically different among used strains (p = 0.000); the pair-wise comparison between the extra ethanol amount produced by *A. oryzae* and *F. venenatum* was the only one found to be not statistically different. Reports on ethanol production from thin stillage are scarce in literature; one exception relates to the work performed with a metabolically engineered *Escherichia coli* strain performed by Gonzalez *et al*. [17]. An ethanol yield of 0.42 g·g^{-1} based on consumed glycerol, maltose and glucose present in thin stillage was reported. Altogether, the reduction of total solids was around 32%, 21%, 16% and 34%, and for suspended solids, the reduction was around 55%, 40%, 58% and 69% during cultivation with *A. oryzae*, *F. venenatum*, *M. purpureus* and *N. intermedia*, respectively. The reduction of total solids was found to be statistically different among strains (p = 0.007). No statistically significant differences were found between *A. oryzae* and *N. intermedia* and between *F. venenatum* and *M. purpureus*. On the other hand, no statistical differences were found for the reduction of suspended solids (p = 0.079).

The results support the degradation of arabinan by *F. venenatum* and *N. intermedia* and xylan by all examined *Ascomycetes* strains and xylan degradation by *Rhizopus* sp. when cultivated at 35 °C and 40 °C. These polymers are traditionally found in thin stillage [27]. In the present conditions, *Ascomycetes* did not consume lactic acid during cultivation. Reasons for such difference in comparison to the *Rhizopus* sp. might include the presence of other more suitable substrates for the *Ascomycetes*, the initial pH or nitrogen-limitation. During cultivation with *A. oryzae*, the highest amount of biomass (19 $g \cdot L^{-1}$) containing 48% crude protein as well as the highest glycerol reduction (54%) were obtained. Cultivation of *N. intermedia* led to the production of 16 $g \cdot L^{-1}$ biomass containing 56% crude protein and a reduction of around 34% of total solids. Additionally, *N. intermedia* produced the highest amount of ethanol (*ca*. 5 $g \cdot L^{-1}$). Considering an ethanol facility producing 200 m^3 thin stillage per hour (corresponding to *ca*. 200,000 m^3 ethanol per year), the inclusion of *N. intermedia* could potentially lead to the production of 11,000 m^3 extra ethanol per year. Furthermore, the recovery of this extra ethanol would not need additional steps since it could be sent back into the process after the series of evaporations as is carried out in the present established industrial processes [18].

4. Conclusions

In this work, five food-grade filamentous fungi, including a *Zygomycete* and four *Ascomycetes* strains were successfully grown in mostly wheat-based thin stillage containing 9% total solids. *N. intermedia*, *A. oryzae and Rhizopus* sp. proved to have high potentiality for inclusion in the industrial process of ethanol production. *A. oryzae* cultivation resulted in the highest amount of biomass (19 $g \cdot L^{-1}$) containing 48% (w/w) crude protein and the highest glycerol reduction (54%). *N. intermedia* cultivation resulted in *ca*. 16 $g \cdot L^{-1}$ biomass containing 56% (w/w) crude protein. Cultivation with *Rhizopus* sp. resulted in up to 85% and 68% reduction of the thin stillage lactic acid and glycerol, respectively. The produced protein-rich biomass can be considered for animal feed purposes such as for fish feed. In an industrial ethanol production process (200,000 m^3 ethanol/year), inclusion of *N. intermedia* can potentially lead to the production of 11,000 m^3 extra ethanol per year.

Acknowledgments

This work was financially supported by the Swedish Energy Agency. The authors would like to express their gratitude to Johan Westman, Päivi Ylitervo and Adib Kalantar for their technical assistance and valuable discussions.

Author Contributions

Jorge A. Ferreira, Patrik R. Lennartsson and Mohammad J. Taherzadeh developed the idea of cultivating *Zygomycetes* and *Ascomycetes* in thin stillage and have contributed for the discussion. Jorge A. Ferreira performed the experiments and wrote the majority of the paper. All authors have given approval to the final version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Energies **2014**, *7*, 4199-4201; doi:10.3390/en7074199

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Correction

Correction: Ferreira, J.A.; Lennartsson, P.R.; Taherzadeh, M.J. Production of Ethanol and Biomass from Thin Stillage Using Food-Grade *Zygomycetes* and *Ascomycetes* Filamentous Fungi. *Energies* 2014, *7*, 3872–3885

Jorge A. Ferreira *, Patrik R. Lennartsson and Mohammad J. Taherzadeh

Swedish Centre for Resource Recovery, University of Borås, Borås SE-50190, Sweden;
E-Mails: Patrik.Lennartsson@hb.se (P.R.L.); Mohammad.Taherzadeh@hb.se (M.J.T.)

* Author to whom correspondence should be addressed; E-Mail: Jorge.Ferreira@hb.se;
Tel.: +46-33-435-4638; Fax: +46-33-435-4008.

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We have found two inadvertent errors in our paper [1], and thus would like to make the following corrections to this paper:

On page 3880, one of the subfigures in Figure 3 was missing. Figure 3 should be changed from:

Figure 3. Concentration of lactic acid, glycerol, ethanol, arabinose and xylose during cultivation of *Rhizopus* sp. in thin stillage at 30 °C (black), 35 °C (grey) and 40 °C (white).

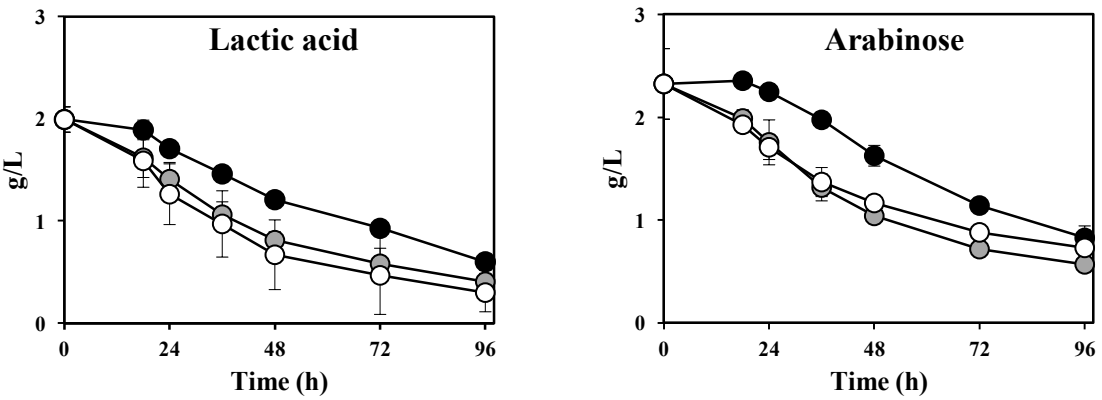
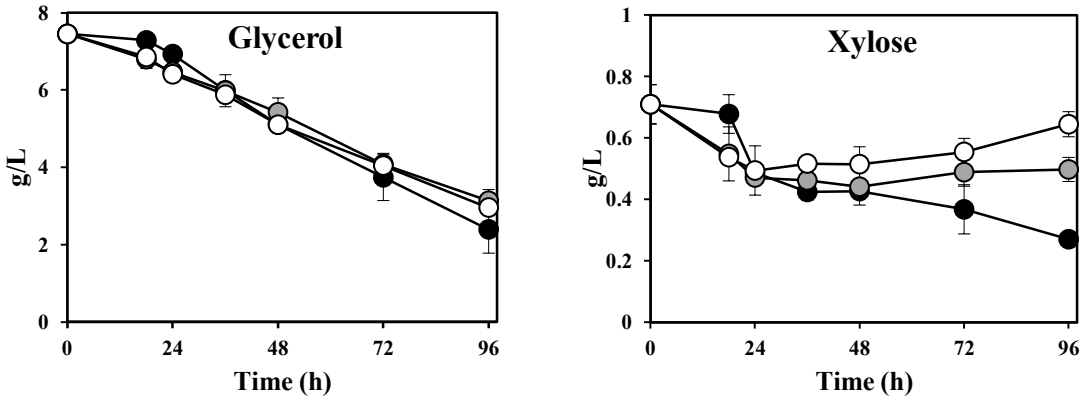
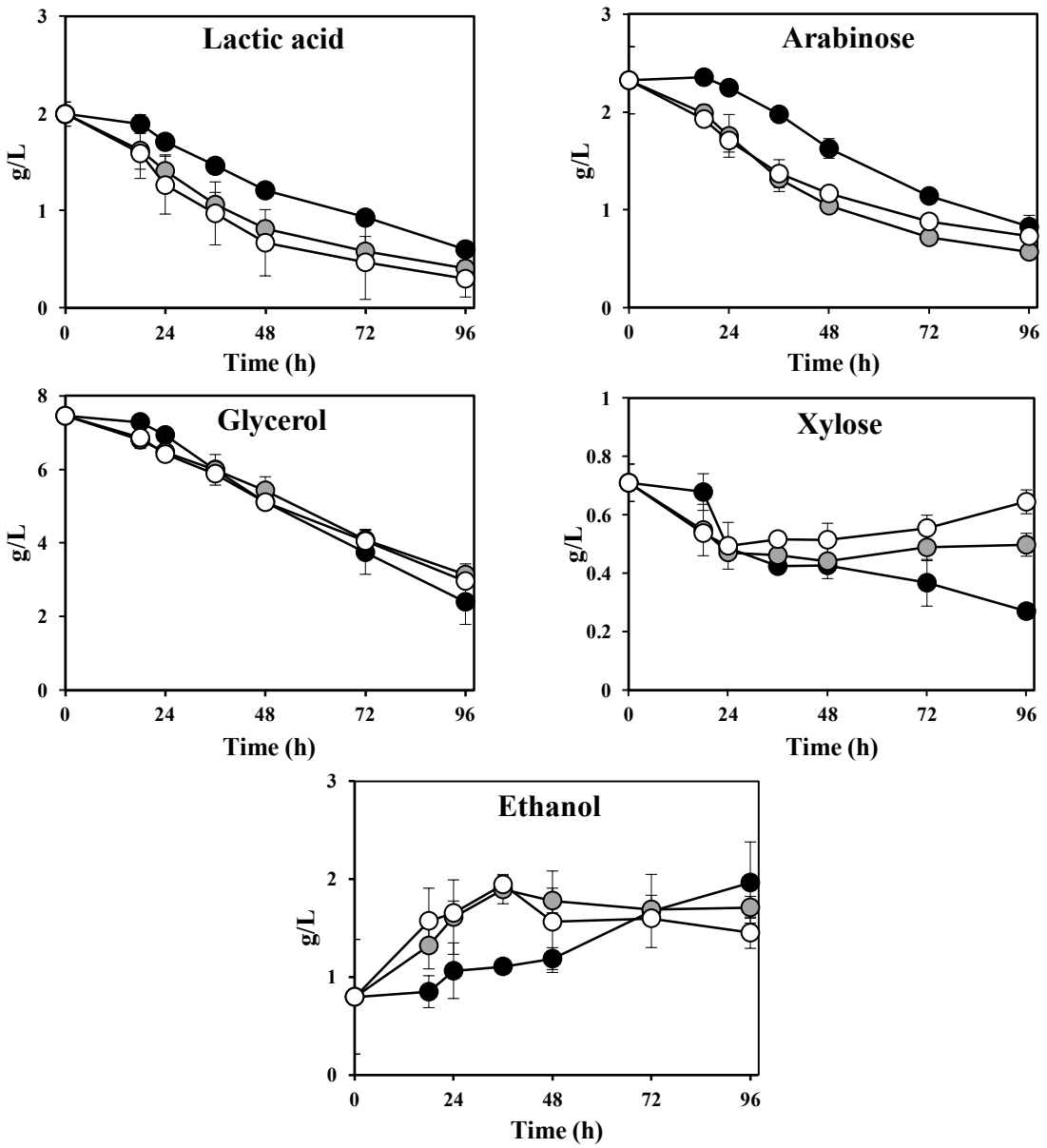


Figure 3. *Cont.*



to the following correct version:

Figure 3. Concentration of lactic acid, glycerol, ethanol, arabinose and xylose during cultivation of *Rhizopus* sp. in thin stillage at 30 °C (black), 35 °C (grey) and 40 °C (white).



Furthermore, Table 2 was missing and should be included in the paper:

Table 2. Final biomass and spent thin stillage characteristics after 96 h and 72 h cultivation with *Zygomycetes* and *Ascomycetes* fungi, respectively.

Fungal Class	***Zygomycetes***			***Ascomycetes***			
Fungal Strain	***Rhizopus* sp.**			***A. oryzae***	***F. venenatum***	***M. purpureus***	***N. intermedia***
T (°C)	30	35	40	30			
Fungal biomass							
Biomass dry weight ($g \cdot L^{-1}$)	15 ± 3	13 ± 2	13 ± 1	19 ± 1	14 ± 1	12 ± 2	16 ± 2
% Crude protein ($g \cdot g^{-1}$)	55 ± 1	55 ± 5	49 ± 1	48 ± 0	56 ± 0	44 ± 2	56 ± 3
AIM ($mg \cdot g^{-1}$)	145 ± 26	106 ± 14	124 ± 8	ND [a]	ND	ND	ND
GlcN ($mg \cdot g^{-1}$)	229 ± 94	234 ± 117	245 ± 95	ND	ND	ND	ND
GlcNAc ($mg \cdot g^{-1}$)	207 ± 59	249 ± 24	254 ± 36	ND	ND	ND	ND
Spent thin stillage							
pH	5.7 ± 0.4	5.6 ± 0.2	5.9 ± 0.2	6.0 ± 0.0	5.4 ± 0.1	5.5 ± 0.1	6.0 ± 0.1
Lactic acid reduction (%)	70 ± 4	80 ± 9	85 ± 9	0	0	0	0
Glycerol reduction (%)	68 ± 8	58 ± 4	60 ± 2	54 ± 0	14 ± 2	7 ± 1	10 ± 3
Ethanol ($g \cdot L^{-1}$)	2.0 ± 0.4	1.7 ± 0.1	1.4 ± 0.2	1.7 ± 0.2	2.4 ± 0.3	1.9 ± 0.1	5.5 ± 0.1
Xylose ($g \cdot L^{-1}$)	0.3 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	1.1 ± 0.1	1.0 ± 0.0	1.2 ± 0.0	0.3 ± 0.1
Arabinose ($g \cdot L^{-1}$)	0.8 ± 0.1	0.6 ± 0.0	0.7 ± 0.0	1.2 ± 0.1	2.0 ± 0.0	0.6 ±0.1	1.5 ± 0.4
TS reduction (%) [b]	20 ± 6	16 ± 5	21 ± 2	32 ± 1	21 ± 3	16 ± 5	34 ± 9
SS reduction (%) [c]	37 ± 15	41 ± 3	54 ± 4	55 ± 6	40 ± 1	58 ± 4	69 ± 20

The Editorial Office would like to apologize for any inconvenience caused to the readers by these changes.

Reference

1. Ferreira, J.A.; Lennartsson, P.R.; Taherzadeh, M.J. Production of Ethanol and Biomass from Thin Stillage Using Food-Grade *Zygomycetes* and *Ascomycetes* Filamentous Fungi. *Energies* **2014**, *7*, 3872–3886.

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PAPER IV

Engineering in Life Sciences

Eng. Life Sci. **2015**, *0*, 1–9 www.els-journal.com

Jorge A. Ferreira
Patrik R. Lennartsson
Mohammad J. Taherzadeh

Swedish Centre for Resource Recovery, University of Borås, Borås, Sweden

Research Article

Production of ethanol and biomass from thin stillage by *Neurospora intermedia*: A pilot study for process diversification

Dry mill ethanol processes produce ethanol and animal feed from whole grains, where the wastewater after the distillation and separation of solid materials is called "thin stillage." In this work, similar production of ethanol (3.5 g/L) and biomass (5 g/L) from thin stillage was obtained during batch cultivation of the edible fungus *Neurospora intermedia* in a 2-m high airlift reactor and bubble column. The fungal biomass, containing 50% w/w protein and 12% w/w lipids, was rich in essential amino acids and omega-3 and -6 fatty acids. In a continuous mode of fermentation, dilution rates of up to 0.2 h^{-1} could be applied without cell washout in the bubble column at 0.5 vvm. At 0.1 h^{-1}, around 5 g/L of ethanol and 4 g/L of biomass containing ca. 50% w/w protein were produced. The fungus was able to assimilate saccharides in the liquid fraction as well as sugar backbones such as xylan and arabinan in the solid fraction. The inclusion of the current process could potentially lead to the production of 11 000 m^3 of ethanol (5.5% improvement vs. normal industrial process) and around 6300 tons of high-quality biomass for animal feed at a typical facility producing 200 000 m^3 ethanol per year.

Keywords: Biomass / Bubble column / Continuous cultivation / Ethanol / *Neurospora intermedia*

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1 Introduction

The process of ethanol production from sugar- and starch-based materials is well developed at industrial scale. Brazil and United States dominate the worldwide production of ethanol, using mostly sugarcane and corn as feedstock, respectively. Beyond ethanol, animal feed nutrients commonly known as distillers dried grains with solubles (DDGS) are also produced during the process when grains (e.g. corn and wheat) are the substrates. The ethanol sector has produced ca. 86 million tons of ethanol and 68 million tons of DDGS in 2011 [1, 2]. However, fluctuations on the price of the feedstock and produced products together with the energy-intensive character of the process might impose constraints to its overtime feasibility [3]. Accordingly, research towards process improvement and diversification has been carried out mostly via valorization of thin stillage. Thin stillage corresponds to the liquid fraction after whole stillage centrifugation (distillation leftovers), 15% of which is normally recycled as backset water, while the remaining goes through a series of evaporations. The resulting condensate is also sent back to the process, whereas the concentrated syrup is normally dried together with the centrifuged solids to give rise to the DDGS [4]. Therefore, the processing steps leading to the production of DDGS are responsible for a large fraction of the overall process energy requirements [3]. The production of a variety of products including ethanol, cell-oil, feed nutrients, biogas, and eicosapentaenoic acid has been investigated using thin stillage as cultivation medium [5]. In addition to the production of new products, the employed microorganisms reduce the solid content of the thin stillage. Therefore, the viscosity of the medium is lowered, which facilitates the series of evaporations and the following drying process. More thin stillage could also be used as backset (lowering the load on the evaporators and driers) and the overall process energy consumption would be reduced [6].

