THESIS FOR THE DEGREE DOCTOR OF PHILOSOPHY

Zygomycetes and cellulose residuals: hydrolysis, cultivation and applications

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CHALMERS UNIVERSITY OF TECHNOLOGY

Göteborg, Sweden 2012

UNIVERSITY OF BORÅS

SCHOOL OF ENGINEERING

Borås, Sweden 2012

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ISBN 978-91-7385-626-3

Doktorsavhandlingar vid Chalmers tekniska högskola
Ny serie nr 3307
ISSN 0346-718X

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Skrifter från Högskolan i Borås, nr. 35
ISSN 0280-381X

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Cover: Dark field microscopy photograph of submerged growth of *Mucor indicus*.

Photographs by Patrik R. Lennartsson.

Printed in Sweden
Repro-service, Chalmers University of Technology
Göteborg, Sweden 2012

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Abstract

Zygomycetes is a class of fungi living worldwide as saprobes, as part of mycorrhizae, and as parasites. Humans have used some zygomycetes for centuries in the production of traditional foods, e.g. Indonesian tempe. In the present thesis, the experimental focus was on two zygomycetes strains, *Mucor indicus* CCUG 22424 and *Rhizopus sp.* IT.

One of the distinguishing features of *M. indicus* is its dimorphism. The different cell forms were influenced by the culturing conditions. After inoculation, when the initial spore concentration was high (6-8×10^6 spores/ml), yeast-like growth dominated under anaerobic conditions. With a smaller inoculum, yielding 1-2×10^5 spores/ml, and access to oxygen, filamentous forms dominated. Only negligible differences in ethanol yield (390-420 mg/g hexoses), productivity (3-5 g/l/h), and inhibitor tolerance were observed. Differential expressions of probably four genes were observed between the yeast-like and filamentous growth forms.

Lignocelluloses are a suitable substrate for cultivating zygomycetes, as they occur in abundance, particularly since zygomycetes, unlike *Saccharomyces cerevisiae,* can utilise pentoses. Lignocelluloses require pretreatment to achieve efficient hydrolysis of the cellulose. N-methylmorpholine-N-oxide (NMMO) was tested for pretreatment of spruce and birch. Reducing wood chip size and/or prolonged pretreatment, promoted hydrolysis yield. Best yields were achieved from <2 mm chips and 5 h pretreatment. The hydrolysate was used for fermentation with *M. indicus*, resulting in 195 and 175 mg ethanol/g wood, and 103 and 86 mg fungal biomass/g wood, from spruce and birch respectively.

Orange peel is another potential substrate. However, the hydrolysate contained 0.6 % (v/v) D-limonene, ten times higher than the concentration inhibiting *S. cerevisiae*. *M. indicus* was more resistant and successfully fermented the hydrolysate, producing 400 mg ethanol/g hexoses and 75 mg fungal biomass/g sugars. Both *M. indicus* and *Rhizopus* sp. grew in 1.0 % and 2.0 % D-limonene, although the latter was unable to grow in the hydrolysate.

A third substrate was also used, spent sulphite liquor (SSL), which is a by-product from sulphite paper pulp mills. The SSL was diluted to 50 % and used for airlift cultivations of *Rhizopus* sp. In 1.0 vvm aeration, up to 340 mg biomass/g sugars was produced. Prolonged cultivations generally decreased the protein (from 500 to 300 mg/g) and lipid (from 70 to 20 mg/g) contents. In contrast, the cell wall fraction, measured as alkali-insoluble material (AIM), increased (160-280 mg/g), as did the glucosamine (GlcN) content (220-320 mg GlcN/g AIM). The produced fungal biomass could serve as animal feed, e.g. for fish.

Keywords: Zygomycetes, fungi, lignocelluloses, ethanol, fish feed, animal feed, dimorphism, airlift, pretreatment

List of publications

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

I. **Patrik R. Lennartsson**, Keikhosro Karimi, Lars Edebo, and Mohammad J. Taherzadeh. Effects of different growth forms of *Mucor indicus* on cultivation on dilute-acid lignocellulosic hydrolyzate, inhibitor tolerance, and cell wall composition. *Journal of Biotechnology*, 2009. **143**(4): p. 255-261.

II. **Patrik R. Lennartsson**, Claes Niklasson and Mohammad J. Taherzadeh, A pilot study on lignocelluloses to ethanol and fish feed using NMMO pretreatment and cultivation with zygomycetes in an air-lift reactor. *Bioresource Technology*, 2011. **102**: p. 4425-4432.

III. **Patrik R. Lennartsson**, Päivi Ylitervo, Christer Larsson, Lars Edebo and Mohammad J. Taherzadeh, Growth tolerance of *Zygomycetes Mucor indicus* in orange peel hydrolysate without detoxification. Submitted.

I V. Jorge A. Ferreira, **Patrik R. Lennartsson**, Claes Niklasson, Magnus Lundin, Lars Edebo and Mohammad J. Taherzadeh, Spent sulphite liquor for cultivation of an edible *Rhizopus* sp. *BioResources*, 2012. **7**(1): p. 173-188.

V. Quang Minh Ho Ky, **Patrik R. Lennartsson** and Mohammad J. Taherzadeh, Detection of differential gene expressions in the dimorphism of *Mucor indicus* by suppression subtractive hybridisation. Submitted.

Statement of contribution

Paper I: Responsible for part of the idea, part of the experimental work, and most of the writing.

Paper II: Responsible for most of the idea, all experimental work, and all writing.

Paper III: Responsible for most of the idea, most of the experimental work, and most of the writing.

Paper IV: Responsible for most of the idea, supervision of the work, and part of the writing

Paper V: Responsible for part of the idea, part of the experimental work, and a major part of the writing.

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1. INTRODUCTION

1.1. Preface and scope

Zygomycetes is a phylogentically early class of fungi encountered worldwide, living as saprobes, part of mycorrhizae, and as parasites. Several species are known to be able to produce ethanol with high yields, utilising pentoses and producing fungal biomass, which can be used for a wide range of applications, such as removal of heavy metals, or production of chitosan and superabsorbents, and as animal feed. Some species produce enzymes or organic acids of commercial interest. Furthermore, several zygomycetes have been isolated from various food sources or from the production of beverages intended for human consumption, and are thus "generally regarded as safe".

Currently, the interest in the utilisation of renewable resources, such as cellulose residuals, is at an all-time high as a consequence of the overexploitation of natural resources, with fossil fuel depletion and concerns regarding green house gas emissions. However, several problems are associated with the utilisation of lignocellulosic materials, such as hydrolysis yield, inhibitor tolerance, and use of pentoses. In specific cases, one problem can be completely dominating, e.g. the inhibitory effect of D-limonene on orange peel waste. Nevertheless, some problems can be circumvented or avoided if other microorganisms than the common yeast, *Saccharomyces cerevisiae*, is utilised, such as zygomycetes strains.