Filamentous fungi have been an important contributor to the global economy via production of a plethora of important products including antibiotics, enzymes, organic acids, human/animal food products among many others [7]. Particularly, in the context of production of biomass for feed applications, filamentous fungi possess an important advantage over, e.g. yeasts, since they can be easily separated from the fermentation broth without requiring a centrifugation step. Both zygomycetes and ascomycetes filamentous fungi have previously been investigated

Correspondence: Prof. Mohammad J. Taherzadeh (Mohammad.Taherzadeh@hb.se), Swedish Centre for Resource Recovery, University of Borås, Allégatan 1, 50190 Borås, Sweden

Abbreviations: AIM, alkali-insoluble material; **DDGS**, distillers dried grains with solubles; **NREL**, National Renewable Energy Laboratory

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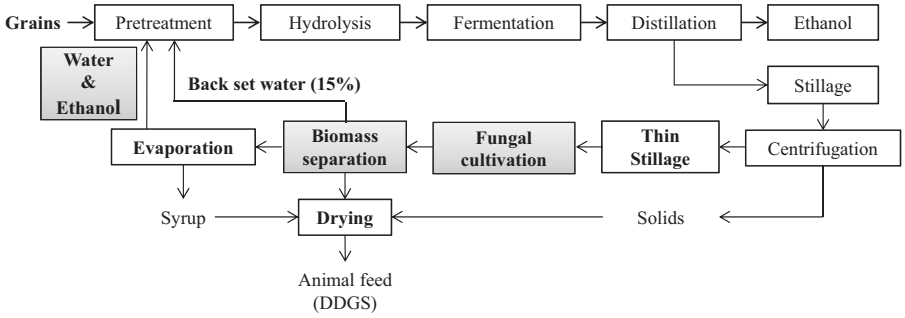

Figure 1. Proposed inclusion of *N. intermedia* in the overall industrial process of ethanol production for production of biomass and ethanol from thin stillage [6], boxes highlighted in gray.

for production of ethanol and protein-rich biomass from mostly wheat-based thin stillage, where the ascomycete *Neurospora intermedia* was found to be the best ethanol producer [5]. This fungus is traditionally used for preparation of oncom, an indigenous Indonesian food, so that it can be classified as generally regarded as safe (GRAS), which is a great advantage if the fungal biomass is produced for animal or human consumption [8]. While *Neurospora crassa* is genetically as well studied as *Saccharomyces cerevisiae* or *Escherichia coli*, biotechnological research studies using *N. intermedia* are scarce in the literature. With its inclusion in the established industrial process, the biomass would join DDGS as animal feed nutrients, while the extra ethanol produced could be sent back to the process with the condensate, as it is already done at industrial scale [6] (Fig. 1). However, the cultivation of filamentous fungi in bioreactors can be troublesome due to its filamentous growth. If stirred-tank reactors are used, the fungal filaments have the tendency to entangle with the inner parts, such as baffles and impellers, leading to suboptimal mass and energy transfer rates. Airlift reactors and bubble columns have been alternatively developed and better process performances have been reported. Their main difference is the absence of inner parts, e.g. baffles and impellers being the medium mixed by the supplied air [9]. The aeration rate greatly influences fungal growth, metabolite production, and assimilation of medium nutrients. For instance, higher aeration gives rise to higher biomass and lower ethanol productions, while assimilation of xylose is not possible under anaerobic conditions [6].

The process of ethanol and biomass production from thin stillage with *N. intermedia* would represent a partial inclusion in the established industrial process of ethanol production from grains. Therefore, after previous bench-scale experiments [5], the present work tries to provide further insights into the process but at a reactor scale. Those include the influence of the supplied air, reactor design, and cultivation mode on the production of ethanol and biomass and the removal of solids from the thin stillage. First, the influence of the aeration rate was studied in a 2-m high airlift reactor. The same reactor was further simplified into a bubble column after removal of the internal-loop tube for comparison purposes at the condition where the highest amount of ethanol was obtained. The cultivation was further studied under a continuous mode in the bubble column where different dilution rates were applied. In all the research steps, special focus was on ethanol and biomass production as well as the final characteristics of the thin stillage after biomass harvesting. To the best of our knowledge, this work reports for the first time, a continuous process in bubble column using thin stillage as cultivation medium and *N. intermedia* as the catalyst.

2 Materials and methods

2.1 Microorganism

The ascomycete *N. intermedia* CBS 131.92 (Centraalbureau voor Schimmelcultures, The Netherlands) was used throughout this study. Strain maintenance and preparation of spore solutions were done according to Ferreira et al. [5]. Spore number was determined by using a counting chamber.

2.2 Thin stillage

Thin stillage used in this work was provided by Lantmännen Agroetanol (Norrköping, Sweden), an ethanol production facility mostly based on wheat grains. The two spaced in time 1 m^3 thin stillage batches were stored in 230 L barrels at 4°C prior to use. Before cultivation, the thin stillage was sterilized at 121°C for 30 min in an autoclave (Systec, Germany).

2.3 Cultivation in bioreactor

Cultivations at different aeration rates, namely 0.5, 1.0, 1.5, and 2 vvm (air volume per culture volume per minute) were randomly carried out in a 2 m high, 15 cm diameter airlift reactor with 26 L total volume of the internal-loop tube reactor model (Bioengineering, Switzerland). The reactor was sterilized in situ with an injection of steam (121°C, 30 min). Twenty liters of sterilized thin stillage was supplemented with 50 μL/L antifoam silicone Snapsil FD10 (VWR International, USA) and adjusted to pH 5.5 with 10 M NaOH. A 24 h inoculum containing 8.8 $\pm$ 0.9 g/L biomass dry weight (average value determined from measurements in triplicate) was prepared in three 1-L Erlenmeyer flasks containing 0.1 L sterilized thin stillage adjusted to pH 5.5 with 10 M NaOH. The inoculum was prepared via incubation in a water bath set at 35°C and shaking at 125 rpm. The spore concentration was 8.7 ($\pm$ 1.6) $\times$ 10^8 spores/L. The cultivation temperature in airlift was maintained at 35 $\pm$ 0.4°C. Cultivation was followed by taking 0.25 L samples every 12 h for a period of 48 h. The thin stillage was poured through a sieve to

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recover the biomass, and stored at −20°C; the harvested biomass was extensively washed with continuous flow of distilled water in order to remove most of the remaining thin stillage solids until a clear effluent was obtained. For comparative purposes, the reactor was transformed in a bubble column by removing the internal-loop tube. The cultivation was carried out as described earlier at the aeration rate of 0.5 vvm.

Continuous cultivation was also carried out in the bubble column reactor at 0.5 vvm. Three dilution rates, namely 0.1, 0.15, and 0.2 h^{-1}, were sequentially applied after 1-day batch cultivation. The sterilized thin stillage was adjusted to pH 5.0–5.5 with 10 M NaOH and supplied using the reactor-coupled peristaltic pump. Cultivation conditions, thin stillage storage, and biomass harvesting were done as described earlier except that samples of 1 L volume were taken. Four 20 L volumes were replaced at each dilution rate. All batch and continuous cultivations were done in duplicate.

2.4 Cultivation in shake flasks

Neurospora intermedia was cultivated in semisynthetic medium containing either 5 g/L acetic acid (Sharlau Chemie), L-(+)-arabinose (Sigma–Aldrich), D-(+)-galactose (Acrös organics), D(-)-glucose (Fisher Chemical), glycerol (Sharlau Chemie), DL-lactic acid (Acrös organics), D(+)-mannose (Fluka), or 10 g/L D(+)-xylose (Sigma–Aldrich). The ascomycete was also cultivated in a mixture of these carbon sources containing (in g/L): acetic acid 1.0, lactic acid, and glycerol 10, and the remaining carbon sources 2. The medium was supplemented with yeast extract (ratio of carbon source:yeast extract of four), salts, and trace metals according to Millati et al. [10]. The pH of sugar-containing and acid-containing media was adjusted to 5.5 with 1 or 10 M NaOH, respectively, prior to sterilization for 20 min in an autoclave (Systec). Cultivations were carried out in 250-mL Erlenmeyer flasks containing 50 mL of medium shaking at 125 rpm using a water bath set at 35°C for 42 h. The spore concentration was 2.3×10^8 spores/L. At the end of the cultivation, biomass was harvested using a sieve, except for that grown in acetic acid, arabinose, and lactic acid-containing medium, which was recovered by vacuum filtration, and washed extensively with distilled water. All cultivations were performed in duplicate.

2.5 Analytical methods

The harvested biomass, reported as biomass dry weight in gram per liter, was dried until constant weight in an oven for 24 h at 70°C. The protein content of the biomass and thin stillage reported as crude protein and Kjeldahl nitrogen, respectively, was determined according to the Kjeldahl method using block digestion and steam distillation. The equipment included an InKjel P digestor and a behrotest® S1 distiller (behr Labor-Technik, Germany). Digestion was carried out by adding 20 mL of 98% H_2SO_4, antifoam, and KT1 tablets (Thompson & Capper Ltd, UK) to 0.4 ± 0.0 g material for a total duration of 100 min at 100% power (of which 10 min were for heating up the system). Digestion was followed by neutralization of the digested solution with 32% NaOH and distillation for 5 min. The distillation vapor was trapped in 50 mL of 4% H_3BO_4. Final titration was carried out with 0.1 M of HCl until pH 4.6. A factor of 6.25 was used to calculate the crude protein content. Determination of amino acid composition of the biomass and thin stillage (SS-EN ISO 13903:2005) and fatty acid composition of the biomass (Karlshamn's analysis methods Nr 2.5.1 and Nr 2.5.2) was performed by Eurofins (Lidköping, Sweden). The biomass cell wall fraction reported as alkali-insoluble material (AIM) was determined according to Zamani and Taherzadeh [11].

The total solids, suspended solids, and their structural composition were determined according to the National Renewable Energy Laboratory (NREL) methods, including "preparation of samples for compositional analysis" [12], "determination of structural carbohydrates and lignin in biomass" [13], and "determination of total solids in biomass and total dissolved solids in liquid process samples" [14]. The dissolved saccharides in thin stillage were determined according to the NREL method including "determination of sugars, by-products, and degradation products in liquid fraction process samples" [15].

The liquid fractions from thin stillage and NREL protocol-resulting samples were analyzed using HPLC (Waters 2695, USA) according to Ferreira et al. [5]. In addition to xylose and arabinose, the lead (II)-based column was also used for analysis of glucose, galactose, and mannose. All samples for HPLC analysis

Table 1. Characteristics of the thin stillage used in batch and continuous culture of *N. intermedia*

Parameter	Batch[a)]	Continuous[b)]
pH	3.5 ± 0.0	4.3 ± 0.0
Kjeldahl nitrogen (g/L)	4.4 ± 0.2	5.3 ± 0.1
Total solids (g/L)	77.5 ± 3.4	90.1 ± 3.3
Suspended solids (g/L)	26.0 ± 0.9	30.5 ± 0.2
Ash (g/L)	10.3 ± 0.5	8.8 ± 0.4
Dissolved monomers		
Arabinose (g/L)	0.6 ± 0.1	1.0 ± 0.1
Galactose (g/L)	ND[c)]	0.7 ± 0.1
Glucose (g/L)	0.5 ± 0.1	0.9 ± 0.1
Xylose (g/L)	0.6 ± 0.2	0.7 ± 0.1
Glycerol (g/L)	7.6 ± 0.5	9.9 ± 0.1
Acetic acid (g/L)	0.9 ± 0.1	0.3 ± 0.1
Ethanol (g/L)	1.7 ± 0.2	0.4 ± 0.1
Lactic acid (g/L)	11.6 ± 0.9	2.8 ± 0.1
Dissolved saccharides[d)]		
Arabinose (g/L)	4.4 ± 0.2	6.2 ± 0.0
Galactose (g/L)	1.6 ± 0.2	1.7 ± 0.0
Glucose (g/L)	9.8 ± 0.7	16.4 ± 0.3
Mannose (g/L)	1.4 ± 0.2	1.7 ± 0.2
Xylose (g/L)	6.1 ± 0.4	6.8 ± 0.1
Solid fraction		
Arabinan (mg/L)	242 ± 140	374 ± 46
Galactan (mg/L)	175 ± 42	193 ± 32
Glucan (mg/L)	3182 ± 383	4553 ± 531
Mannan (mg/L)	488 ± 234	979 ± 83
Xylan (mg/L)	907 ± 419	920 ± 129

[a)] Thin stillage used for batch cultivation.
[b)] Thin stillage used for continuous cultivation.
[c)] Not detected.
[d)] Dissolved monomers included.

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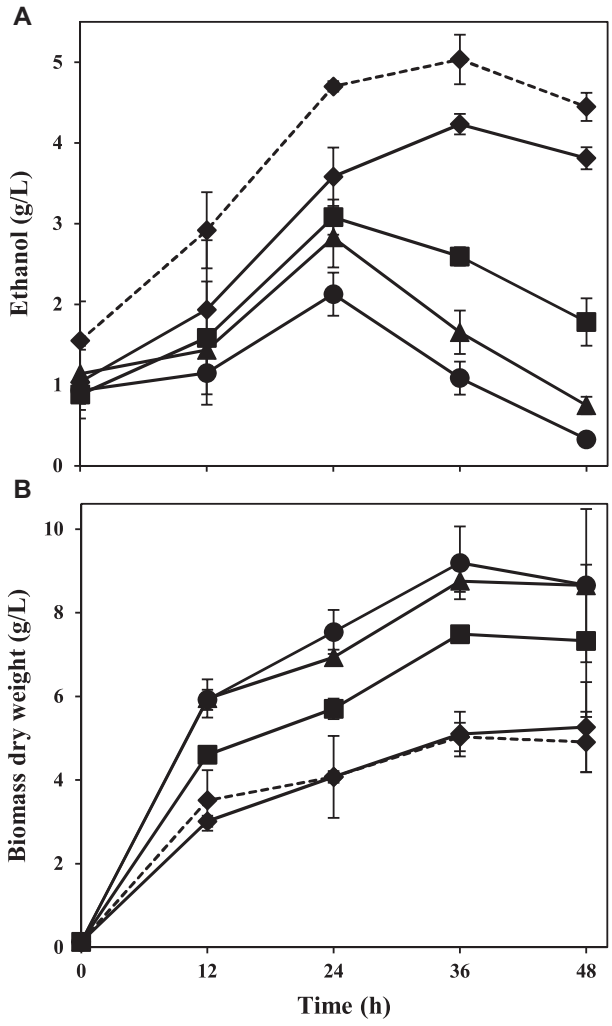

Figure 2. (A) Ethanol and (B) biomass dry weight profiles obtained during 48 h of cultivation of *N. intermedia* in airlift (straight lines) and bubble column (dashed line) at 0.5 (♦), 1 (■), 1.5 (▲), and 2 vvm (●). All conditions were tested in duplicate and all error bars represent two SDs.

were centrifuged for 10 min at 10 000 × *g*, and the supernatant was stored frozen at −20°C.

3 Results

3.1 Ethanol and biomass production from thin stillage

3.1.1 Aeration rate effect

The effect of the aeration rate on the production of ethanol and biomass from thin stillage was studied in a 2-m high airlift reactor (Fig. 2A and B). A clear trend was observed where higher the aeration rate applied, the lower ethanol and the higher biomass production achieved and vice-versa. The highest amount of produced ethanol of around 3 g/L was achieved at 0.5 vvm while the highest amount of produced biomass of around 9 g/L was achieved at 2 vvm. It was also observed that at aeration rates higher than 0.5 vvm, the ethanol concentration decreased after 24 h of cultivation. The biomass crude protein slightly decreased during cultivation at all tested aeration rates applied, where crude protein ranges of 48–53% w/w were found.

3.1.2 Reactor design effect

For comparative purposes, the airlift reactor was simplified into a bubble column by removing the internal-loop tube and the cultivation was performed at 0.5 vvm. As shown in Fig. 2A and B (dashed line) the ethanol and biomass production profiles between airlift and bubble column were similar. The highest ethanol production and production rate were 3.5 ± 0.2 g/L after 36 h and 131 ± 20 mg/L/h after 24 h of cultivation, respectively. The highest biomass production was 5.0 ± 0.3 g/L after 36 h of cultivation. Considering that using a bubble column reactor instead of an airlift represents cost savings, a more detailed study on the composition of the biomass harvested after 48 h of cultivation was carried out and compared to that of the DDGS (Table 2). All nine essential amino acids to humans were present in *N. intermedia* biomass and accounted to 40% of its amino acid composition. The most striking differences between DDGS and fungal biomass were the amounts of glutamic acid, lysine, and proline. The lipid content of the biomass was mainly composed of linoleic acid (47.6 ± 0.7%), palmitic acid (20.5 ± 1.5%), oleic acid (15.3 ± 0.4%), α-linoleic acid (4.6 ± 0.1%), and stearic acid (3.8 ± 0.2%; Table 2). The cell wall fraction as AIM of *N. intermedia* showed roughly a 60% increase during cultivation; it composed 18.9 ± 0.4% w/w and 30.3 ± 1.6% w/w of the biomass after 12 and 48 h of cultivation, respectively. A maximum of 1.5 ± 0.1 g/L of AIM was obtained at the end of the cultivation. The ash content of the biomass was 5.1 ± 0.1% w/w at the end of the cultivation.