The main goal of the present thesis was to investigate and develop zygomycetes fungi for two main purposes, production of ethanol and production of fungal biomass, starting from cellulosic materials. To accomplish these goals, the work was divided into five topics:

- Effect of different growth forms of *Mucor indicus* (Paper I).
- Pretreatment and enzymatic hydrolysis of birch and spruce, which has received comparatively little attention in terms of ethanol and fungal biomass production (Paper II).
- Utilisation of orange peels for production of ethanol and fungal biomass (Paper III).
- Production and properties of fungal biomass from spent sulphite liquor (Paper IV).
- Differences in gene expression between the different growth forms of *M. indicus*, with the possibility of further studies on regulation of the growth forms (Paper V).

1.2. Outline

The thesis comprises five main chapters and five papers, summarised as follows:

- Chapter 1 introduces the thesis and the research is motivated.

- Chapter 2 briefly describes the kingdom *Fungi* and the class *Zygomycetes*, and the two zygomycetes strains investigated, are presented. Incorporating results from Papers I and V, great emphasis is placed on the dimorphic behaviour of *Mucor*, in terms of induction, germination, growth, and gene expression. The zygomycetes cell wall is also discussed as well as the effects of dimorphism on its characteristics.

- Chapter 3 presents the raw materials for cultivation of zygomycetes, such as lignocelluloses in general, but also more specifically those of birch and spruce, orange peels, and spent sulphite liquor. Furthermore, pretreatment and hydrolysis procedures of the raw materials are included. This chapter involves Papers I-IV.

- Chapter 4 describes the ethanol production, starting with the current world market and continuing with ethanol from *M. indicus*, including ethanol from pentoses. Data from Papers I-III are incorporated.

- Chapter 5 depicts various possible applications and characteristics of zygomycetes biomass. This includes fish feed, chitosan extraction, and production of biological superabsorbents. Papers I-IV are included in this chapter.

2. ZYGOMYCETES

2.1. The kingdom *Fungi*

Five decades ago, there was no such thing as a fungal kingdom. Fungi were rather considered to belong to the plant kingdom [1], or to bacteria and microscopic algae, as protists. In 1969, Wittaker [2] suggested a novel five kingdom system, specifically plants, animals, fungi, eubacteria and archaebacteria. Today, the kingdom *Fungi* is considered to comprise four phyla, *Chytridiomycota*, *Ascomycota*, *Basidiomycota* and *Zygomycota* [3]. An estimated total of 1.5 million species exists, including fungi-like species from other kingdoms [4]. All fungi share certain properties; they are absorptive heterotrophs, they lack photosynthesis as well as phagotrophy, their spores are usually chitinous and they are often growing as filaments with several nuclei, one notable exception being yeasts [5]. Due to their diverse nature, fungi can be found almost anywhere in the environment, growing as saprobes, parasites or mutualists [6], where they play crucial, if not essential, roles in the ecosystem [4]. Examples include lignin degradation [7], formation of mycorrhizae [8], and they were possibly playing a crucial part in the first colonization of land by plants [9].

The kingdom *Fungi* is monophyletic, i.e. the species have developed from a common ancestor, which supposedly happened at least 400-500 million years ago [1], possibly 1 000 million years ago [10]. However, fungal properties have evolved in several other groups of microorganisms, which only recently have been distinguished from the true *Fungi* as a result of advances in molecular science. These include the *Oomycota* (water moulds), *Dictyosteliomycota* (cellular slime moulds), and *Myxomycota* (plasmodial slime moulds) [6].

The growth of filamentous fungi is of special interest since it differs quite dramatically from yeasts and bacteria. The most obvious characteristic is the filament, the hypha, which in submerged cultivations can cause elevation of broth viscosity, and considerable mass and heat transfer problems [11]. The fungi can also attach to impellers, baffles, and walls of fermenters [12] or grow as spheres of intertwined hyphae, called pellets (Fig. 2.1), a few mm in diameter. Fungal species differ explicitly in terms of form and colour of the colonial growth on solid substrates, where the growing fungus might be viewed as “the growth of a multicellular integrated organism” [13]. This probably holds true for submerged growth as well, which could have major implications for cultivation methods, particularly regarding inoculation.

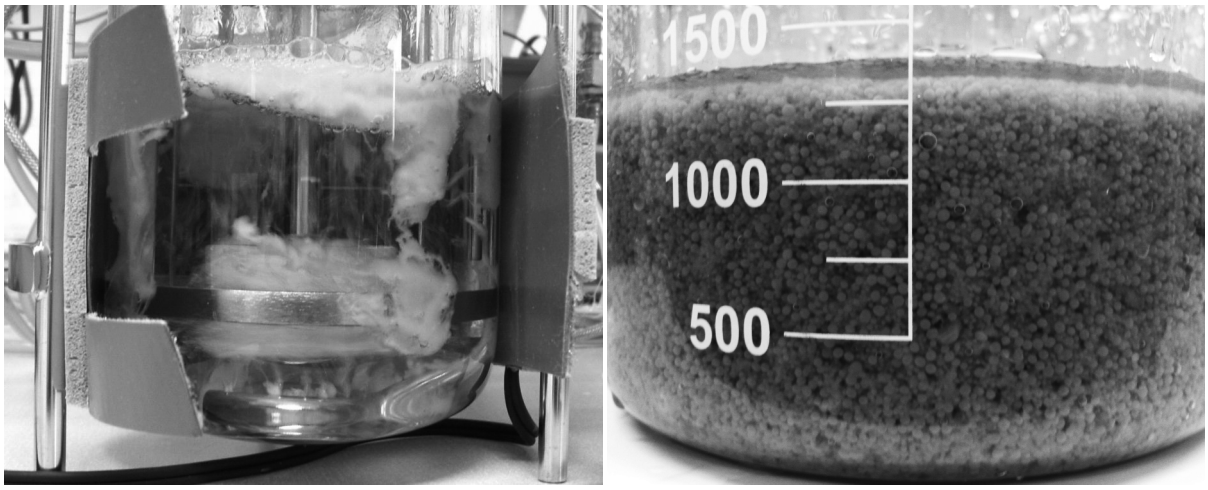

Figure 2.1: Submerged filamentous fungal cultivation with two different outcomes: attachment of mycelia to reactor equipment (left) and freely suspended pellets (right, photo by Jorge A. Ferreira).

2.2. An overview of *Zygomycetes*

The phylum *Zygomycota* comprises two classes; *Trichomycetes*, mainly living in the guts of arthropods and *Zygomycetes*, found worldwide as saprobes, part of mycorrhizae, and as parasites. Species belonging to *Zygomycetes* are either growing as filaments (usually without septa) or as yeasts. Asexual reproduction occurs by production of spores or conidia, while sexual reproduction (and sometimes also asexual reproduction) is accomplished by production of zygospores [14], the structure after which the class *Zygomycetes* is named [15]. The class is further divided into ten orders, *Basidiobolales*, *Dimargaritales*, *Endogonales*, *Entomophothorales*, *Geosiphonales*, *Glomales*, *Kickxellales*, *Mortierellales*, *Mucorales*, and *Zoopagales* [14]. The classification of the entire phylum is presently discussed by the scientific community [1, 3, 16]. Of particular interest, from an environmental point of view, is the mycorrhizae forming *Glomales*, which has been proposed to be a separate phylum (*Glomeromycota*) [17]. Figure 2.2 illustrates the fungal hierarchy, focusing on *Zygomycetes*.

Modern phylogenetic studies have disclosed *Zygomycetes* and the entire phylum *Zygomycota* (closely associated with *Chytridiomycota*) as the earliest emerging *Fungi* [18]. This is also reflected by their relatively simple structure, rendering them to be considered “evolutionary primitive”. The simpler structure holds some advantages, however, allowing zygomycetes to rapidly extend their hyphae and quickly colonise new areas in the search of substrates. It

should be noted, though, that some orders, including the *Mucorales*, possess the ability to produce septa under certain conditions, similar to the higher fungi [19].

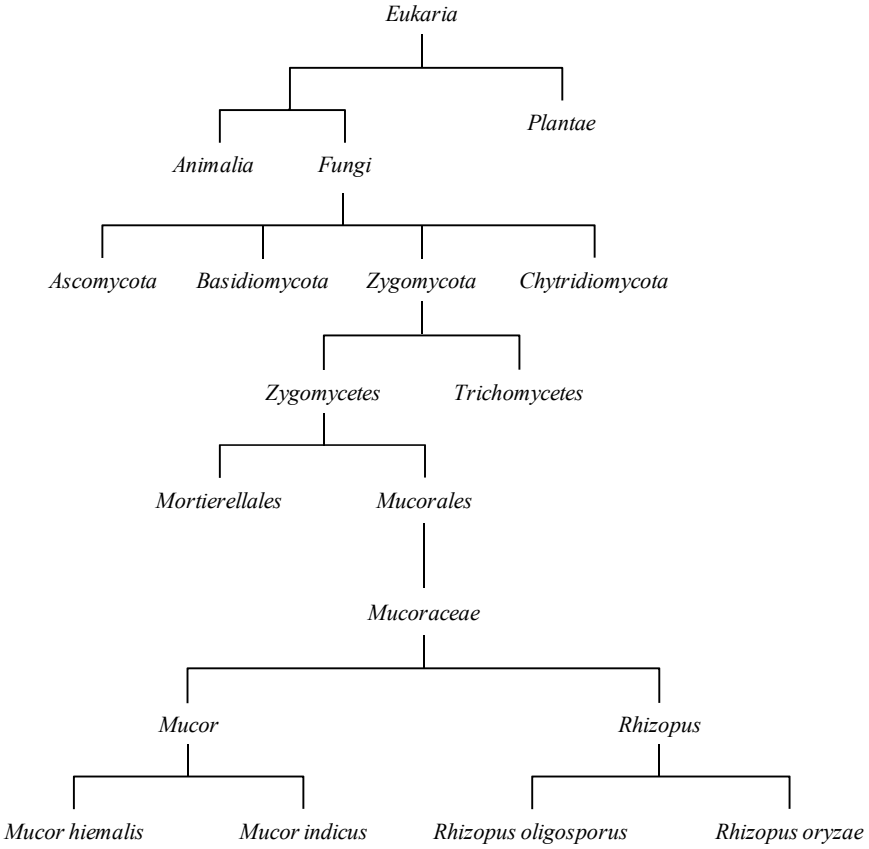

Figure 2.2: Classification of the organisms of biotechnological interest within the fungal kingdom, with a focus on *Zygomycetes*, and specifically the microorganisms studied in the current work. The classification of the entire phylum is presently being discussed by the scientific community [1-3, 14, 16].

The various zygomycetes are able to grow in a wide range of environments. Some species 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 [15]. Some species of the order *Mucorales* are able to grow under anaerobic conditions, while others require aerobic conditions. Furthermore, while the majority of the zygomycetes only grow at high water activities, some of them are able to grow in salt concentrations of at least 15 % [15].

In the present thesis, two different strains of zygomycetes were studied: *Mucor indicus* and *Rhizopus* sp.

2.2.1. Zygomycetes: Mucor indicus

Among the zygomycetes and especially the *Mucor* species, *M. indicus* (Fig 2.3) (formerly *M. rouxii*) is one of the most investigated species and has thus been subjected to a number of studies.

One of the more promising study fields concerning *M. indicus* is production and conversion of polyunsaturated fatty acids. In particular, the production of γ-linoleic acid (C18:3) has been popular. Linoleic acid is an important fatty acid and is also a precursor of long-chain polyunsaturated fatty acids. When using *M. indicus*, no special considerations need to be applied since it is produced under normal growth conditions. By using conditions supporting lipid production, γ-linoleic acid contents of ca 14 % of the fungal biomass have been acquired in specific wild-type strains [20]. Solid-state fermentation of the zygomycetes has also resulted in promising results; production of approximately 6 mg linoleic acid/g rice bran has been reported [21].

Another research focus involving *M. indicus* is the search for antimicrobial compounds. This includes attempts of biotransformation, in which *Mucor* species have been tested. Using *M. indicus* for transformation of oleanolic acid, a well known drug with antimicrobial, antiviral, anti-inflammatory, and antitumor properties, into derivatives with higher antimicrobial activities, was successful [22]. Since the cell walls of zygomycetes contain chitosan (Section 2.4.1), attempts have also been made to use chemically treated biomass of *M. indicus* for antimicrobial applications. For instance, after treatment with sodium hydroxide and acetic acid, the remaining biomass was added to a cotton fabric, which then exhibited antibacterial properties [23].