3.1.3 Cultivation mode effect

Under continuous cultivation in the bubble column at 0.5 vvm, dilution rates up to 0.2 h^{-1} could be applied without cell washout (Fig. 4A). The production of ethanol decreased at higher dilution rates; the highest ethanol production of 5.1 ± 1.0 g/L was achieved at 0.1 h^{-1} (Fig. 3A). However, the ethanol production rate showed an inverse trend where the highest value of 778 ± 59 mg/L/h was achieved at a dilution rate of 0.2 h^{-1}, which was 27% higher than that at a dilution rate of 0.1 h^{-1} (Fig. 3B). The trend for biomass production was similar to that for ethanol where the highest value of 3.6 ± 0.9 g/L was achieved at a dilution rate of 0.1 h^{-1} (Fig. 4A). The highest biomass production rates were similar at 0.1 and 0.15 h^{-1} (363 ± 93 and 366 ± 61 mg/L/h, respectively) and decreased when the highest dilution rate was applied (Fig. 4B). The crude protein of the biomass after 66 h of cultivation (end of the first dilution rate) was analyzed and found to be 50.1 ± 3.8% g/g on a dry basis.

3.2 Thin stillage

The two commercial 1 m^3 thin stillage batches used during this study were composed of around 60% w/w total solids of potential carbon sources and of around 6% w/w total solids of nitrogen. The main differences between the two batches were the contents

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Table 2. Protein and lipid composition of industrial DDGS and *N. intermedia* biomass obtained from a 48-h cultivation in bubble column reactor at 0.5 vvm

Component (mg/g)	DDGS	*N. intermedia*	Component (mg/g)	DDGS	*N. intermedia*
Protein content and amino acid profile			Lipid content and fatty acid profile		
Crude protein	514 ± 13	529 ± 65	Lipid	77 ± 2	116 ± 11
Alanine	20.2 ± 0.5	32 ± 4	C 14:0	ND[a)]	< 1
Ammonia	17.2 ± 0.4	13 ± 3	C 15:0	ND	< 1
Arginine	23.1 ± 0.6	29 ± 8	C 16:0	ND	24 ± 5
Aspartic	28.6 ± 0.7	39 ± 9	C 16:1 *n*-7	ND	< 1
Cysteine	11.1 ± 0.3	6 ± 1	C 17:0	ND	< 1
Glutamic acid	143.2 ± 3.6	52 ± 10	C 17:1 *n*-7	ND	< 1
Glycine	19.3 ± 0.5	22 ± 6	C 18:0	ND	4 ± 0
Histidine[b)]	11.2 ± 0.3	12 ± 3	C 18:1 *n*-9	ND	18 ± 1
Isoleucine[b)]	21.8 ± 0.6	21 ± 7	C 18:2 *n*-6	ND	55 ± 4
Leucine[b)]	37.7 ± 1.0	32 ± 10	C 18:3 *n*-3	ND	5 ± 0
Lysine[b)]	15.7 ± 0.4	33 ± 9	C 20:0	ND	< 1
Metionine[b)]	8.4 ± 0.2	8 ± 3	C 20:1 *n*-9	ND	< 1
Ornitine	0.3 ± 0.0	6 ± 1	C 20:2 *n*-6	ND	< 1
Proline	46.0 ± 1.2	19 ± 3	C 22:0	ND	< 1
Phenylalaline[b)]	25.2 ± 0.6	18 ± 6	C 24:0	ND	< 1
Serine	26.2 ± 0.7	21 ± 5	C 24:1 *n*-9	ND	< 1
Threonine[b)]	16.8 ± 0.4	21 ± 5			
Tryptophan[b)]	5.7 ± 0.1	7 ± 2			
Tyrosine	18.1 ± 0.5	15 ± 4			
Valine[b)]	26.1 ± 0.7	27 ± 9			

[a)] Not determined.
[b)] Essential amino acids to humans.

of lactic acid, glucose, and arabinose in the liquid fraction and glucan and mannan in the solid fraction (Table 1).

During batch cultivation, either in the airlift at different aeration rates or in the bubble column, the acetic acid present in the thin stillage was consumed after 12 h of cultivation, glycerol and lactic acid concentrations remained constant and the total solids were reduced by 12–17% w/v (10–15 g/L). At the end of cultivation in the bubble column, the thin stillage pH was 6.2 ± 0.1 and its ash content had been reduced by 31.9 ± 13.1% w/v.

The analysis of the dissolved saccharides and sugar polymers in the solid fraction was carried out for bubble column batch and continuous cultivations and the findings are depicted in Fig. 5. During batch cultivation in the bubble column, the concentration of arabinose-, glucose- and xylose-based saccharides has been reduced by more than 70%. The total amount of carbon sources reduced in the liquid fraction was 16.0 ± 0.1 g/L (Fig. 5A). Under continuous cultivation the reduction of dissolved glucose- and xylose-based saccharides at the stable phase decreased gradually at higher dilution rates (Fig. 5B); at 0.1 h^{-1} their reduction was 14.7 ± 1.6 g/L while that of arabinose, galactose, and mannose was < 0.5 g/L. *Neurospora intermedia* could also assimilate sugar polymers where a reduction range of 57–91% (2.5 ± 0.2 g/L) was achieved during batch cultivation. Under continuous cultivation, the reduction of xylan in the suspended solids decreased gradually at higher dilutions rates whereas such a clear trend was not clearly observed for other sugar backbones present in the suspended solids (Fig. 5C and D). Total reduction amount was 2.4 ± 0.2 g/L at 0.1 h^{-1}. During continuous cultivation at a dilution rate 0.1 h^{-1}, the reduction of measured sugars both in liquid and solid fraction (18.1 ± 1.4 g/L) was comparable to that of total solids (16.9 ± 3.8 g/L). Thus, at this dilution rate, the yield of ethanol was 303 ± 9.5 mg/g of reduced solids.

4 Discussion

4.1 Ethanol and biomass production from thin stillage

4.1.1 Aeration rate effect

At the reactor scale, the supplied air plays a crucial role on the fungal cultivation performance since it influences the fungal growth, metabolite production, and assimilation of medium nutrients [9]. The supplied air was found to influence the production of ethanol and biomass in a 2-m high airlift reactor, where a clear trade-off between the production of ethanol and biomass when varying the aeration rate was found. Although the condition that achieved the highest ethanol production was chosen for further studies, economical evaluations are needed in order to ascertain which aeration rate would be more positive for the process. Moreover, it is worth noting that the dissolved oxygen was not monitored during this work. The dissolved oxygen would provide further insights into the oxygen uptake rate of the fungal culture and oxygen supply rate by the culture vessel, rendering its status of a key factor during process scale-up [16].

Also, due to better oxygen transfer rates achieved at reactor scale in comparison to that at bench-scale experiments with shake flasks, higher production of biomass was expected to be achieved. The maximum biomass value achieved during batch cultivation in the airlift was lower than that achieved in a previous work with shake flasks experiments with the same fungal

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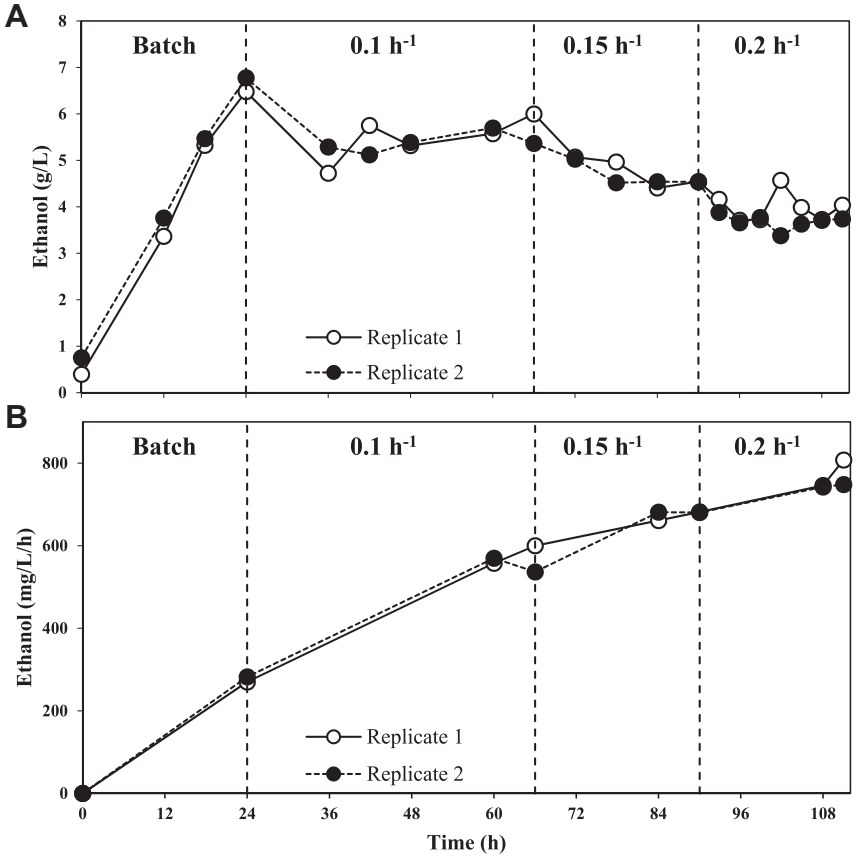

Figure 3. (A) Ethanol concentration and (B) ethanol production rate during continuous cultivation of *N. intermedia* in thin stillage at dilution rates of 0.1, 0.15, and 0.2 h^{-1}. The cultivation was shifted to continuous mode after 24 h of batch cultivation.

strain [5]. The observed differences might be related to differences in the thin stillage composition used or inefficient wash stage of the biomass, since *N. intermedia* grew as massive mycelial suspensions in shake flasks [5]. In the airlift, the ascomycete grew as small clumps of decreasing size for progressively higher aeration rate. Ferreira et al. [17] have observed the effect of better oxygen transfer rates when cultivating a *Rhizopus* sp. in spent sulfite liquor. They achieved clear improvement in biomass production when changing from shake flasks to the airlift reactor.

4.1.2 Reactor design effect

Due to their growth morphology, the cultivation of filamentous fungi has been widely explored using reactors with simpler design, such as airlifts and bubble columns alternatively to the traditional stirred-tank reactors. This study shows that a simpler reactor, such as a bubble column can be used for production of ethanol and biomass with the same performance as an airlift, which at a starting point represents cost savings. Airlift reactors have been developed as an alternative to bubble column reactors. The internal-loop tube of the airlift promotes a different mixing pattern that has been shown to lead to comparatively better mass and oxygen transfer rates [18]. However, such effect was not observed in this study. Perhaps, such effect would be seen if the process had been compared at higher aeration rates, using other reactor size and/or experimental setup.

4.1.3 Cultivation mode effect

This study also shows the possibility of setting a continuous cultivation, which means that a smaller size reactor can be used. Dilution rates up to 0.2 h^{-1} could be applied without cell washout. At progressively higher dilution rates namely from 0.1 to 0.15 and 0.2 h^{-1}, the ethanol production was reduced by around 1 g/L and the production rate increased by around 100 mg/L/h. As shown in Fig. 3, a threshold in production rate seems to be achieved when increasing the dilution rate from 0.1 to 0.15 h^{-1}. However, the production rate almost doubled when setting the cultivation in a continuous instead of batch mode. The implication of using this dilution rate of 0.1 h^{-1} is that a reactor of 2000 m^3, around four times smaller than that needed for a batch process of 36 h, would be sufficient considering a thin stillage flow of 200 m^3/h. Reasonably, developing a cell-retention system for the current process would potentially have positive effects on achieving higher dilution rates and thus, much smaller reactors and less investment would be needed. In contrast to the advantageous higher productivities and reduction of the reactor size, continuous processes have contamination as the major drawback. The risk of contamination is increased due to the additional flows to and from the reactor. However, the potential loss due to contamination is usually much smaller than the gain with the productivity [19]. In this work, during continuous cultivation of *N. intermedia* in thin stillage, growth of lactic acid bacteria was detected after 72 and 36 h of cultivation for the replicates 1 and 2, respectively. However, the bacterial growth did not

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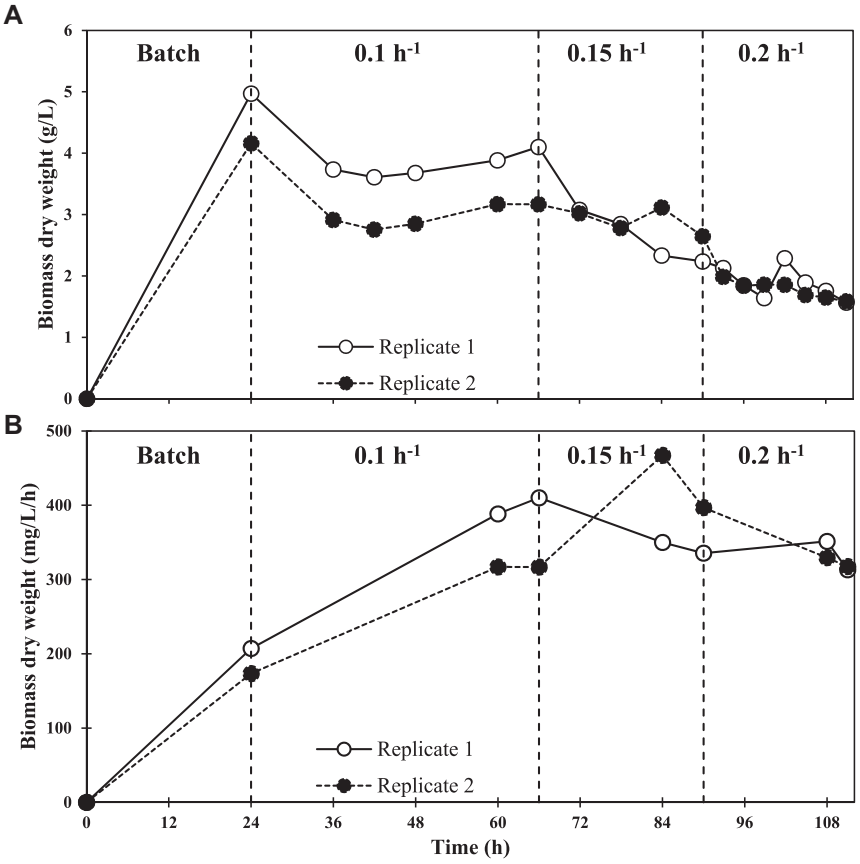

Figure 4. (A) Biomass dry weight and (B) biomass production rate during continuous cultivation of *N. intermedia* in thin stillage at 0.1, 0.15, and 0.2 h^{-1}. The cultivation was shifted to continuous mode after 24 h of batch cultivation.

affect the production of ethanol: As shown in Fig. 4A, there is a difference regarding biomass production (around 1 g/L) among replicates between 24 and 66 h of cultivation, which was not observed for the ethanol production (Fig. 3A). Moreover, lactic acid bacteria did not take over the cultivation; the lactic acid concentration in the medium, which increased from 3 to 5 g/L with bacterial contamination, remained constant at all dilution rates applied. The yield of ethanol obtained was 303 ± 9.5 mg/g of reduced solids at a dilution rate of 0.1 h^{-1}. To the best of our knowledge, the only work on production of ethanol from thin stillage was by Gonzalez et al. [20]. The authors have reported an ethanol yield of 0.42 g/g based on consumed glycerol, maltose, and glucose by *E. coli*.

4.2 Production of ethanol and biomass from thin stillage — implications

The treatment and use of thin stillage is crucial in order to achieve cost-effective and environmentally friendly ethanol production facilities. In dry mill processes, typically 6–7 L of thin stillage is produced per liter of ethanol [21]. Therefore, thin stillage is available in large quantities and can be used for production of other value-added products considering its relevant amount of nitrogen and carbon sources [22].

The inclusion of the current process in the established industrial ethanol plant could lead to the production of two value-added products, namely ethanol and biomass for feed. Taking into account the present industrial setup, it is hypothesized that such process inclusion would not trigger high capital investments. For instance, all needed apparatus for the distillation of the ethanol produced by *N. intermedia* is already available at the industrial plant. The alcohol would follow the normal stream of condensate after the series of evaporations back to the normal process and then to the distillation column as it is done nowadays at the industrial scale (Fig. 1).