M. indicus has also been tested for adsorption of heavy metal contaminants in industrial wastewaters. Dead biomass treated with sodium hydroxide as well as live biomass were tested for adsorption of single and multiple heavy metal ions, with successful results [24]. Ion exchange, strongly dependent on the carboxyl, amino, and phosphate functional groups in the fungal cell wall, has been suggested to be the main mechanism behind the adsorption [25]. The initial adsorption process was very fast; ca 70 % of the maximal adsorption was reached within 10 minutes. Notwithstanding, the total adsorption was strongly dependent on pH and temperature, with optimal results at pH 5.0-6.0 and at a temperature below 30 °C [26].

Adsorption has further been shown to be influenced by the dimorphic nature of *M. indicus* (Section 2.3), and was most efficient in the filamentous growth form [27]. Furthermore, *M. indicus* has been tested for the adsorption of oil contaminants in water. Also this effect was found to be influenced by pH, but with a maximum at pH 3.0 [28]. The adsorption capacities were nevertheless below those exhibited by chitosan and walnut shell media [29].

Significant research on the phenomenon of dimorphism in *M. indicus* is described in detail in Section 2.3.

In this thesis, the *M. indicus* strain CCUG 22424 (Culture Collection University of Gothenburg) originally isolated from rice fermentations, was used (Papers I-III and V).

Figure 2.3: A mixture of yeast-like and filamentous growth forms of *M. indicus* in submerged cultivation. The bar corresponds to 100 µm. Picture taken with dark field microscopy.

2.2.2. Zygomycetes: Rhizopus sp.

Another well-known family within the zygomycetes is *Rhizopus* (Fig 2.4), whose members have been subjected to numerous studies.

One of the better studied characteristics of the *Rhizopus* family is lactic acid production, which has a long history [30]. The research interest in lactic acid production by this family is due to the *Rhizopus'* process holding a major advantage over the currently employed bacterial processes: high optical purity of the product is obtained, when using the correct *Rhizopus* strains [31]. However, problems associated with yield, productivity, and control of the fungal morphology, have retained lactic acid production by *Rhizopus* in the research phase. Nevertheless, optimisation of process parameters and studies on the metabolism of the family might solve these problems and result in a feasible process [32].

Rhizopus has further been studied for production of other organic acids, most notably fumaric acid, which is produced in a redox process running the TCA cycle in reverse. Other TCA associated organic acids are also produced but in lower quantities. Nonetheless, production of these acids requires triggering in the form of stress to acquire high productivity and yield. The most important stress factor determined thus far is severe nitrogen limitation. When the nitrogen source is completely consumed, the fumaric acid production may reach 100 g/l. Furthermore, since production of fumaric acid includes fixation of carbon dioxide, additional carbonate stimulates the process. To satisfy the naturally high demands of energy of the process, aerobic conditions are required which, when accounted for, results in a theoretical yield of 1.5 mol fumaric acid per mol consumed glucose. The actual production by *Rhizopus* has been reported to be at least 1.32 mol fumaric acid per mol consumed glucose [33, 34].

Members of the *Rhizopus* family have also been targeted for extracellular production of carbohydrate degrading enzymes, which has been investigated with satisfactory results; most significantly, cellulases have been identified, albeit comparably few and with properties diverging from those previously identified in other species [35]. A more thorough investigation [36] confirmed these findings, augmenting the research scope to include other carbohydrate degrading enzymes as well. Hence, an enzyme production significantly different from that of higher fungi was revealed. Evidently, *Rhizopus*, and most likely several other

zygomycetes, displays arrays of enzymes associated with the more easily degradable carbohydrates, such as hemicelluloses and cell wall material belonging to other fungi [36].

Rhizopus is also known to play key roles in the retting of flax fibres, a process dependent on the removal of pectin and hemicellulose from flax stems. In the natural process, several species are involved, as illustrated by the various fungi that have been isolated [37]. The most effective was a strain of *Rhizopus*, mainly because of its production of a pectinase, or more specifically a polygalacturonase [38].

The *Rhizopus'* specialization towards easily degradable materials has its drawbacks from a human standpoint, as is illustrated by *Rhizopus stolonifer*. This infamous fungus is encountered worldwide and is the main cause of *Rhizopus*-soft rot, a major starting point of food spoilage in harvested ripe fruits and vegetables. Remarkably, *R. stolonifer* can cause food spoilage even after considerable heat treatment as its produced enzymes are very stable; enzymatic activity has been detected even after 40 min of heat treatment at 100 °C [15].

In the present thesis, the *Rhizopus* strain used is consistent with Zygomycete IT, isolated by Millati et al. [39]. The strain, originally isolated from Indonesian tempe, was used in Papers III and IV.

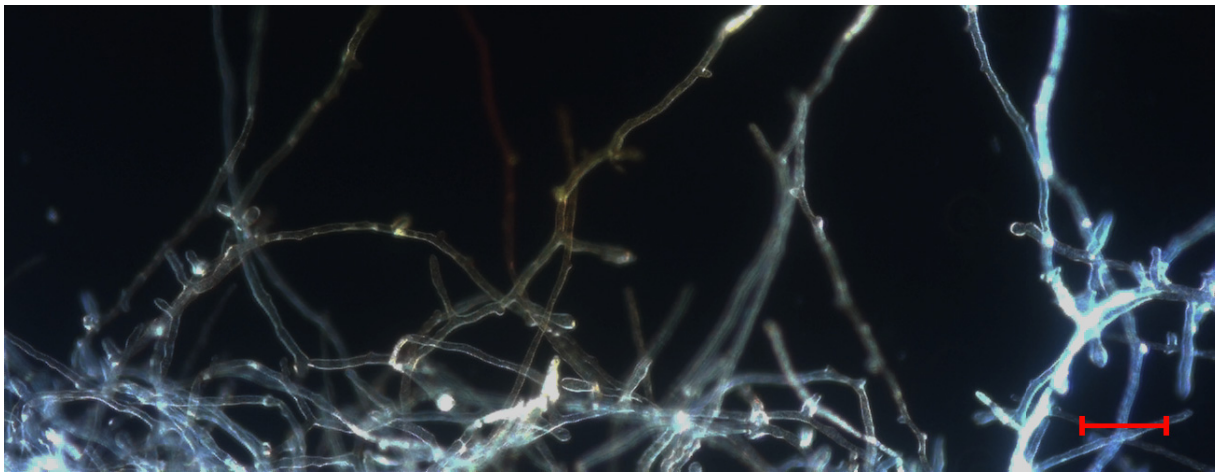

Figure 2.4: *Rhizopus sp.* in submerged cultivation. The bar corresponds to 100 µm. Picture taken with dark field microscopy.