The produced biomass could be dried together with the solids from the stillage centrifugation and the syrup from the series of evaporations (Fig. 1). The use of filamentous fungi is also attractive, since the separation of the biomass from the medium is easier when compared with that for yeast or bacterial cells since a centrifugation step is not needed. In view of its protein, amino acid, lipid, and fatty acid composition as well as its comparable profile of amino acids with that of DDGS, *N. intermedia* biomass is a potential source of high-quality nutrients for feed application. The ascomycete lipid fraction was to a large extent composed of polyunsaturated fatty acids (52.3 ± 0.8%) of which 47.8 ± 0.7% w/w were omega-6 fatty acids and 4.6 ± 0.1% w/w were omega-3 fatty acids. Those polyunsaturated fatty acids are considered essential to human diet [23]. Moreover, the cell wall of *Ascomycetes* has chitin as main component and although the presence of native chitosan has not been reported for these fungi, the extraction of chitosan has previously been carried out [24, 25]. Chitosan applications have steadily been

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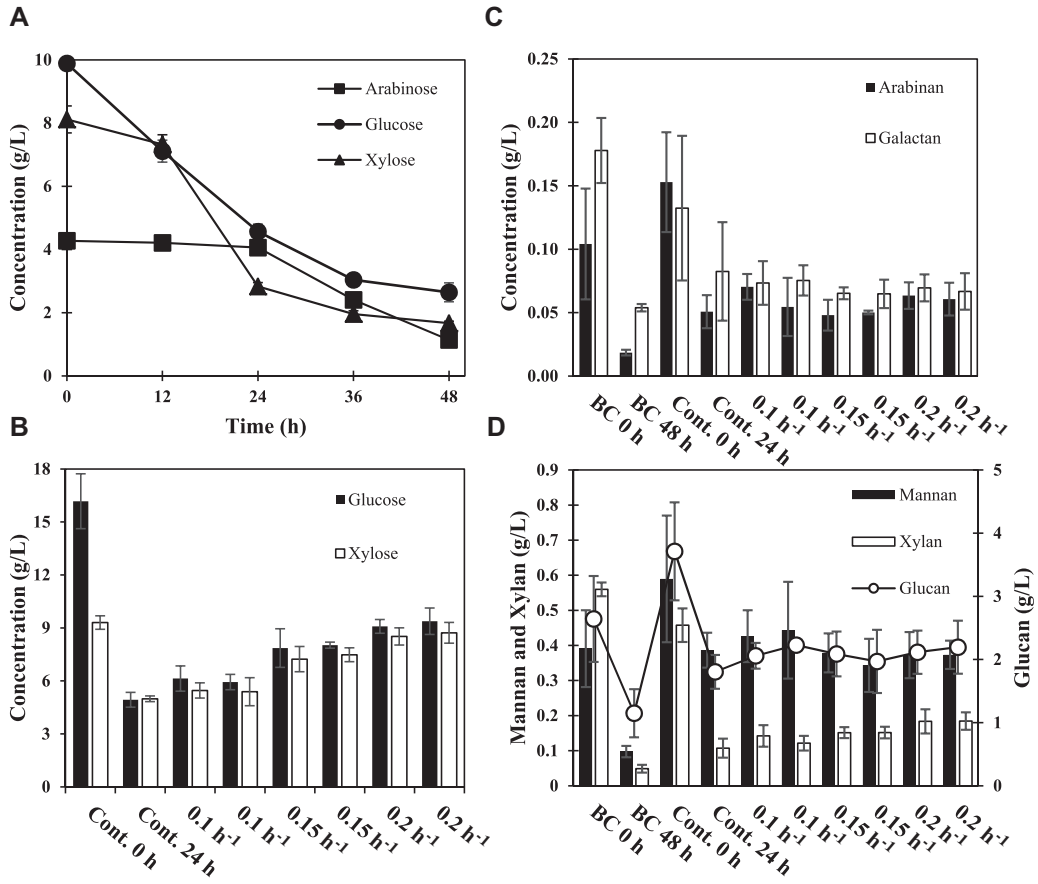

Figure 5. Profiles of (A and B) dissolved saccharides in the liquid fraction and (C and D) sugar polymers in the solid fraction of thin stillage during batch and continuous cultivation of *N. intermedia* in the bubble column. Error bars represent two SDs. "BC" stands for the batch process in the bubble column, "Cont." stands for the continuous cultivation, and "Cont. 0 h" and "Cont. 24 h" represent the 24 h of batch cultivation preceding the continuous cultivation. The graph (A) includes the sugar profiles during batch cultivation in the bubble column.

increasing, e.g. in the health sector [26]. Therefore, the extra biomass produced via inclusion of the present process would increase and diversify the amount of animal feed nutrients of the ethanol industry.

In addition to the production of ethanol and fungal biomass, the inclusion of *N. intermedia* in the industrial ethanol process can have a positive impact on thin stillage evaporation and drying steps via assimilation of organic matter. Continuous cultivation of *N. intermedia* at a dilution rate of 0.1 h^{-1} led to the reduction of 18% w/v of thin stillage solids including dissolved saccharides and sugar polymers in the solid fraction. Other potential carbon sources, such as glycerol and lactic acid remained constant at all conditions tested in this work. Clearly, *N. intermedia* preferred carbon sources than glycerol or lactic acid. In semisynthetic medium, the ascomycete had consumed all glycerol after 42 h of cultivation when it was the single carbon source in the medium. However, when the fungus was cultivated in a mixture of carbon sources, glycerol started to be consumed just when all hexoses and pentose sugars were depleted. *Neurospora intermedia* hardly consumed lactic acid though; only 1 g/L of lactic acid had been consumed after 42 h of cultivation. Beyond being converted to mainly ethanol, CO_2, and biomass, the reduction of solids can have a positive impact on the energy savings of the overall production process. Removing water during the series of evaporations would be easier and so the amount of water to be removed when in the driers would be lower. Furthermore, more thin stillage could be sent back to the process lowering the load on the evaporators and driers [6].

5 Concluding remarks

In this work, up to 5 g/L of ethanol and 4 g/L of biomass were obtained during *N. intermedia* growth in thin stillage under continuous mode using a 2-m high bubble column of 26 L capacity. Similar results regarding maximum ethanol and biomass production were obtained when the same reactor was used as airlift or bubble column in a batch mode. Moreover, the production of ethanol and biomass was found to be intimately related to the assimilation of both dissolved saccharides and sugar polymers present in the thin stillage suspended solids. The inclusion of the present process under a continuous mode at 0.1 h^{-1} in a bubble column can potentially lead to the production of around 11 000 m^3 of ethanol (5.5% improvement vs. the normal process) as well as 6300 tons of biomass that can be used for animal

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or fish feed at a facility producing 200 000 m^3 of ethanol per year.

Practical application

The present research can potentially have a positive impact on the overall process economics of the established industrial process of ethanol production from grains. Thin stillage, a wastewater obtained after distillation and solid separation, can be used for production of ethanol and biomass for feed using the edible filamentous fungus *Neurospora intermedia*. Less investment is needed since the produced ethanol can follow the normal stream towards the distillation column after the series of evaporations, which is already being done in the industry. The produced biomass can be dried in the same way as the animal feed products produced by the dry mill ethanol facilities, commonly known as DDGS (distillers dried grains with solubles). Considering the data obtained in this study, an improvement of 5.5% on ethanol production as well as the production of around 6300 tons of biomass could potentially be achieved at a facility producing 200 000 m^3 of ethanol per year.

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The authors have declared no conflict of interest.

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PAPER V

Ethanol and protein from ethanol plant by-products using edible fungi *Neurospora intermedia* and *Aspergillus oryzae*

Veronika Bátori [a,b], Jorge A. Ferreira [a*], Mohammad J. Taherzadeh [a], Patrik R. Lennartsson [a]

[a] Swedish Centre for Resource Recovery, University of Borås, SE 501 90, Borås, Sweden

[b] The University of West-Hungary, Faculty of Forestry, H-9400, Sopron, Hungary

***Corresponding author at:** Swedish Centre for Resource Recovery, University of Borås, SE 501 90, Borås, Sweden

Tel: +46-33-4354638; **fax:** +46-33-4354003

Email address: Jorge.Ferreira@hb.se

Abstract

Feasible biorefineries for production of second-generation ethanol are difficult to establish due to the process complexity. An alternative is to partially include the process in the first-generation plants. Whole stillage, a by-product from dry-mill ethanol processes from grains, is mostly composed of undegraded bran and lignocelluloses can be used as a potential substrate for production of ethanol and feed proteins. Ethanol production and the proteins from the stillage were investigated using the edible fungi *Neurospora intermedia* and *Aspergillus oryzae*, respectively. *N. intermedia* produced 4.7 g/L ethanol from the stillage and increased to 8.7 g/L by adding 1 FPU of cellulase/g suspended solids. *Saccharomyces cerevisiae* produced 0.4 and 5.1 g/L ethanol, respectively. Under a two-stage cultivation with both fungi, up to 7.6 g/L of ethanol and 5.8 g/L of biomass containing 42% (w/w) crude protein were obtained. Both fungi degraded complex substrates including arabinan, glucan, mannan and xylan where reductions of 91, 73, 38 and 89 % (w/v) were achieved, respectively. The inclusion of the current process can lead to the production of 44,000 m^3 of ethanol (22 % improvement), around 12,000 tons of protein-rich biomass for animal feed, and energy savings considering a typical facility producing 200,000 m^3 ethanol/year.

1. Introduction

Currently bioethanol is the dominant biofuel in the transport sector. Corn and sugarcane are the most widely used feedstocks in the bioethanol industry, following other materials such as wheat and crop roots [1]. In addition to ethanol, dry-mill starch-based processes also produce DDGS (Distillers Dried Grains with Solubles), a source of protein for animal feed from the fermentation residues. After the fermentation and distillation of ethanol, the slurry leaving the bottom of the column (whole stillage) contains the oil, protein, fibre and other unfermented components of the grains and yeast cells [2]. Up to 20 litres of whole stillage is produced per litre of ethanol from corn or wheat grains [3], which means a global production of more than a billion tons per year. Whole stillage undergoes a centrifugation step to produce a liquid fraction (thin stillage), 15% or more of which is used as backset water and a solid fraction (wet distiller's grains). The syrup resulting from the series of evaporations of the remaining thin stillage is dried with the wet distiller's grains to produce DDGS (**Figure 1**) [2]. In 2011, the ethanol sector produced ca 86 million metric tons of ethanol and 68 million tons of DDGS [4, 5]. Reasonably, the revenues from the DDGS play an important role for the overall process economics [1] due to its large volume, which make up for the relatively low value which also presents an opportunity, since relatively small improvements can have a large impact. The large economic importance of the DDGS also puts a limit on the potential solutions, since the feed quality of DDGS cannot be compromised. In practice, this means that any retrofitting must fulfil feed quality standards, which also includes any introduced microorganism [6]. A potential solution is to use edible filamentous fungi to produce ethanol and protein-rich fungal biomass.

Filamentous fungi are known to be able to produce a wide array of enzymes enabling them to degrade complex substrates. This is one of the reasons that some filamentous fungi such as

Neurospora intermedia and *Aspergillus oryz*ae are used for production of fermented food such as oncom [7] and enzymes for feed, beverage, textile, and paper and pulp industries, respectively [8]. Many fungi also have the ability to utilize sugars such as xylose that baker's yeast (*Saccharomyces cerevisiae*) cannot assimilate. These advantages have sparked a research interest in developing new processes based on filamentous fungi. Related examples include fungal biomass production from spent sulphite liquor [9] and corn-based thin stillage [10] with *Rhizopus* sp., and ethanol and fungal biomass production from wheat-based thin stillage with *N. intermedia* [11]. *N. intermedia* was found to produce the highest amount of ethanol (5 g/L) in comparison to other ascomycetes such as *A. oryzae*, *Fusarium venenatum*, *Monascus purpureus* and the zygomycete *Rhizopus* sp. and its biomass was 56 % (w/w) crude protein. Implemented in a bioethanol process both production of ethanol and fungal biomass from the residues have the advantage of being relatively easy to separate through filtration and evaporation. The latter is already in use in the drying process for the DDGS, with the condensate being sent back to the beginning of the ethanol production process. The ethanol produced by *N. intermedia* would therefore only influence the overall industrial process by increasing the final ethanol concentration in the mash and would be separated in the normal distillation step.

Whole stillage has a theoretically higher potential for ethanol production than thin stillage as a higher solid content is available. This solid content consists mainly of undegraded bran if whole-meal wheat is used as feedstock, and is thus lignocellulosic material. Ethanol production from whole stillage could therefore not only result in even more ethanol being produced from current resources, but also serve as an important step towards second generation ethanol. Information regarding production of ethanol from whole stillage is currently lacking in the scientific literature. Being of lignocellulosic origin, addition of

enzymes might enhance the production of ethanol via degradation or increase of the degradation rate of complex substrates that *N. intermedia* might naturally assimilate. The commercial available cellulase has widely been used for conversion of cellulose to fermentable sugars [12-14]. However, the high solid content of the whole stillage hampers a clear separation of the produced fungal biomass if a purified protein-rich fungal product for feed applications is desired. A two-stage cultivation including ethanol production followed by biomass production after separation of the suspending fibres might represent an alternative.

In this study, the potential of whole stillage as a substrate for production of ethanol was investigated. Special focus was on the influence of enzyme loading and different whole stillage fractions on ethanol production. A comparison between production of ethanol from whole stillage between *N. intermedia* and baker's yeast *S. cerevisiae* was also carried out in order to investigate the need to introduce a new microorganism in the established industrial process. Furthermore, a novel strategy based on a two-stage cultivation was also investigated including ethanol production by *N. intermedia* under simultaneous saccharification and fermentation followed by production of protein-rich biomass by *Aspergillus oryzae* after the medium had been distilled and sieved.

2. Materials and Methods

2.1. Substrate

The whole stillage, originating from a dry-mill bioethanol production process based mainly on wholemeal wheat, was kindly provided by Lantmännen Agroetanol (Norrköping, Sweden). The whole stillage, originated from a single batch, was autoclaved for 30 minutes at 121 °C and stored at 4 °C until use.

2.2. Microorganisms

The ascomycetes *Neurospora intermedia* CBS 131.92 and *Aspergillus oryzae* var. *oryzae* CBS 819.72 (Centraalbureau voor Schimmelcultures, the Netherlands) were used and maintained on potato dextrose agar (PDA) medium containing 20 g/L glucose, 15 g/L agar and 4 g/L potato extract. New plates were prepared via incubation at 30 °C for 3-4 days and stored at 4 °C. Spore solutions for inoculation were prepared by flooding each plate with 20 ml sterile distilled water and disposable plastic spreaders were used to release the spores into the liquid. Spore concentration was determined by using a counting chamber. Ethanol Red® (Fermentis, France), a specially selected strain of *Saccharomyces cerevisiae* for industrial ethanol production was also used and maintained on yeast peptone dextrose agar (YPDA) medium, containing 20 g/L glucose, 20 g/L agar, 20 g/L peptone and 10 g/L yeast extract. New plates were prepared via incubation at 30 °C for 2 days and stored at 4 °C. For inoculation one yeast colony was added to the medium.

2.3. Cultivations in shake-flasks

All cultivations were, unless otherwise noted, carried out in cotton plugged 250 mL wide-necked Erlenmeyer flasks containing 150 mL of medium autoclaved at 121 °C for 20 min. A water bath was used to maintain the temperature at 35 °C and continuous shaking at 125 rpm. Each flask was inoculated with 3 mL of *N. intermedia* spore solution, resulting in 1.14 (± 0.53) × 10^5 spores/mL. Stillage was adjusted to pH 5 prior to cultivations with 10 M NaOH and 6 M HCl. Samples were taken from the fermentation broth at predetermined times and centrifuged at 10,000×g for 10 minutes. The supernatant was kept at -20 °C until analysis. Unless otherwise stated, all cultivations were done in duplicate.

2.3.1. Whole stillage

Undiluted whole stillage was treated with 0, 1, 5 and 10 FPU Cellic® Ctec2/g SS (Novozymes, Denmark). Enzyme was added along with spore solution and cultivation was carried out for 120 h. The enzyme activity was 94 FPU/mL.

2.3.2. Fractionation

Whole stillage was divided into 3 fractions, large particles by sieving, small particles by centrifugation using a semi-continuous centrifuge (CEPA, Germany) at 29,000×g and a flow of 5 L/h, and the remaining liquid phase. The solid particles were washed with distilled water and were finally resuspended in distilled water to achieve the same concentration as in the whole stillage. Cultivations in triplicate were carried out with *N. intermedia* for 120 h, both with and without addition of 1 FPU Cellic® Ctec2/g SS.

2.3.3. Comparison with S. cerevisiae

Cultivation of whole stillage was also carried out using *S. cerevisiae*, both as mono-cultures and co-cultures with *N. intermedia*. Flasks that were inoculated with mono-cultures were done with and without enzyme treatment. Enzyme load was 1 FPU/g SS. Cultivations were carried out for 120 h.

2.3.4. Carbohydrate assimilation

Carbohydrate assimilation was examined using semi-synthetic medium containing 7.5 g/L $(NH_4)_2SO_4$, 3.5 g/L KH_2PO_4, 1.0 g/L $CaCl_2 \cdot 2H_2O$, 0.75 g/L $MgSO_4 \cdot 7H_2O$, 10 mL/L trace metal solution, 5 g/L yeast extract, and different carbon sources. Carbon sources were: cellobiose, starch, cellulose (Avicel) and xylan (30 g/L respectively). Cultivation time was 144 h for cellobiose and starch and 166 h for Avicel and xylan.

2.4. Cultivation in a Bioreactor

Whole stillage was cultivated in a 2.5 L continuous-stirred tank reactor (CSTR) (Biostat A, B. Braun Biotech International, Germany). The cultivation was carried out in two 72-h stages. In both cases temperature was controlled and kept at 35 °C, stirring at 250 rpm, air flow rate at 25 L/h and pH at 5.0 with addition of 2 M NaOH or 2 M H_2SO_4. The 24 h inoculums were prepared in 250 mL wide-necked Erlenmeyer flasks containing 100 mL of yeast peptone dextrose medium (YPD) composed of 20 g/L glucose, 5 g/L peptone and 5 g/L yeast extract. Incubation was carried out as described before. In the first stage of cultivation, 1 FPU cellulase/g SS and the inoculum of *N. intermedia* (dry weight: 1.82 ± 0.31 g/L) were added to 2.01 (± 0.01) kg of whole stillage. After 72 h cultivation, ethanol in the broth was separated from the remaining liquid using a rotary evaporator (Laborata 20 eco, Heidolph, Germany) at 110 °C, 20 rpm and 400 mbar. The evaporated water was compensated for by addition of distilled water and the medium was sieved. In the second stage of the experiment, 1 L of the obtained liquid was inoculated with previously grown *A. oryzae* biomass (dry weight: 2.70 ± 0.25 g/L). All cultivations were carried out in duplicate.