2.3. Dimorphism – two growth forms

The phenomenon dimorphism, the ability of a microorganism to form either yeast cells (henceforth referred to as yeast-like to avoid misunderstanding) or hyphae, both true and pseudohyphae, is found in several phyla. A few examples include: *Candida albicans*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Ophiostoma ulmi*, *Paracoccidioides brasilienses*, *Sporothrix schenckii*, and *Wangiella dematitidis* [40]. Nevertheless, dimorphism amongst the zygomycetes is a fairly rare phenomenon, with the exception of the genus *Mucor* [40]. The microorganism of choice was thus *M. indicus*, correctly identified as dimorphic by Pasteur, Reess, and Fitz, independently of each other around 1870 [41].

2.3.1. Induction of yeast-like and filamentous growth

Different *Mucor* strains react very differently to various environmental conditions. In general, anaerobic conditions induce yeast-like growth, while aerobic conditions induce filamentous growth [42]. Similarly, compounds inhibiting mitochondrial processes involved in energy production, such as oxidative phosphorylation, have been confirmed to force *Mucor* into yeast-like growth even under aerobic conditions [42].

For growth of *M. indicus*, several factors have been identified. By using a partial pressure of at least 0.3 atm CO_2 under anaerobic conditions, Bartnicki-Garcia succeeding in inducing *M. indicus* to grow as yeast-like cells [43]. However, the presence of oxygen negated this effect, allowing the zygomycete to develop filamentous growth, and partial pressures of CO_2 below 0.3 atm caused *M. indicus* hyphae to produce spores, not yeast-like cells [43]. The concentration of hexose sugars also had profound effects. Increasing the sugar concentration, resulted in shorter filaments, and at glucose concentrations above 80 g/l only yeast-like cells developed [44]. Furthermore, the choice of sugar was important; glucose, fructose, mannose and galactose, in falling potency, guided the fungus towards yeast-like growth [44]. The nitrogen source has also been shown to influence the growth forms of *M. indicus*. Depending on the amino acid used, the microorganism alternated between an even mix of yeast-like and filamentous growth and purely filamentous growth [45]. Another important environmental factor influencing the growth of *M. indicus* has been identified: a culture starting with a high spore concentration has a tendency to induce the fungus to grow predominantly in the yeast-like form [46].

By varying the initial spore concentration (Table 2.1) it was possible to provoke *M. indicus* to grow aerobically in three different forms: purely filamentous, mostly filamentous with a few yeast-like cells, and mostly yeast-like with a few shorter filaments. In order to attain a culture with purely yeast-like cells, anaerobic conditions had to be employed. Figure 2.5 illustrates the different morphological mixtures (Paper I).

Table 2.1: Conditions employed to induce different growth forms of *M. indicus* (Paper I).

Growth morphology	Spore concentration (spores/ml)	Aerobicity
Purely filamentous	$1\text{-}2 \times 10^5$	Aerobic
Mostly filamentous	$6\text{-}18 \times 10^5$	Aerobic
Mostly yeast-like	$6\text{-}8 \times 10^6$	Aerobic
Purely yeast-like	$6\text{-}8 \times 10^6$	Anaerobic

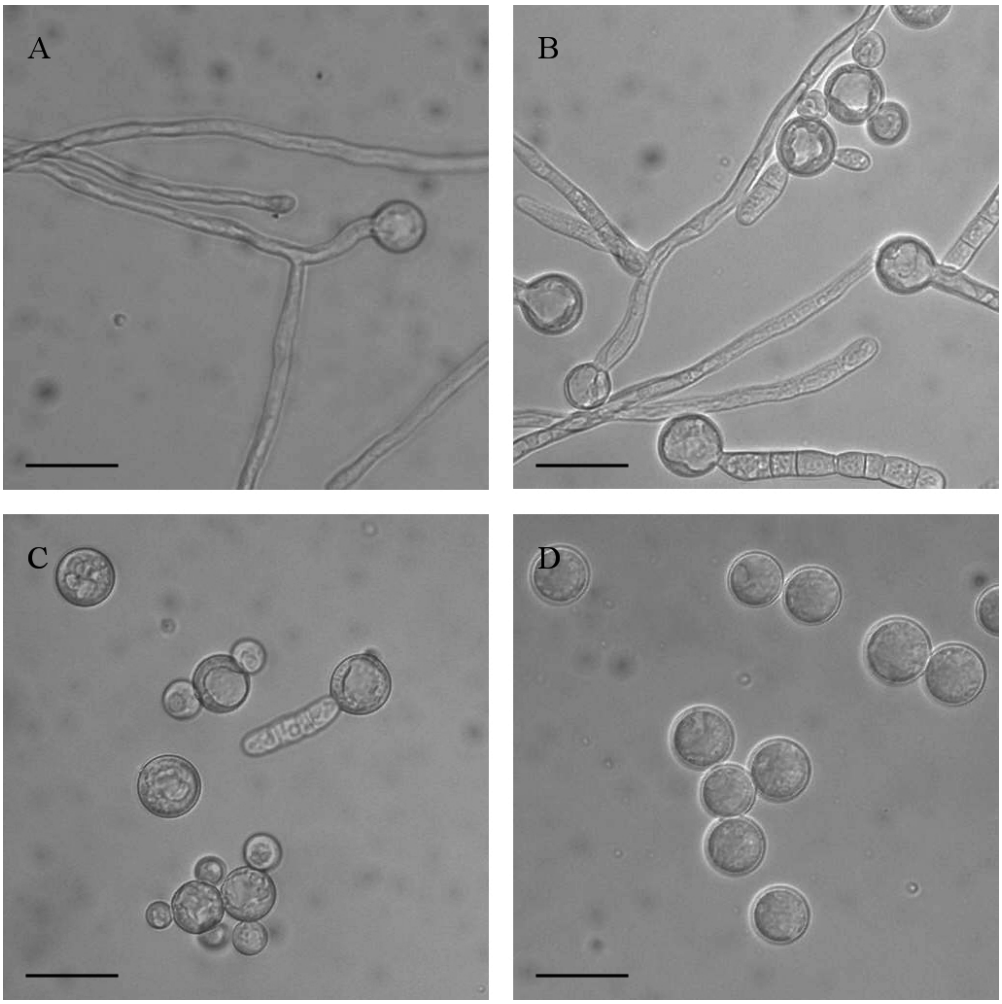

Figure 2.5: Different growth forms of *M. indicus*. (A) Purely filamentous, (B) mostly filamentous, (C) mostly yeast-like and (D) purely yeast-like (Paper I). The bars correspond to 25 µm.