2.5. Analytical methods

Stillage suspended solid content was determined by washing and vacuum filtration with Büchner funnel and Whatman (Cat No 1001-070) filter paper, followed by drying at 70 °C for 24 hours. Total solid and dry content was determined by drying the material in oven at 70 °C for 24 h, achieving constant weight. Harvested biomass was dried following the same protocol.

Crude protein content was determined according to the Kjeldahl method applying digestion, distillation and acid-base titration using an InKjel P digestor and a behrotest® S1 distiller (behr Labor-Technik, Germany). Digestion was carried out by adding 20 mL of 98 % (v/v) H_2SO_4, antifoam and KT1 tablets (Thompson & Capper ltd, United Kingdom) to 0.51 ± 0.04

g material for a total duration of 100 minutes at 100 % power (of which 10 min for heating up the system). Digestion was followed by neutralization of the digested solution with 32 % (w/w) NaOH and distillation for 5 min. The distillation vapour was trapped in 50 mL of 4 % H_3BO_4. Final titration was carried out with 0.100 M of HCl until pH 4.6. A factor of 6.25 was used to calculate the crude protein content.

Spore concentration was determined using a Bürker's counting chamber. The spores were counted in 144 E-squares (1/250 µL) and a final concentration of solution was calculated.

For identifying and quantifying different components of the broth samples high performance liquid chromatography (HPLC) (Waters 2695, Waters, Milford, USA) analysis was used. Acetic acid, ethanol, glucose, glycerol, lactic acid and xylitol were analysed using an analytical ion exchange column based on hydrogen ions (Aminex HPX-87H, Bio-Rad, USA) operated at 60 °C with 0.6 ml/min of 5 mM H_2SO_4 as eluent. Arabinose, galactose, glucose, mannose, and xylose were analysed using a lead (II)-based column (HPX-87P, Bio-Rad) with two Micro-Guard Deashing (Bio-Rad) pre-columns operated at 85 °C with 0.6 mL/min ultrapure water as eluent.

Structural carbohydrates of the solid biomass samples were prepared for determination according to NREL/TP-510-42618 [15]. Total amount of dissolved carbohydrates was determined according to NREL/TP-510-42623 [16]. The cellulase Cellic® Ctec2 activity was measured according to NREL/TP-510-42628 [17] using a Biochrom Libra spectrophotometer (Biochrom, United Kingdom).

All analyses were carried out in duplicate and reported intervals and error bars are ±2 standard deviations, unless otherwise noted.

2.6. Statistical analysis

Statistical analysis of the data obtained from the enzyme loading experiments was performed using the software package MINITAB® 17. Results were analysed with ANOVA (analysis of variance) using general linear models with a 95% confidence interval.

3. Results and Discussion

The economical robustness of established biorefineries can greatly rely on its intrinsic capacity to further improve the process. The strategy normally involves the valorisation of side-streams via production of value-added chemicals. The process of a typical ethanol plant (200,000 m^3 of ethanol/year) from corn or wheat grains can give rise to up to 4 million m^3 of whole stillage [3]. Moreover, the additional steps (**Figure 1**) in order to produce the DDGS including centrifugation of the whole stillage, evaporation of the thin stillage and drying are responsible for a large fraction of the overall process energy expenses [18]. Thin stillage, originating from a whole-wheat ethanol process, has been successfully investigated for further ethanol production [19] using *N. intermedia*. Further studies have shown that dissolved saccharides and sugar polymers (e.g. xylan) present in the suspended solids are among the carbon sources that *N. intermedia* can assimilate and convert to ethanol (unpublished data). Considering that the whole stillage has not gone through a first centrifugation step yet, its suspended solid content is higher and so it is its potential for production of ethanol. Moreover, the changes and low investment needed for the inclusion of production of ethanol by *N. intermedia* from thin stillage would be similar to those needed if the cultivation medium is whole stillage (**Figure 1**).

From the parameters evaluated and presented in **Table 1**, the stillage used in this work was composed of around 60 g/L of potential carbon sources in addition to its relevant amount of crude protein. Glucose- and xylose-based dissolved saccharides and the polymers glucan and xylan, all potential substrates for ethanol production, made up to around 38 % and 14 % of the measured carbon sources, respectively. The use of enzymes in order to increase the ethanol production from lignocellulosic substrates has been widely investigated and demonstrated in the literature [20]. Therefore, research on the effect of cellulase complex Cellic® Ctec2 addition on ethanol production from whole stillage using *N. intermedia* was carried out in this work. A comparison performance between the widely used baker's yeast *S. cerevisiae* and *N. intermedia* towards ethanol production was also carried out. Research on production of a second value-added product namely fungal biomass for feed applications was further studied by applying a two-stage cultivation strategy.

3.1. Effect of cellulase loading on ethanol production

Undiluted stillage was treated with different loadings of cellulase and cultivations were carried out in simultaneous saccharification and fermentation mode. As depicted in **Figure 2**, the addition of enzyme led to clear improvements in the maximum ethanol production in comparison to that when no enzyme was added (p-value = 0.000). The highest amount of ethanol (11.6 ± 0.8 g/L) and the highest production rate (232 ± 6 mg/L/h) were achieved when the highest enzyme loading was used. However, the most striking differences were observed between the absence of enzyme and its use at 1 FPU/g SS where an increase of 85 % and 98 % was achieved in the ethanol production and production rate, respectively. Moreover, remarkable differences were observed when cellulase was added to the medium regarding the release of sugars (glucose and other sugars), production of xylitol and carbon source consumption patterns during cultivation. Within the sampling time used in this work up to 14 g/L of glucose were detected when the highest concentration of enzyme was used, up to 160

% increase in xylitol production and up to 324 % increase in the release of other sugars when enzyme was used, total consumption of acetic acid and no significant changes in the glycerol concentration at all conditions examined. The consumption of xylitol and of the other sugars showed a decreasing and increasing trend, respectively, at gradually higher enzyme loadings. The xylitol production indicates that *N. intermedia* had consumed xylose and that its conversion occurs probably via the general fungal pathway [21]. Moreover, the higher consumption of other sugars when enzyme is added might be related to the higher amount of free glucose in the medium. Davis, et al. [3] have noticed a higher consumption of xylose when the stillage medium was supplemented with glucose.

The amount of enzyme added during hydrolysis of lignocellulose-based or derived substrates contributes to a large fraction of the process costs and therefore its amount must be minimised. In this work, the effect of the addition of enzyme diminished at gradually higher concentrations since 0.35 ± 0.02, 0.13 ± 0.00 and 0.08 ± 0.00 g ethanol/L/FPU were obtained when 1, 5 and 10 FPU were used, respectively. Therefore, considering the amount of ethanol produced when progressively higher concentrations of cellulase were used, 1 FPU of enzyme/g SS was chosen and used in further studies.

3.2. Whole stillage fractions contribution to ethanol production

Whole stillage is a complex medium in which both solid and liquid fractions contain carbon sources which *N. intermedia* can use to produce ethanol. Therefore, unveiling which fraction contributes the most to the production of ethanol gives an important input towards process understanding of ethanol production from this side stream. Whole stillage was divided into three fractions namely sieved solids named “large particles”, solids after centrifugation named “small particles” and the supernatant. The production of ethanol was studied with addition or

absence of 1 FPU cellulase/g SS and the findings are presented in the **Figure 3**. Evidently, the supernatant gave the highest contribution (75 %) to the ethanol production, while the ethanol produced from the “large particles” and “small particles” accounted to 20 and 5 % of the total amount when no enzyme was added to the medium, respectively. Addition of cellulase led to similar maximum ethanol production, but at a higher production rate (211 ± 6 vs 125 ± 1 mg/L/h) when the supernatant was the cultivation medium. Moreover, no further improvement in the ethanol production from “large particles” was observed, while three times more ethanol was produced when “small particles” were used. An analysis of dissolved saccharides was performed for all different fractions after cultivation with and without enzyme (**Figure 4**). The most striking differences were the reduction of glucose-based saccharides with a similar reduction of 82 – 87 %, while the reduction of xylose-based saccharides reached 51 % when the supernatant was used with or without cellulase. Moreover, higher final concentrations of arabinose- and xylose-based saccharides were obtained when “large particles” were used when compared with those from cultivation with “small particles”. The addition of yeast extract (5 g/L) to the medium containing “large particles” did not lead to further improvements on ethanol production (data not shown).

Interestingly, using just the supernatant as cultivation medium, a higher maximum ethanol production was obtained than that when whole stillage was used as cultivation medium (6.0 vs 4.7 g/L). The reason for the observed behaviour is unknown and so further studies are needed. Moreover, the similar maximum ethanol production when the supernatant was used both with and without addition of cellulase points out the relevant enzymatic capabilities of *N. intermedia*.

3.3. Comparison with yeast

The research with filamentous fungi has been greatly stimulated by their metabolic diversity, easier separation from the medium and ability to consume pentose sugars namely xylose and arabinose when compared to the baker's yeast [22]. In this work, a performance comparison towards ethanol production between *N. intermedia* and *S. cerevisiae* was carried out and the results are presented in the **Figure 5**. When *N. intermedia* was used as the fermenting agent, higher ethanol production was achieved both at absence of cellulase and when 1 FPU of the enzyme/g SS was used. A more clear difference was observed when no enzyme was added to the medium; *N. intermedia* produced 10.5 times more ethanol that *S. cerevisiae*. Such difference should be related to the higher enzymatic capability of *N. intermedia* to consume and convert glucose- and xylose-based saccharides present in the whole stillage liquid fraction to ethanol. The higher production of ethanol when cellulase was added to the medium might be related to the filamentous fungus ability to convert xylose to ethanol contrary to *S. cerevisiae*. The co-culture of both microorganisms also led to a lower amount of ethanol being produced. Therefore, the performance of *N. intermedia* towards ethanol production from whole stillage was clearly superior to that of *S. cerevisiae* pointing out the beneficial potential of the inclusion of *N. intermedia* in the established industrial process of ethanol production.

3.4. Two-stage cultivation for production of ethanol and fungal biomass

The production of ethanol with addition of 1 FPU cellulase/g SS was also studied using a 2.5 L bench-scale continuous stirred-tank reactor. However, an innovative two-stage cultivation process in order to produce fungal biomass in addition to ethanol was investigated. During the first stage of the process, the maximum ethanol production of 6.9 ± 0.1 g/L was achieved after 36 h and the maximum production rate of 235 ± 13 mg/L/h was reached after 24 h of cultivation with *N. intermedia* (**Figure 6**). Therefore, the value of ethanol produced in the

bioreactor was somewhat lower (6.9 versus 8.7 g/L) than that when cultivations were performed in shake-flasks which indicates that aeration and mixing optimization is needed. During the three-day cultivation, the amount of xylitol increased 233 %, the amounts of glycerol and lactic acid did not change significantly and acetic acid had been totally consumed after 24 h. The main monomeric sugars, arabinose, glucose and xylose, exhibited different concentration patterns during the first stage of cultivation: the amount of arabinose increase continuously during cultivation; the maximum amount of glucose (7.7 g/L) was measured after 12 h and was depleted after 36 h of cultivation; and xylose increased by 386 % after 12 h and 49 % of it had been consumed at the end of the first cultivation stage.

The use of the bulk medium whole stillage does not allow fungal biomass to be separated from other medium components. Therefore, the second stage of the process was preceded by a harvesting stage in addition to the evaporation of the medium ethanol and *Aspergillus oryzae* was used for production of biomass. After 72-h of cultivation, 5.8 ± 0.8 g/L of biomass containing 42.3 ± 1.7 % crude protein on a dry weight basis was obtained. Besides, up to 0.7 g/L of ethanol were produced and total consumption of arabinose and xylitol as well as 63 % of the xylose was achieved by the end of the cultivation. The lactic acid and glycerol concentrations were reduced by 13 and 9 %, respectively. *A. oryzae* was chosen for this second step of the process based on its outstanding capacity to consume glycerol from thin stillage [11]. The low reduction of glycerol might be related to C/N ratio or other more preferable carbon sources for the ascomycete fungus. An analysis of dissolved saccharides and sugar polymers in the liquid and solid fraction, respectively, was also conducted in this part of the study and the main changes are represented in **Figure 7**. After the two stage cultivation, the arabinose-, glucose- and xylose-based saccharides had been reduced by 86, 51 and 40 % (w/v), respectively, while the sugars polymers arabinan, glucan, mannan and xylan

present in the suspended solids had been reduced by 91, 73, 38 and 89 % (w/v), respectively. The fibres recovered by sieving after the first stage of cultivation had their glucan, mannan and xylan content reduced by 21, 72 and 9 % (w/v), respectively. The spent stillage after the second stage cultivation contained 8.3 ± 0.4 and 0.9 ± 0.1 % (w/v) total and suspended solids, respectively. Cultivation of *N. intermedia* in semi-synthetic medium containing single carbon sources corroborated the assimilation of more complex substrates observed in whole stillage. The ascomycete fungus was able to produce ethanol when the sugar polymers Avicel, starch and xylan were used as cultivation substrate. Maximum ethanol productions of 0.4 ± 0.1 after 144 h and 3.0 ± 0.2 g/L after 48 h were obtained during cultivation in xylan and starch, respectively. Ethanol production from Avicel was slower and after 166 h of cultivation 1.2 ± 0.2 g/L had been produced. *N. intermedia* was also able to consume fully cellobiose after 60 h where the maximum ethanol yield of 0.15 g/g was reached.

Altogether, after a two-stage cultivation, 7.6 g/L of ethanol and 5.8 g/L of biomass containing around 42 % (w/w) crude protein were produced. Moreover, throughout the present study both filamentous fungi showed their self-ability to degrade more complex substrates enzymatically unaided. In a process inclusion context, the produced ethanol could simply be sent back to the process together with the condensate, while the degradation and assimilation of carbon sources of the whole stillage can have an important positive impact on evaporation and drying costs of the industrial process.

4. Conclusions

The valorisation of whole stillage towards ethanol production was improved by addition of cellulase; more 4 g/L of the alcohol were obtained with addition of 1 FPU enzyme/g SS. By applying an innovative two-stage cultivation with *N. intermedia* and *A. oryzae*, 7.6 g/L of

ethanol and 5.8 g/L of biomass containing around 42 % (w/w) crude protein were obtained. Both filamentous fungi were able to degrade complex substrates in the medium such as arabinan, xylan and glucan which together with those carbon sources assimilated in the liquid fraction will potentially have a positive impact on evaporation and drying costs of the industrial process. *N. intermedia* was also shown to be superior to *S. cerevisiae* regarding ethanol production from whole stillage both with and without addition of cellulase to the medium.

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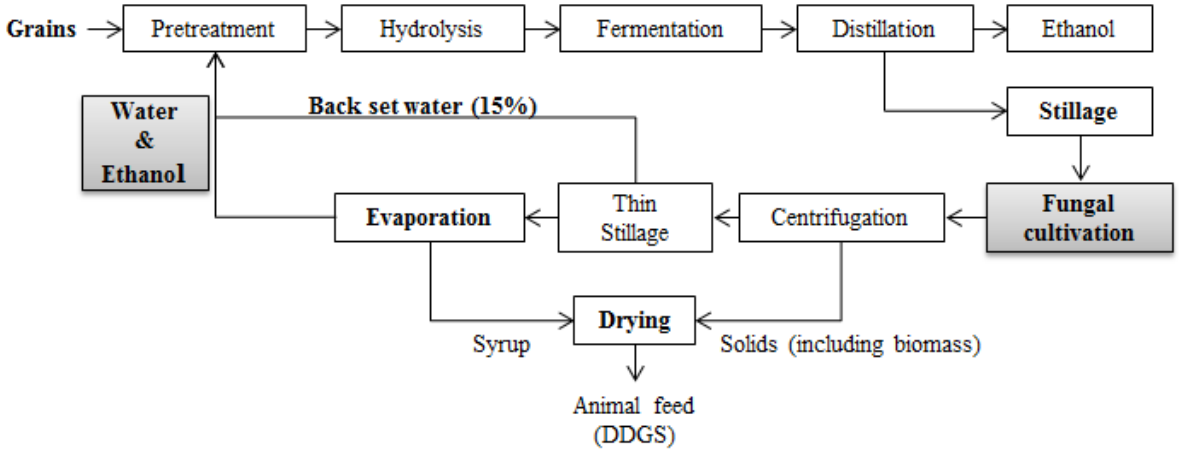
467

468 **Table 1.** Characteristics of the mostly wheat-based stillage used in the study.
469

		Dissolved monomers (g/L)		Dissolved saccharides (g/L)[c]		Sugar polymers (g/L)[d]	
Parameter	**Value**	**Parameter**	**Value**	**Parameter**	**Value**	**Parameter**	**Value**
pH	4.3 ± 0.0	Acetic acid	0.4 ± 0.1	Arabinose	6.3 ± 0.1	Arabinan	1.8 ± 0.1
Total Solids (% w/w)	15.6 ± 0.1	Arabinose	1.6 ± 0.1	Galactose	1.7 ± 0.0	Galactan	0.3 ± 0.0
Suspended Solids (% w/w)	8.8 ± 0.0	Ethanol	0.7 ± 0.0	Glucose	12.0 ± 0.3	Glucan	4.7 ± 0.1
Sieved solids (% w/v)	3.2 ± 0.2	Glucose	1.4 ± 0.1	Mannose	2.4 ± 0.1	Mannan	0.6 ± 0.0
Crude protein (% w/w)[a]	32.0 ± 0.6	Glycerol	12.0 ± 0.1	Xylose	9.7 ± 0.1	Xylan	3.6 ± 0.1
Crude protein (% w/w)[b]	15.1 ± 3.9	Lactic acid	1.7 ± 0.0				
		Xylitol	0.6 ± 0.1				
		Xylose	0.7 ± 0.1				

470 [a] Based on dry total solids.
471 [b] Based on dry sieved solids
472 [c] Dissolved monomers included
473 [d] From dry sieved solids
474



475

476 **Figure 1**

477 Overall industrial scheme of production of ethanol from grains with process modifications via

478 inclusion of the process of ethanol production from whole stillage by *N. intermedia*

479 (highlighted boxes).