2.3.2. Spore germination and initial growth

The first step in the germination process of *M. indicus* spores, i.e. the volumetric expansion of each activated spore [47], is initiated without an apparent lag phase and continues exponentially [48]. The step does not entail swelling of the spores, but active biosynthesis of RNA and proteins, utilising stored mRNA as template. The first step can be divided into two parts: change from ellipsoidal to spherical form, and expansion of the spherical spore by a factor of approximately 20 [48]. The initial biosynthesis also includes building a vegetative cell wall and rupture of the old spore wall [47]. The synthesis of DNA is not immediate, but is initiated 30-45 minutes prior to polarized growth, i.e. budding or production of hyphae [48]. The polarized growth is initially not detectable morphologically [49], but on the genetic level, the fungus should already be on the path toward that particular growth form.

2.3.3. Yeast-like and filamentous growth

The initiated polarized growth (Section 2.3.2) can either be maintained to acquire filamentous growth or be made to cease, leading to yeast-like growth [48]. Several differences between the two growth forms have been discovered, most of them related to the production and the characteristics of the cell wall (Section 2.4). However, no major differences have been observed in the metabolite production between the two growth forms, or mixtures of them. Smaller differences were detected in terms of inhibitor tolerance in *M. indicus*, and the conversion of furfural and acetic acid differed between the growth forms in this species as well (Paper I). Interestingly, in the same experiment, the initial growth morphology of *M. indicus*, ranging form purely yeast-like to purely filamentous, was maintained for more than one day, regardless of whether the aerobicity was maintained or changed from aerobic to anaerobic or vice versa (Paper I). Thus, once the growth form had been established, probably on a genetic level, it seemed to be stable.

Two other differences between the growth forms should also be mentioned. First, the intracellular cAMP levels differ between the two growth forms [50]; anaerobic yeast-like cells hold three to four times higher concentrations than aerobic hyphae [51] and addition of cAMP constrained the growth of *M. indicus* to the yeast-like form, regardless of whether aerobic or anaerobic conditions were employed [51]. The other difference concerns the lipid composition of *Mucor*. In *M. hiemalis*, filamentous cells show higher concentrations of

unsaturated fatty acids, in contrast to higher concentrations of saturated fatty acids in yeast-like cells [52, 53]. The same trend was also observed in *Mucor circinelloides* [54]. Hence, it is not unreasonable to assume that the same behaviour may be found within other species of *Mucor*. Furthermore, it has been confirmed that inhibition of lipid synthesis via cerulenin prevents yeast-like cells of *Mucor recemosus* from changing into the filamentous growth form [55].

The underlying reason for the development of the two different growth forms of *Mucor* is yet to be unveiled. The thicker cell wall of the yeast-like form (Section 2.4) along with the tendency of *M. indicus* to form yeast-like cells in highly toxic media, such as orange peel hydrolysate (Paper III), make it conceivable that the yeast-like form may represent a "survival mode" for the fungus. Compared to the more durable spores, the advantage of the yeast-like form is possibly the highly active growth capacity and the capacity of the fungus to adapt to new conditions.

2.3.4. Gene expression and dimorphism

The great differences between yeast-like and filamentous growth forms of *Mucor*, imply a gene regulation regime [56]. Information is however scarce, particularly concerning transcription, and very little information is available in the literature. What is obtainable mostly concerns various kinases, not uncommonly associated with cAMP, their activation [57], and the resulting molecules [42]. Nevertheless, a study on the expression of the two genes coding for the different subunits of one specific cAMP-dependent kinase [58], revealed that in yeast-like cells grown anaerobically, the expression of both genes had increased considerably. During transition from yeast-like to filamentous growth, the expression of the gene coding for the regulatory subunit approximately doubled.

In order to study the differential gene expression between the yeast-like and the filamentous growth forms of *M. indicus*, the suppression subtractive hybridisation (SSH) technique, presented by Diatchenko et al. [59], was employed (Paper V). According to Diatchenko et al. [59], the technique allows only exponential amplification of mRNA sequences, expressed differentially between the two investigated mRNA populations. Consequently, even rare sequences can be compared between samples, with the background noise significantly reduced.

By employing the SSH method for yeast-like and filamentous forms of *M. indicus*, and performing a BLAST analysis, some interesting information was revealed (Table 2.2, Fig. 2.6). In the yeast-like growth form, a sequence was detected that was not expressed in the filamentous growth form. However, this could not be confirmed in the control experiments. Notwithstanding, four sequences were detected, showing a higher expression in the filamentous growth form than in the yeast-like form. The full sequences are listed in Paper V, Table 2. One of the sequences did not have any matching sequence in the Genbank®. The remaining sequences shared similarities with sequences originating from another member of the *Mucor* genus, *M. circinelloides*. Unfortunately, information on which genes these sequences code for was not available.

Table 2.2: Results from the BLAST analysis of cDNA sequences from SSH, comparing yeast-like and filamentous growth morphologies of *M. indicus*. Only sequences with a higher gene expression in the filamentous growth form were detected. Sequences failing the quality controls have been excluded (Paper V).

Sequence	Similar to accession (Genbank®)	Organism	mRNA length (bp)	Similarity (%)	Obtained cDNA length (bp)
1.2	GR543143.1	*M. circinelloides*	396	89	681-737
2.9	Unknown				680
2.13	GR550798.1 & GR535467.1	*M. circinelloides*	560 – 726	93	396
2.21	GR550259.1	*M. circinelloides*	770	91	365

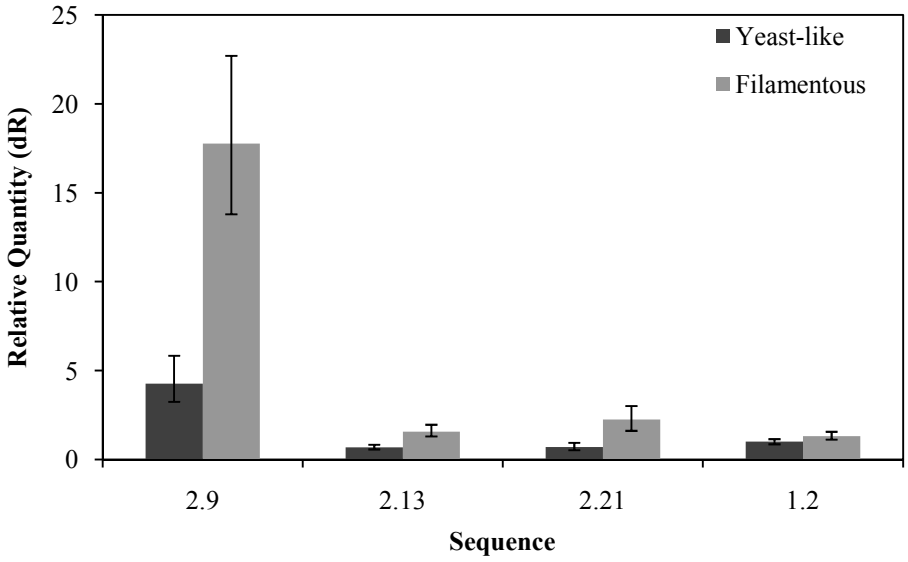

Figure 2.6: Relative differences in gene expression between yeast-like and filamentous growth morphologies of *M. indicus* analysed with quantitative qPCR (Paper V).

The complete gene, containing fragment 2.9, was identified using RACE-PCR and knocked down by siRNA. The tendency of the cells to develop yeast-like or filamentous growth was not changed, but a shape abnormality was detected in the hyphae emerging from previously yeast-like cells, possibly indicating that the gene is involved in the development of new hyphae and/or the formation of yeast-like cell buds. The remaining confirmed sequences might be used for identification of the complete genes, which in turn may lead to improvements in two different fields. If they contain regulatory gene(s) for filamentous or yeast-like growth, these could be knocked out [60], allowing only one type of growth, which may be advantageous in industrial applications. Furthermore, if all genes involved in the regulation of dimorphism were mapped, considerable progress in that particular field could probably be made. Even if all identified sequences would be originating from genes differentially expressed between the different forms of growth, and not from the regulatory genes, the data would still be useful. For instance, important information on the differences between yeast-like and filamentous growth, e.g. possible differences in their metabolism, would still be revealed.

2.4. The cell wall of *Zygomycetes*

The cell wall is a component of the fungal cell of utmost importance since it is crucial for maintaining the integrity of the cell. The general main components are carbohydrates (80 %), proteins (3-20 %), lipids, pigments, and inorganic salts [61]. The structure of the cell wall can be divided into two main types depending on their function: fibrillar, consisting of chitin, cellulose, and β-glucans, and matricidal, consisting of β-glucans, α-glucans, chitosan, polyuronides, glycoproteins, lipids, inorganic salts, and pigments [61]. One of the hallmark characteristics of some orders of *Zygomycetes* (including *Mucorales*), is the presence of high amounts of chitosan, which in some species have been known to exceed 30 % of the total cell wall mass [62].

2.4.1. Chitin and chitosan in the cell wall

Chitin and chitosan are two structurally similar compounds; chitin is mainly made up of N-acetyl-glucosamine (GlcNAc) residues, while chitosan mainly consists of glucosamine (GlcN) residues (Fig. 2.7) [61]. The biosyntheses of chitin and chitosan are also very similar. In both cases [63], chitin is produced from uridine diphosphate-N-acetyl-glucosamine via a group of

different trans-membrane chitin synthetases [64], associated with the micro-vesicle chitosomes [65]. For production of chitosan, the newly produced chitin is deacetylated by chitin deacetylase before crystallisation [66, 67]. Chitosan is thus produced by the "tandem action" of chitin synthetase and chitin deacetylase, as aptly put by Davis and Bartnicki-Garcia [66]. Plenty of research concerning the utilisation of fungal chitosan has been carried out in recent years (Section 5.4.).

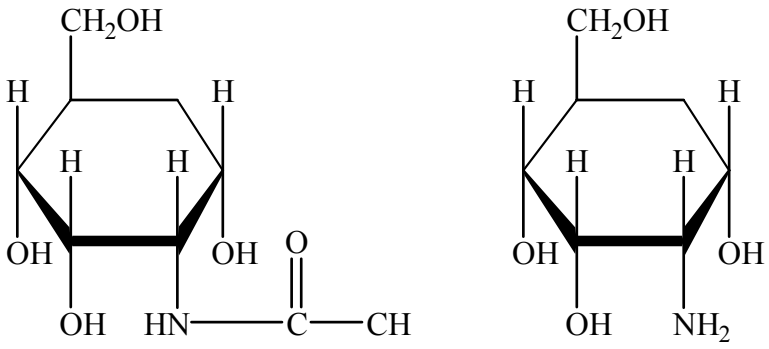

Figure 2.7: Structure of GlcNAc (left) and GlcN (right), the main constituents of chitin and chitosan, respectively [61].

2.4.2. Influence of dimorphism on the zygomycetes cell wall

A major factor having an impact on the cell wall composition of *M. indicus* is the dimorphic behaviour of the fungus. The most obvious effect of this is probably the amount and thickness of the cell wall. Electron microscopy has revealed that the cell wall of the yeast-like cell is comparatively thick, being more than 0.5-1 μm across, and having a double-layered appearance. The filamentous cell wall is thinner, 0.05-0.1 μm across, and is clearly single-layered [68]. The same trend was also observed in the alkali insoluble material (AIM), comparing well with the total amount of cell wall (Fig 2.8) (Paper I).

Interestingly, the proportions between chitosan and chitin are known to be influenced by the dimorphism of *M. indicus*, together making up a smaller portion of the cell wall of the yeast-like growth form (27.9 % and 8.4 % respectively) than of the cell wall of the filamentous growth form (32.7 % and 9.4 % respectively) [68]. The influence of dimorphism on the composition could be greatly enhanced (Fig 2.8), leading to a maximum of chitosan in mixed cultures (Paper I). However, the analytical methods differed and the growth conditions were different, which naturally has an impact on the final biomass yield and morphological

behaviour [69]. Other compounds worth mentioning that differ in concentration between the growth forms include mannose, lipids, protein and purines and pyrimidines [68].