480

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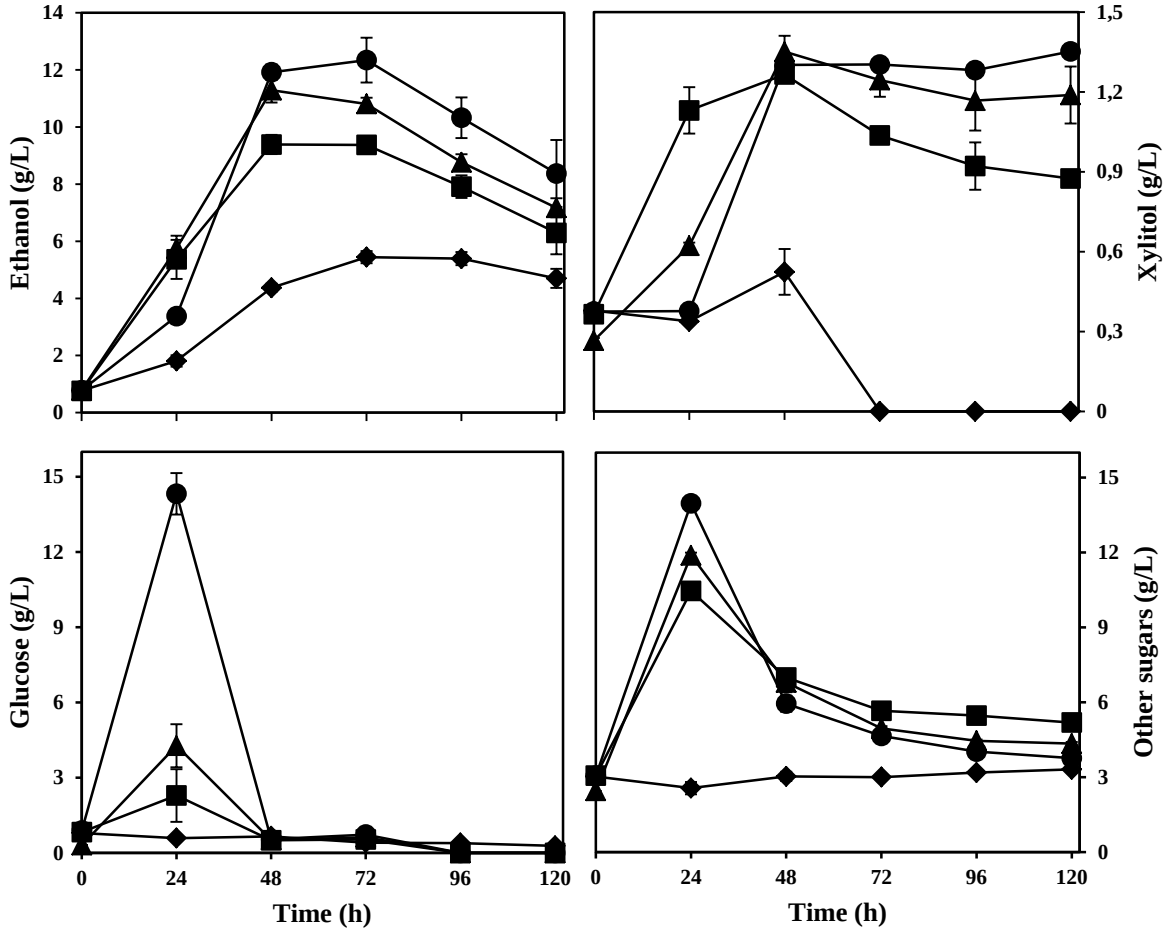
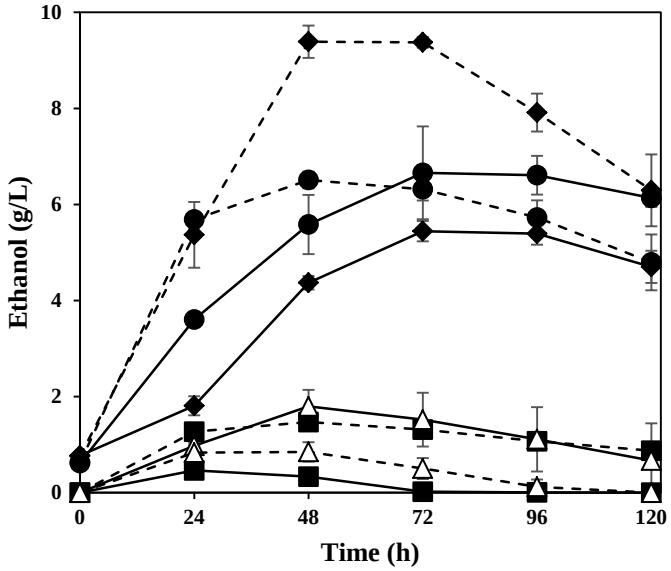


Figure 2

Profiles of ethanol, xylitol, glucose and other sugars during cultivation of *N. intermedia* in whole stillage with 0 (diamonds), 1 (squares), 5 (triangles) and 10 (circles) FPU of cellulase per g of suspended solids. Error bars represent two standards deviations.



499

500 **Figure 3**

501 Ethanol production profiles during *N. intermedia* cultivation in undiluted whole stillage
502 (diamonds) and its fractions (supernatant (circles), “small particles” (squares) and “large
503 particles” (triangles)) with (dashed lines) and without (straight line) cellulase addition. Error
504 bars represent two standard deviations.

505

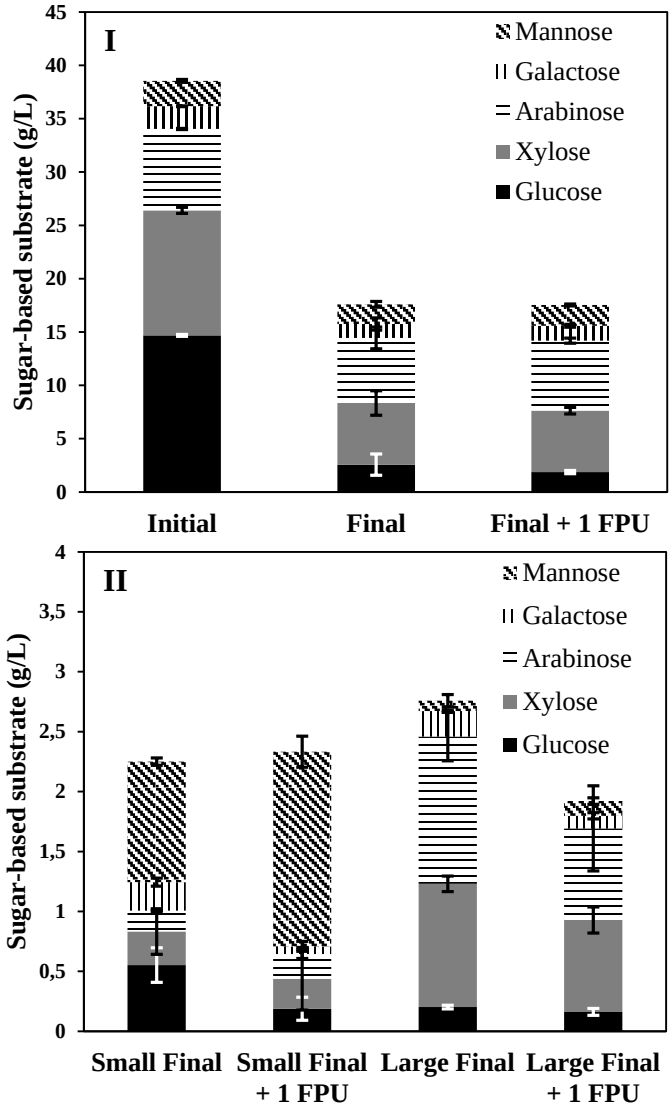
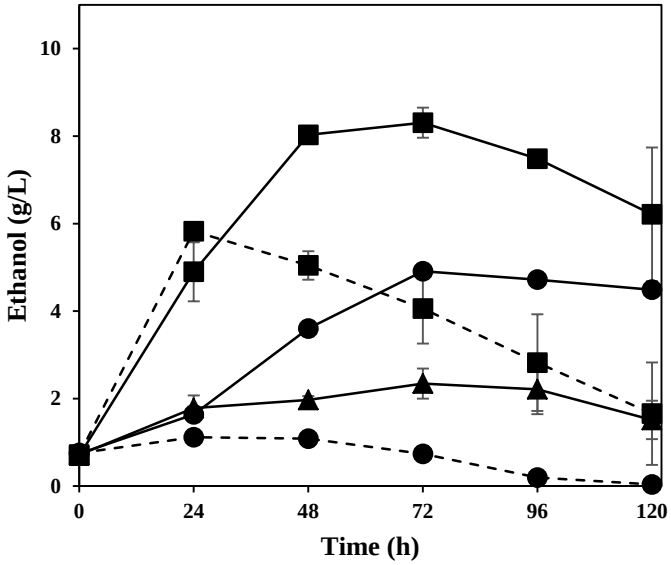


Figure 4

Profiles of dissolved sugar-based substrates after *N. intermedia* cultivation in whole stillage supernatant (**I**) and small and large particles (**II**) with addition or not of cellulase. Error bars represent two standard deviations.

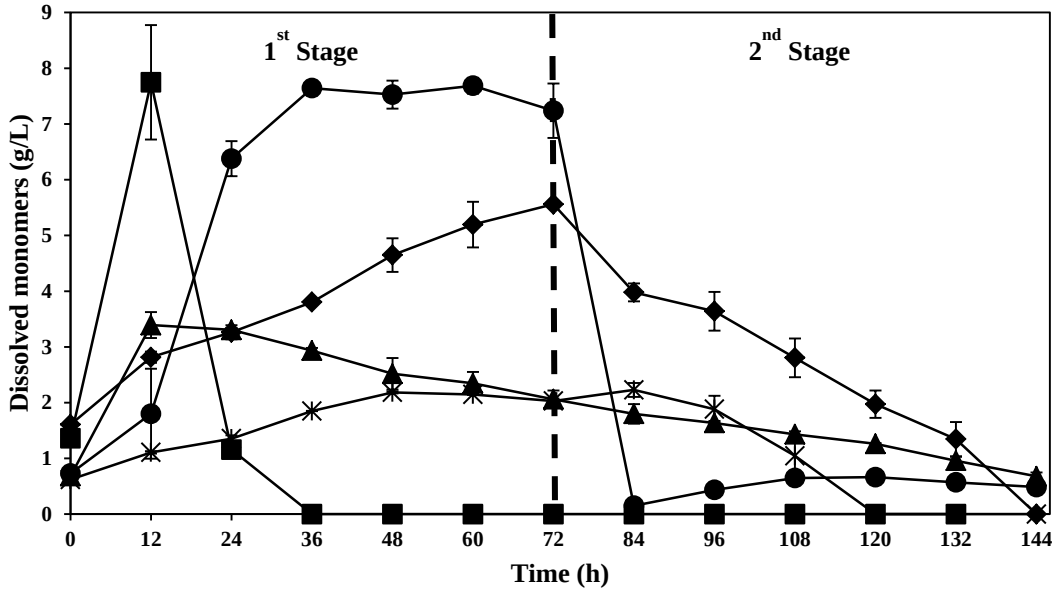


521

522 **Figure 5**

523 Ethanol profiles during cultivation in whole stillage without cellulase (circles), with addition
524 of 1 FPU cellulase/g SS (squares) with *N. intermedia* (straight lines) and *S. cerevisiae* (dashed
525 lines) or their co-culture (triangles). Error bars represent two standard deviations.

526



527

528 **Figure 6**

529 Concentration profiles of arabinose (diamonds), ethanol (circles), glucose (squares) and
530 xylose (triangles) during a two-stage cultivation in a 2.5 L continuous stirred tank reactor. The
531 first 72 h stage corresponds to the production of ethanol by *N. intermedia* with 1 FPU
532 cellulase/g SS and the second 72 h stage corresponds to the production of biomass by *A.*
533 *oryzae* after the medium had been distilled and its solids sieved. Error bars represent two
534 standard deviations.

535

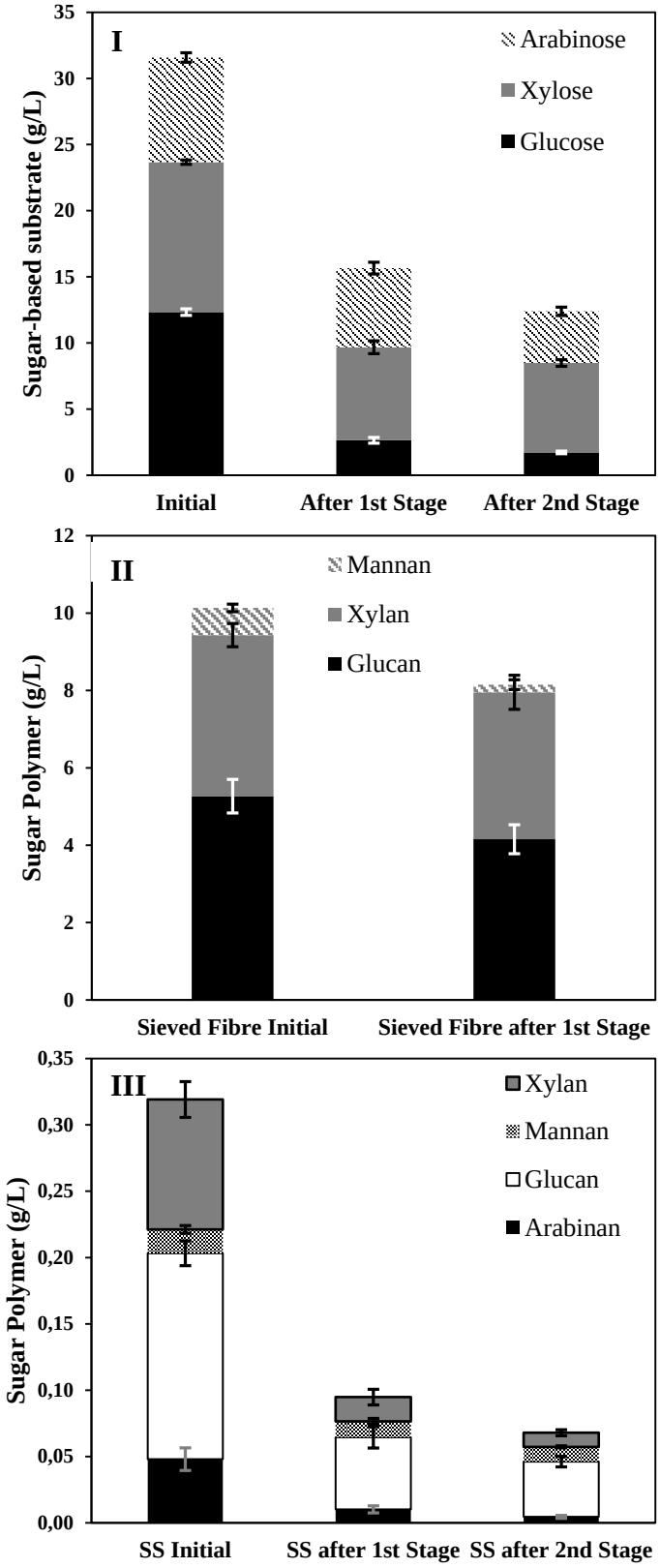


Figure 7

Sugar profiles from the supernatant (**I**), sieved fibres (**II**) and suspended solids (**III**) in a two-stage whole stillage cultivation in a bench-scale reactor. Error bars represent two standard deviations.

PAPER VI

Fungal protein and ethanol from lignocelluloses using *Rhizopus* pellets under simultaneous saccharification, filtration and fermentation (SSFF)

Somayeh FazeliNejad [a], Jorge A. Ferreira [a,] *, Tomas Brandberg [a], Patrik R. Lennartsson [a], Mohammad J. Taherzadeh [a]

[a] Swedish Centre for Resource Recovery, University of Borås, SE 501 90, Borås, Sweden

*Corresponding author:

Tel: +46-33-4354638

Fax: +46-33-4354003

E-mail: Jorge.Ferreira@hb.se

Abstract

The feasibility of the process for production of 2nd generation bioethanol cannot rely on a single product but instead a biorefinery around it needs to be built. In this work, both ethanol and fungal biomass that can be used for animal feed were produced from acid pretreated wheat straw slurry under an innovative simultaneous saccharification, fermentation and filtration (SSFF) strategy. A membrane unit was used to separate the solids from the liquid where the latter was converted to biomass from residual sugars or to both biomass and ethanol in the fermentation reactor containing *Rhizopus* sp. pellets. Biomass yields of up to 0.34 g/g based on consumed monomeric sugars and acetic acid were achieved. A surplus of glucose in the feed resulted in ethanol production and reduced the biomass yield, whereas limiting glucose concentrations resulted in higher consumption of xylose and acetic acid. The specific growth rate was in the range of 0.013-0.015 h^{-1} and did not appear to be influenced by the composition of the medium carbon sources. Under anaerobic conditions, an ethanol yield of 0.40 g/g and an ethanol productivity of 0.023 g/g/h were obtained with *Rhizopus* sp. pellets. The membrane filtration unit did not need to be regenerated where no fouling was observed for cultivation periods of up to 168 h. This work represents the first work combining a SSFF strategy with fungal pellets for both ethanol and biomass production from lignocellulosic materials.

Keywords: Cellulosic ethanol; fish-feed; fungal pellets; *Rhizopus* sp.; SSFF; wheat straw

Introduction

Nowadays, the production of 1st generation bioethanol from agricultural sugar- or starch-rich crops, as a replacement to gasoline, is well established at commercial scale. The leading ethanol-producing countries, USA and Brazil, use corn and sugarcane as main feedstocks, respectively [27]. However, due to ethical issues related to the use of sugar- or starch-rich feedstocks for fuel production instead of being directed to human consumption has put pressure on finding alternative feedstocks [1].

The production of ethanol from lignocellulose-based materials has been considered for several decades [68]. Nevertheless, due to their recalcitrant structure, a feasible commercial facility producing the so called 2nd generation bioethanol is presently inexistent being limited to some pilot plants [22]. Constraints include the cost-intensive pretreatment needed to open up the lignocellulosic structure, the cost of enzymes needed in the post-pretreatment stage, and the lack of robust microorganisms that can cope with inhibitors as well as robust cultivation strategies that can meet all requirements for feasible 2nd generation bioethanol [261]. Another conclusion of the intensive studies over the years is that a facility having lignocelluloses as feedstock cannot rely on a single product (i.e. ethanol) for feasibility [22]. The most commonly used strategies for production of ethanol include simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF). Running a SSF instead of a SHF circumvents the product inhibition of cellulase enzymes due to glucose accumulation [258]. However, SSF disadvantageously requires the use of new microorganism at each batch since it is difficult to separate it from the medium [260]. A new cultivation strategy, simultaneous saccharification, filtration and fermentation (SSFF), was developed by Ishola, et al. (2013). This new concept consists of a membrane unit connecting a hydrolysis reactor to a fermentation reactor. The enzyme-slurry mixture from the hydrolysis reactor is

filtered with the sugar rich permeate continuously reaching the fermentation reactor. The fermented medium is pumped back to the hydrolysis reactor for volume balance in both reactors (**Figure 1**). Therefore, SSFF combines the advantages of both SSF and SHF solving at same time their limitations: both hydrolysis and fermentation can be carried out at optimal conditions; the end-product inhibition is avoided and there is also the possibility to reuse the cells.

Wheat straw is a cheap agricultural by-product available at huge amounts with potential for production of ethanol in view of its cellulose (33%) and hemicellulose (33%) dry weight contents [270]. Various studies have been carried on the pretreatment and hydrolysis of wheat straw [42]. For instance, during acid pretreatment, the hemicellulose is dissolved releasing xylose, the inhibitor acetic acid among other compounds [271]. Combined with enzymatic hydrolysis significant amounts of glucose can be released from the cellulose that can be used for ethanol production [272].

The fact that the biomass is free from solids in the fermentation reactor under SSFF, opens up the opportunity for production of biomass from pretreated wheat straw slurry residual glucose, pentose sugars and other components as second value-added product of the process. This would be a similar situation as it exists for the 1st generation bioethanol plants from starch grains where both ethanol and animal feed products are produced [44]. Edible filamentous fungi have been previously used for production of protein-rich biomass from various types of substrates [144]. The tempe-isolated zygomycete *Rhizopus* sp. used in this work has previously been found to cope with inhibitors from a lignocellulosic hydrolysate [273], to form pellets easily separated from the medium [274] and to consume pentose sugars contrary to baker's yeast *Saccharomyces cerevisiae* [136].

In the present work, SSFF was developed for ethanol production from glucose and biomass for animal feed from residual carbon sources using *Rhizopus* sp. in pellet form. The focus was on the effect of the enzyme addition to the hydrolysis reactor, the temperature in the hydrolysis and fermentation reactors, and aeration for assimilation of carbon sources in the filtered permeate for production of biomass. This is the first work reporting the use of SSFF with filamentous fungi towards production of two value-added products from lignocelluloses.

Materials and Methods

Microorganisms

A *Rhizopus* sp. CCUG 61147 (Culture Collection University of Gothenburg, Sweden) isolated from Indonesian leaves traditionally used for preparation of tempe, was used in this work. The strain has been identified as RM4 in a previous publication [136]. The fungus was kept in PDA (Potato Dextrose Agar) plates and its incubation and preparation for inoculation were carried out according to FazeliNejad, et al. (2013). A strain of *Saccharomyces cerevisiae*, Ethanol Red, kindly provided by Fermentis (France) in dry form, was also used.

Pretreated wheat straw slurry

Slurry of wheat straw, delivered by SEKAB E-Technology (Örnsköldsvik, Sweden), was produced by continuous treatment of wheat straw at 22 bars overpressure during 5-7 minutes. The resulting slurry, a liquid fraction with fine particles, had 14.6% suspended solids (SS) and 23.8% total solids (TS). The liquid had pH 2.0 and contained (in g/L): glucose 7.2, xylose 22.1, galactose 2.3, arabinose 4.6, acetic acid 5.9, 5-hydroxy methyl-furfural (HMF) 2.1, and furfural 4.2. The solid fraction contained 34.7% (w/w) glucan and 4.6% (w/w) xylan.

Enzyme Cocktail

Cellic® CTec2, kindly provided by Novozymes (Denmark), was used in the experiments with enzymatic treatment. The product had an activity of 168 FPU (filter paper units) per milliliter.

Cultivations in shake-flasks

A complex medium, containing (in g/L): xylose 20, potato extract 4, soybean peptone 6, and $CaCO_3$ 6, was used for preparation of *Rhizopus* sp. pellets. The medium (50 mL) was transferred to 250 mL cotton-plugged Erlenmeyer flasks followed by sterilisation in an autoclave at 121 °C for 20 min, where the xylose was autoclaved separately. After mixing and inoculation with 1.0×10^5 spores/mL of *Rhizopus* sp., the flasks were kept in a water-bath at 30 °C and 150 rpm for 72 hours.

The produced pellets were transferred to new cultivations to a cell concentration of 1.65±0.10 g/L (dry weight, ± 1 SD) for further biomass determination. New cultivations were carried out in 250-mL Erlenmeyer flasks containing 100 mL of autoclaved medium composed of (in g/L): $(NH_4)_2SO_4$ 7.5, KH_2PO_4 3.5, $CaCl_2 \cdot 2H_2O$ 1, $MgSO_4 \cdot 7H_2O$ 0.75, and one of the following carbon sources namely acetic acid 5.0, ethanol 10, glucose 10, lactic acid 10, and xylose 10. The cultivations were kept in a water-bath at 30 °C shaking at 150 rpm.

With similar preparation of the initial biomass, 2.20 ± 0.12 g/L pellets (dry weight, ± 1 SD) were transferred to a new medium and cultivated as described above but a combination of the carbon sources at 3.5 g/L was used. This experiment was carried out in duplicate.

Liquid samples were withdrawn and stored at -20 °C for subsequent analysis. At the end of cultivation, the pellets were harvested using a sieve, washed with distilled water, and dried in an oven at 70 °C to constant weight for 24 h. The cultivations using single-carbon sources or their combination were performed in quadruplicate and duplicate, respectively.

Cultivations under SSFF

Simultaneous saccharification, fermentation and filtration (SSFF) previously described by Ishola et al. [269] was employed. The cellulosic feedstock was hydrolysed enzymatically in a separate vessel (hydrolysis reactor) and the resulting sugar-rich liquid was circulated through a bioreactor, where the fungal biomass production took place. The solid fraction was separated from the sugar-rich stream by cross-flow filtration. However, a cell retention system was not needed in this work since 5 mm spherical pellets of *Rhizopus* sp. were used and they maintained this morphology throughout the cultivations.

For SSFF trials, pellets were prepared as described above and transferred to a 750 mL fermentor (Ant, Belach Bioteknik AB, Sweden) containing sterilised salt solution as described above and 0.1 g/L antifoam. The transferred biomass of *Rhizopus* sp. had an initial dry weight within the range 1.5-2.1 g (based on wet weight) and the volume was adjusted to a total volume of 500 mL. Wheat straw slurry was transferred to a parallel hydrolysis reactor (Memma, Belach Bioteknik AB, Sweden) and diluted with deionized water to 5.0% SS to a total volume of 3.5 L. The salt and antifoam content was the same as in the bioreactor. The cross-flow filtration unit was set up according to Ishola et al. [269]. After integration (**Fig. 1**), the flow of filtrate through the fungus bioreactor was 40 mL/h.

In a first experiment, the integration of the SSFF system was preceded by 24 h enzymatic decomposition by addition of Cellic®CTec2, corresponding to 10 FPU/g SS. The pH was initially adjusted to 5.5 in both reactors and regulated to 5.5 in the bioreactor by on-line addition of 2.0 M NaOH. The temperature was kept at 50 °C in the hydrolysis reactor and 35 °C in the bioreactor. The stirring was 350 rpm in the hydrolysis reactor and 100 rpm in the fungus bioreactor, which was aerated at 1 vvm (volume of air per volume of liquid per minute). The experiment was carried out in duplicate where the integration phase lasted 140 h and 168 h, respectively. Samples were withdrawn directly from the tubes channeling medium in and out of the biomass production vessel. The final biomass content was analysed by weighing it after 24 h at 70 °C. The experiment was then repeated with the same parameters but with the following differences; no enzymes were added and the integration phase lasted 72 h.

A subsequent experiment was initiated in the same way as that without enzyme addition described above. That experiment was used for indirect assessment of the biomass content after 72 h of biomass cultivation with SSFF integration. In a similarly initiated experiment, the air supply was switched off after 72 h and simultaneously 10 FPU/g SS enzyme was added. This anaerobic fermentation phase lasted 94 h.

Another SSF trial was carried out where the temperature was adjusted to 35 °C in both reactors. Enzyme (10 FPU/g SS) and 15 g of dry baker's yeast were added to the hydrolysis reactor. The duplicate trials lasted 96 and 120 h, respectively. In a different set-up, the cultivation referred to above (35 °C, 10 FPU/g SS, and 15 g dry yeast) was initially performed without any integration for 54 h. The resulting fermented slurry was distilled using a rotary evaporator (Labinett, Sweden) at 140 °C (oil bath), and 30 rpm rotation speed at atmospheric

pressure. The water content lost during distillation was re-adjusted by addition of sterile ultrapure water. The resulting slurry, now without ethanol, was used for integration with SSFF and aerobic production of *Rhizopus* sp. biomass as described above during 96 h.

Analytical methods

The analysis of concentrations regarding glucose, metabolites, and inhibitors as well as the spore counting were performed according to FazeliNejad, et al. (2013). SS were determined by filtration with Munktell filters, Grade 3 (5-8 µm) and the TS were determined by drying a sample to constant weight at 105 °C overnight. The solid fraction of the wheat straw slurry and the enzyme activity were analysed according to the NREL protocols [275, 276].

Results and Discussion

SSFF of wheat straw slurry with Rhizopus sp. pellets

Production of additional products in a biorefinery concept has been proposed to improve the process economy of ethanol production from cellulosic raw materials [277, 278], and fish-feed in the form of *Rhizopus* sp. biomass is suggested as such a by-product. Implementing SSFF for aerobic production of zygomycetes biomass entails the application of cross-flow fermentation to separate available sugars and other organic compounds from a pretreated lignocellulosic slurry (**Fig. 1**). The filtrate is supplied to an aerated fermentor, where carbon source is consumed by zygomycetes in pellet form in order to produce biomass. The pellet morphology is useful in order to prevent leakage of biomass when liquid is pumped back to the hydrolysis reactor. This reflux is necessary in order to maintain the liquid balance between the vessels and prevent the dry matter content of the slurry from increasing. Besides, the glucose concentration, which would increase as a result of enzymatic decomposition of cellulose and inhibit the enzymes, can be controlled in this way. The filtration of the slurry is

in itself a very important operation since the solid fraction must not be mixed with the biomass, which would result in a downstream separation problem. In addition to biomass, the *Rhizopus* sp. used in this work is also a potential producer of ethanol [279].

The implementation of SSFF for production of ethanol and biomass includes the use of continuous cross-flow filtration as described in materials and methods. It was shown that the filtration unit was applied for up to 168 h without regeneration of the membrane and without any fouling that caused interruption of any experiment. In a similar set-up, involving slurry of pretreated spruce, the same operation was performed during 28 days without interruption, regeneration, or fouling [269].

1.1 Specific growth rate and biomass yield

Various SSFF experiments with *Rhizopus* sp. production from wheat straw slurry were carried out in order to validate this concept. The main difference between the different trials was the composition of the feed, notably its glucose content. Enzymatic decomposition of cellulose in the solid fraction prior to integration with SSFF (**Fig. 2**) produced a relatively high initial glucose concentration in contrast to a similar experiment without enzyme addition (**Table 1**). Addition of baker's yeast to the hydrolysis vessel nearly eliminated the glucose in the inflow to the bioreactor. Furthermore, an experiment was carried out where the amount of glucose was reduced by addition of baker's yeast and the ethanol was removed by distillation. The resulting mix was used for zygomycetes production by SSFF (**Table 1**).

The specific growth rate, μ, was calculated according to:

$$\mu = \frac{\ln(\frac{x}{x_0})}{t} \qquad (1)$$

where x_0 denotes the initial biomass concentration and x the biomass concentration after the elapsed time *t*. To assume a constant μ is debatable taking into consideration the dynamic conditions regarding, for instance, the substrate concentrations and the results are sensitive to the accuracy of wet weight measurements of the initial biomass. Growth in the form of pellets is also known to be different from that of free cells [280], but considering the small size of the used pellets this effect can be assumed to be relatively low.

However, **Table 1** shows that μ as measured, hardly changes depending on the substrate composition, the range is $0.013\ h^{-1} < \mu < 0.015^{-1}$ under aerobic conditions. These moderate levels show no tendency to be affected by glucose concentrations as long as alternative carbon sources are present, such as acetic acid, ethanol, and xylose. However, it can be assumed that more efficient aeration would have resulted in a higher growth rate.

The biomass yields reported in **Table 1** ranged between 0.24 and 0.34 gram produced biomass per gram consumed monomeric sugars (arabinose, galactose, glucose, and xylose), acetic acid and ethanol, except for the case with enzymatic hydrolysis (i.e. high initial glucose concentration, **Fig 2**) where the biomass yield drops due to ethanol production. These biomass yields are in harmony with separate batch experiments with synthetic medium, where the biomass yields for different carbon sources (10 g/L of each compound was added except that of acetic acid which was 5 g/L) were assessed individually. The measured yields of biomass for acetic acid, ethanol, and xylose in these trials were 0.30 g g^{-1}, 0.30 g g^{-1} and 0.29 g g^{-1} respectively after 140 h batch cultivation (96 h for acetic acid, data not shown). The corresponding consumption of glucose was faster, less than 42 h, but the resulting biomass yield was merely 0.11 g g^{-1} due to formation of ethanol and glycerol (data not shown),

confirming overflow metabolism (Crabtree effect) for *Rhizopus* sp. [281, 282]. The pooled standard deviation for the biomass yields was 0.042 (1 SD).

1.2 Steering the uptake of carbon sources

In a separate experiment with synthetic medium, the uptake pattern was studied in a cultivation, where acetic acid, ethanol, glucose, lactic acid, and xylose were added to the same cultivation of *Rhizopus* sp. in aerobic shake-flasks. The results (**Fig. 3**) show a relatively rapid consumption of glucose, followed by acetic acid, whereas xylose and ethanol with similar consumption trends were not totally consumed after 72 h of cultivation. Lactic acid frequently occurs as an undesired metabolite produced by contaminants [283] and its uptake by other zygomycetes is documented [284]. However, no measurable consumption of lactic acid by the *Rhizopus* sp. strain was confirmed in this experiment. It is observed that the preference of carbon source, among those examined, under the examined conditions can be ranked as follows: glucose > acetic acid > xylose & ethanol (**Fig. 3**). The measured specific growth rate, μ, was 0.013 h^{-1}, i.e. similar to the level in the SSFF experiments with wheat straw hydrolysate (**Table 1**), but it is difficult to isolate the effects of a less inhibitory environment and different conditions regarding aeration from each other.

In the SSFF experiment with cellulase addition it is clearly visible that the glucose uptake is relatively efficient, which has no visible positive effect on the specific growth rate. Instead, ethanol is produced in a respire-fermentative pattern (**Fig. 2**, **Table 1**). In a biorefinery context, it is probable that glucose, if available, would be used for other purposes, such as ethanol production by fermentation. Therefore, it may be advantageous to utilize other compounds than glucose for biomass formation. By omitting the enzymatic decomposition, the glucose concentration was sharply reduced, resulting in a higher uptake of xylose and

acetic acid. Combining cultivation of zygomycetes by SSFF with addition of *S. cerevisiae* in the hydrolysis bioreactor further reduces the glucose concentration in the feed and increases the consumption of xylose, acetic acid, and ethanol. The use of enzymatic hydrolysis and fermentation followed by distillation prior to SSFF cultivation produces a result remarkably similar to the case with untreated slurry, i.e. without enzyme addition (**Table 1**). In conclusion, reducing the glucose concentration steers the uptake by *Rhizopus sp.* to for instance xylose and acetic acid, which can both be present as residual compounds in a biorefinery, without reducing either the biomass yield or the specific growth rate.

Fermentation by Rhizopus sp.

Rhizopus sp. is furthermore useful as a fermenting organism for ethanol production and the combination of valuable biomass and ethanol is interesting in a biorefinery perspective. To suggest a complete list of process possibilities is beyond the scope of this study, but possibly a process could be based on production of ethanol and fish-feed, with *Rhizopus* sp. as the production organism.

Two experiments were carried out, where *Rhizopus* sp. was initially grown aerobically on straw hydrolysate in order to build up biomass. One of the trials was stopped after 72 h (referred to in **Table 1** as cultivation without enzyme addition), the biomass was harvested and measured (4.4 g). The second experiment was initiated in a similar way, but after 72 h cellulase (10 FPU/g SS) was added and the air was switched off (**Fig. 4**). During the subsequent 94 h, the amount of biomass increased to 6.4 g, suggesting a specific growth rate (μ) of 0.034 h^{-1} during the anaerobic phase. Furthermore, the biomass yield was lower (**Table 1**). In the timespan from 72 to 166 h, at least 11.5 g of ethanol was produced (some may have evaporated), which would suggest an ethanol yield of 0.40 g per gram consumed glucose and

xylose and an ethanol productivity of 0.023 g $g^{-1}h^{-1}$, based on the average biomass concentration as measured at 72 and 166 h, respectively, in the two different experiments. The volumetric productivity of ethanol, 0.24 g $l^{-1}h^{-1}$, was relatively low if compared to optimized fermentations with *S. cerevisiae* [285]. The xylose uptake corresponded to only 5 % of the consumed carbon source and the uptake should be a sign of leakage of oxygen into the fermentation vessel, considering that fungi normally don't consume xylose under anaerobic conditions. According to measurements, the concentration of xylose in the hydrolysis vessel increases after 72 h, indicating release of xylose from the solid fraction as a result of the enzymatic decomposition.

1.3 Impact of inhibitors and infections

After dilution to 5.0 % SS, the concentrations of acetic acid, furfural and HMF were 1.8 g l^{-1}, 0.65 g l^{-1}, and 1.3 g l^{-1} respectively. Considering a previous study [273], these levels should not be very inhibiting by themselves. Hydrolysates of lignocellulosic material usually contain other inhibitors, such as phenolic compounds, the concentrations of which were not measured. It was observed in all SSFF trials that ingoing furfural and HMF are completely converted (i.e. not detected in the outflow) after 6-8 hours of integrated fermentation, confirming *in-situ* conversion of these compounds by *Rhizopus* sp. [273]. Furthermore, the specific growth rate, μ, was similar in the hydrolysate-based SSFF experiments and the shake-flask experiments with synthetic medium. It is thus assumed that the impact of inhibitors was limited. Presence of bacteria was noted in the trial with addition of dry yeast, originating from the dry yeast product. Infections occurred also in the cultivations, which lasted longer than 120 h, but the exact impact on for instance biomass yields was not determined.

Conclusions

Rhizopus sp. in pellet form was successfully used for aerobic production of biomass, which can be used as fish-feed, by SSFF from acid pretreated wheat straw slurry with biomass yields of up to 0.34 g biomass per gram of consumed monomeric sugars and acetic acid. A surplus of glucose in the feed resulted in ethanol production and reduced the biomass yield, whereas limiting glucose concentrations resulted in higher consumption of xylose and acetic acid. The specific growth rate was in the range of 0.013 h^{-1} and 0.015 h^{-1} and did not appear to be influenced by the composition of the carbon source. Under anaerobic conditions, an ethanol yield of 0.40 g/g and an ethanol productivity of 0.023 g/g/h were obtained with *Rhizopus* sp. pellets.

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Conflict of Interest

The authors declare no conflict of interests.

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Table 1. Overview of SSFF trials. "Glucose & ethanol reduced" refers to the experiment with SSF and evaporation. "Glucose removed" refers to trial with yeast added to the hydrolysis vessel. The glucose column refers to the concentration of glucose in the biomass production vessel and how it develops. In the distribution of uptake, the uptake of arabinose and galactose is omitted.

						Distribution of uptake (%)			
	Glucose (g/L)	Cultivation time (h)	$Y_{X/S}$ (g/g)	$Y_{E/S}$ (g/g)	μ $.(h^{-1})$	Glucose	Xylose	Ethanol	Acetic acid
Enzyme addition	~ 27 decreases to ~ 6	140	0.11*	0.21*	0.013	86	9	-	6
		168	0.14*	0.14*	0.014	80	12	-	7
No enzyme addition	2.2 decreases to 0.2	72	0.32*	-	0.015	46	43	-	12
SSF & evaporation†	2.2 to ND	96	0.34*	0.10*	0.015	28	39	-	25
Yeast in hydrolysis‡	<0.3	96	0.24*	cons.	0.015	<1	53	22	25
		120	0.30*		0.013	1	37	28	34
Anaerobic	peaks at 17.5	94	0.034+	0.40+	0.002	95	5	-	<1

* Biomass and ethanol yields related to consumed amounts of acetic acid, arabinose, ethanol, glucose, galactose, and xylose.

† This treatment resulted in reduced amounts of glucose and ethanol prior to SSFF integration

‡ This methods sharply reduced the glucose content in the flux into the cultivation vessel

+ Biomass and ethanol yields related to consumed amounts of glucose and xylose

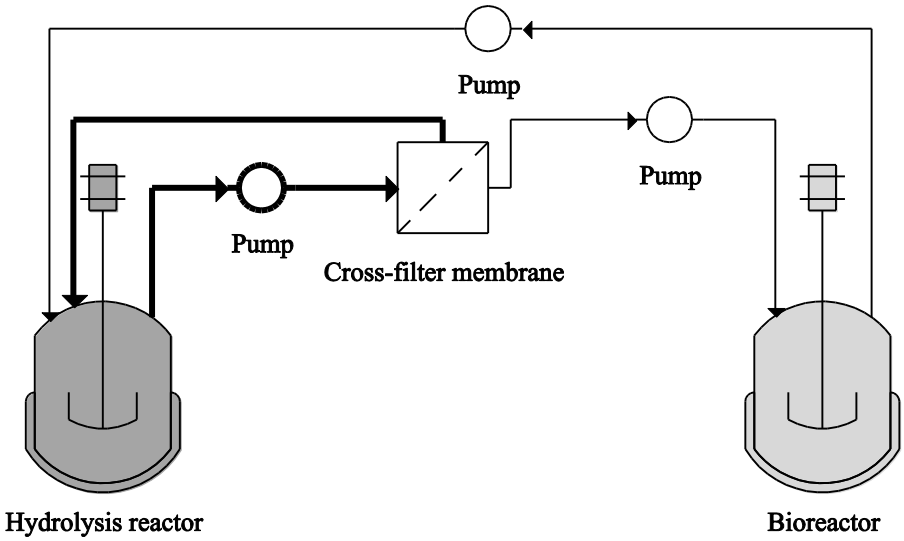

Figure 1: Schematic representation f SSFF for aerobic biomass production.

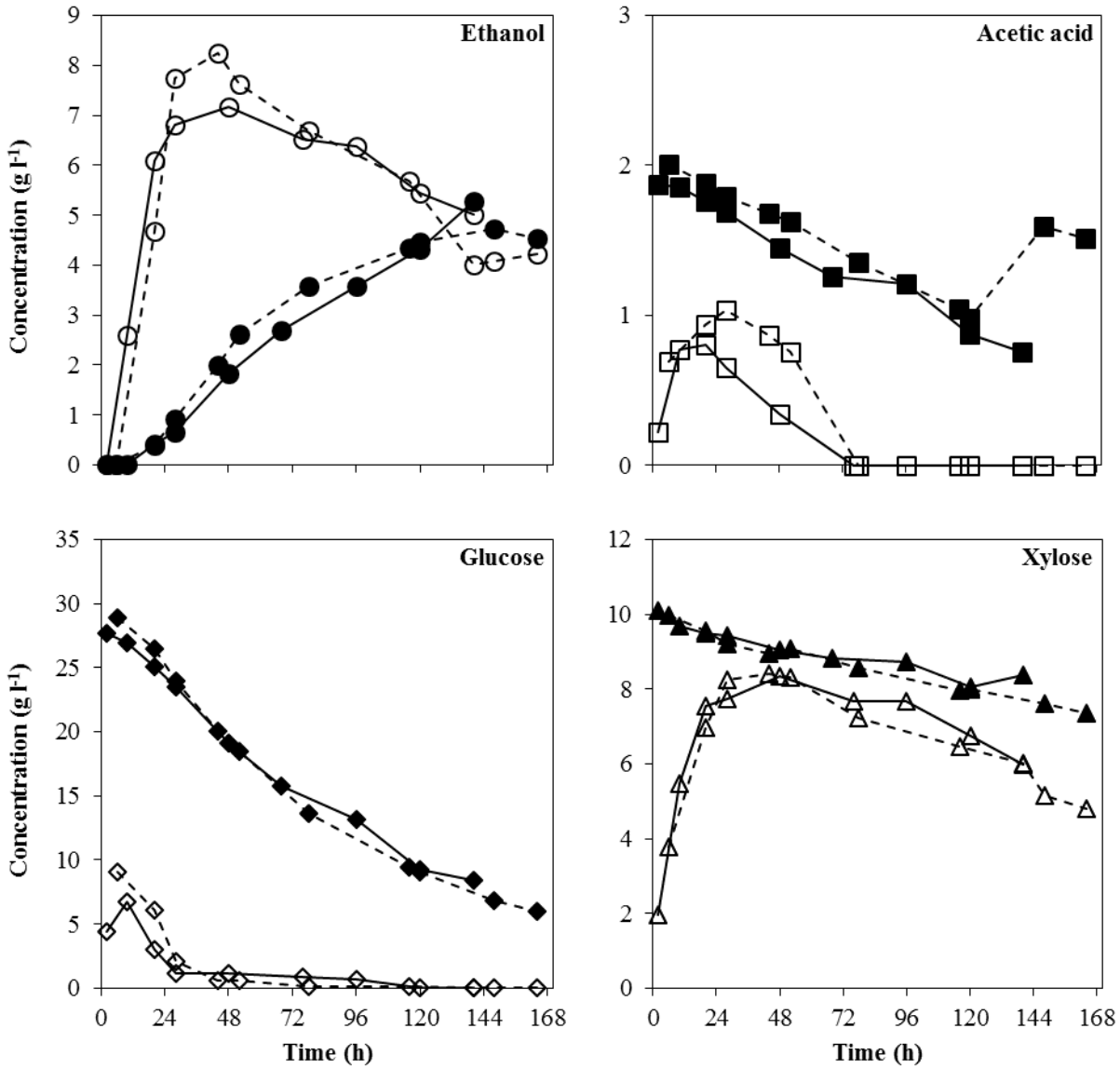

Figure 2: Concentrations of ethanol, acetic acid, glucose, and xylose in SSFF for aerobic production of *Rhizopus* sp. biomass, preceded by 24 h of enzymatic hydrolysis before integration. Closed symbols denote concentrations in the ingoing feed, open symbols denote concentrations in the recirculation feed.

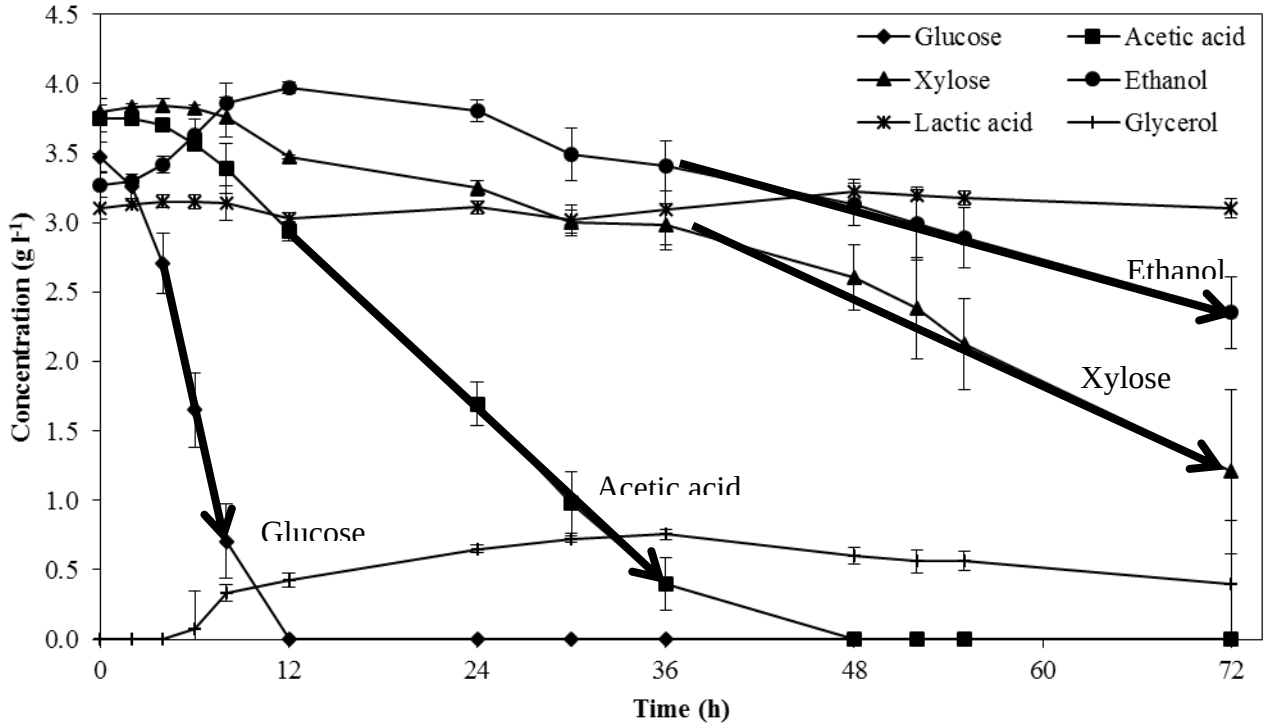

Figure 3: Concentrations of glucose, acetic acid, xylose, ethanol, lactic acid, and glycerol in an aerated shake-flask experiment inoculated with *Rhizopus* sp., initially containing 3.5 g l^{-1} of glucose, acetic acid, xylose, ethanol, and lactic acid respectively. Error bars denote 1 SD.

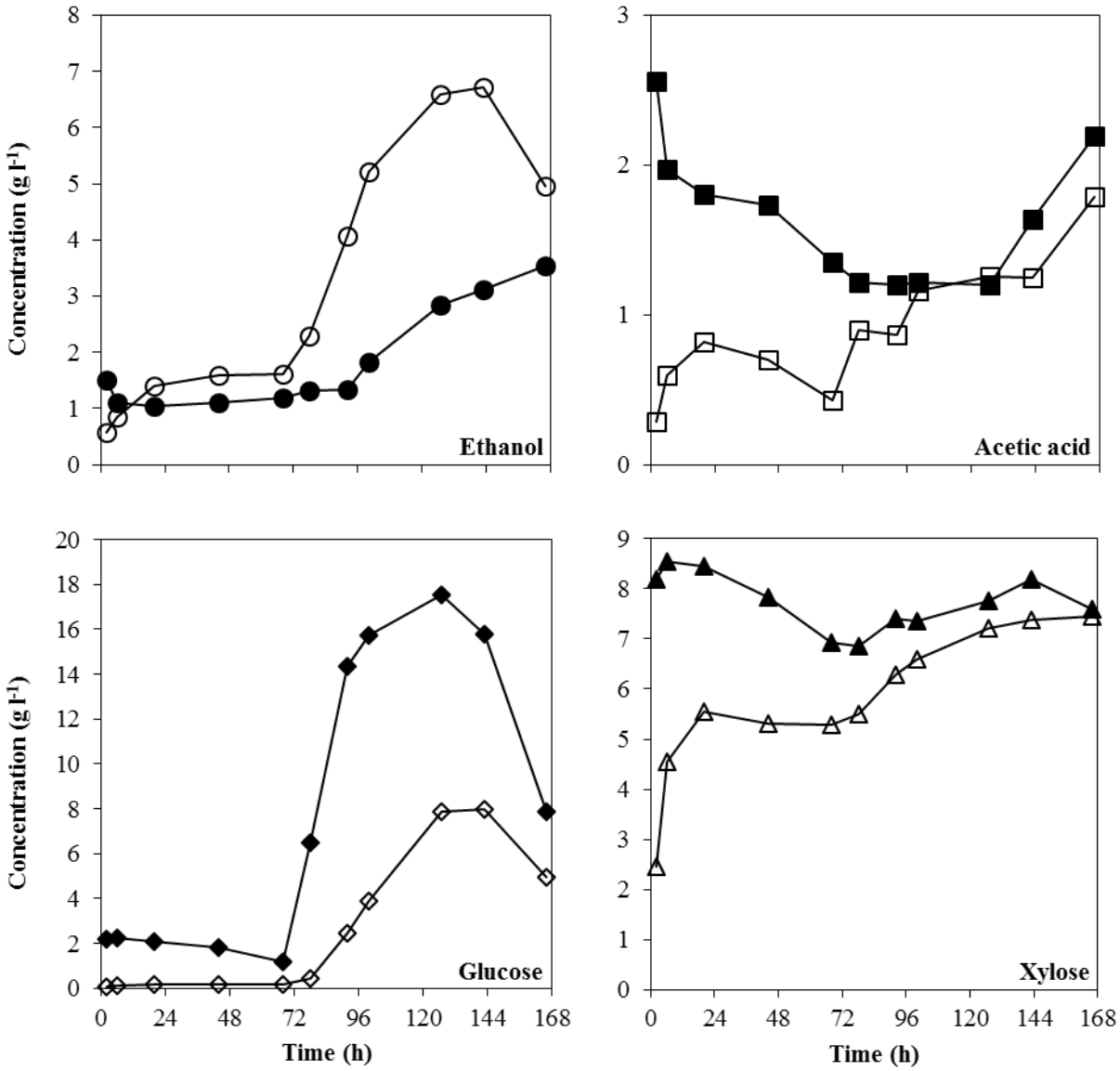

Figure 4: Concentrations of ethanol, acetic acid, glucose, and xylose in SSFF for aerobic production of biomass followed by anaerobic fermentation by *Rhizopus* sp. The air of the biomass vessel is switched off and enzymes are added to the hydrolysis vessel at t = 72 h.