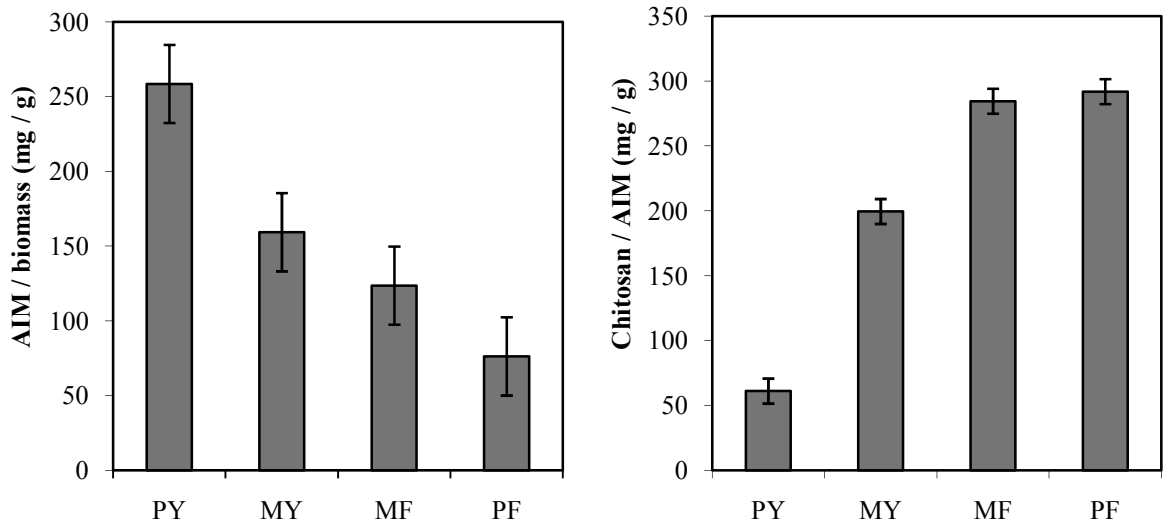

Figure 2.8: AIM content of the biomass (left) and chitosan content of the AIM (right) of purely yeast-like (PY), mostly yeast-like (MY), mostly filamentous (MF) and purely filamentous growth forms of *M. indicus* (Paper I). The error bars represent 95 % confidence intervals, using pooled standard deviations.

2.5. Current applications of zygomycetes

A major application of zygomycetes can be observed in the production of various fermented foods; fermenting is a process traditionally most common in Asia [70] (Table 2.3). Other products heavily relying on zygomycetes are tofu, fermented with *Mucor racemosus*, *Rhizopus chinensis*, and *Actinomucor elegans*, and sofu, a moulded version of tofu, where several identified zygomycetes species are used [15].

Table 2.3: A selection of fermented foods utilising zygomycetes [70].

Dish	Country	Description	Zygomycetes
tou-shi hamanatto	China	condiment	*Mucor sp.*
tempe kedele	Indonesia	side dish	*Rhizopus oligosporus, Rhizopus oryzae, M. indicus*
tempe bongkrek	Indonesia	side dish	*Rhizopus sp.*
yakju and takju	Korea	wine	*Rhizopus sp.*
huan-ju	China	wine	*Rhizopus sp.*
bai-ju	China	spirit	*Rhizopus sp.*
chiang jnard	India, Nepal	beer	*M. circinelloides, M. indicus, R. oryzae*

There are several other commercially important applications of various zygomycetes. These include lactic acid production by *Rhizopus* sp. [71], production of lipases by several species of *Mucor* and *Rhizopus*, i.e. *R. arrhizus*, *R. delmer*, *R. japonicus*, *R. niveus*, *R. oryzae*, *M. javanicus*, and *M. miehei* [72, 73], as well as proteases produced by several species of *Rhizopus* and *Mucor* [72]. The enzymes are used mainly in the detergent industry, food and dairy production, the leather industry, and the medical industry [72].

Novel applications for zygomycetes are presently being developed, which are discussed in the later chapters.

3. SUBSTRATES USED FOR ZYGOMYCETES CULTIVATION

The substrate requirements for cultivation of zygomycetes are very similar to those of *S. cerevisiae*; they should be fermentable, cheap, readily available, and contain as much of the required nutrients as possible. Political and environmental considerations have further included a non-edible feedstock, preferably based on waste products, as a desired prerequisite [74].

The most commonly tested substrates are hexoses and pentoses from lignocellulosic sources. These include pure hydrolysates [75] as well as process streams, such as spent sulphite liquor [76]. Other substrates are molasses (from sugar cane and sugar beet) and sucrose, notwithstanding that invertase was required for the utilisation of sucrose [77]. Starch-based effluents have also been tried [78]. Finally, soy bean and starch materials, e.g. rice, grains, and potato, should also be mentioned as they are used as substrate for cultivation of zygomycetes for human food [70].

3.1. Lignocelluloses

Lignocellulosic materials fit all of the desired characteristics of a next generation feedstock as discussed above, and have been studied as a substrate for bioethanol production for several decades [79]. This is not surprising, considering their abundance. With an estimated annual production of 200×10^9 ton plant biomass and a lignocellulosic content of 90 %, lignocellulose is the most abundant biomass material in the world [80]. An estimated 4-10 % ($7.2\text{-}18\times10^9$ ton), is considered "potentially accessible" [80], which may be compared with the total amount of crude oil, 3.6×10^9 ton, produced in 2008 [81].

3.1.1. Composition of lignocelluloses

Lignocellulosic materials form a heterogeneous group, where the chemical composition is correlated to the plant species as well as the function of the different parts of the plant. They all contain cellulose, hemicellulose, and lignin in varying degrees. Generally, cellulose and hemicellulose comprise 55-75 % of the dry weight, while lignin constitutes 10-30 % of the lignocellulosic material [82].

Cellulose and hemicellulose are polymers made up of sugar monomers, although monomers and bonds differ between the two. Cellulose consists of β-1,4-linked glucose monomers with no branching, forming long (6-14,000 units) ribbon-like microfibrils. In contrast, hemicellulose is the joint name of several different irregular polymers. The most common monomers include glucose, mannose, galactose, xylose and arabinose. Several different bonds are present, and side chains are common in these matrix polymers [83].

3.1.2. Pretreatment

In order to release the sugar monomers, hydrolysis is required. For successful hydrolysis of lignocelluloses, an adequate pretreatment is essential, especially if the hydrolysis is enzymatic [82, 84, 85]. Chemical hydrolysis requires pretreatment as well, at least in the form of size reduction of the feedstock [86]. The pretreatment methods can be divided into physical, physico-chemical, chemical, and biological, depending on their main mode of action (Fig. 3.1) [85].

White-rot fungi are mostly used for biological pretreatment, as this group of fungi possess the ability to degrade lignin, with an extensive array of specialised enzymes. The process increases the yield from the subsequent hydrolysis but requires several weeks, and at least partial loss of cellulose and hemicellulose is to be expected [87]. Biological pretreatment can also be used as a preparatory step prior to other pretreatments, in order to decrease the energy demands of these [88].

Chemical pretreatments include acid hydrolysis, alkaline hydrolysis, ozonolysis, oxidative delignification, and the organosolv process [85]. Acid hydrolysis pretreatment operates at high temperature and low acid concentration, and vice versa, resulting in successful hydrolysis of mainly the hemicellulose sugars, while alkaline hydrolysis mainly removes intermolecular bonds, decreasing the degree of polymerisation, and generally lowers the lignin content. Ozonolysis causes degradation of the hemicellulose and the lignin in lignocellulosic material. The organosolv process involves an organic solvent mixed with an acid catalyst, and mainly attacks the intramolecular lignin and hemicellulose bonds [84].

Physico-chemical pretreatment is a combination of chemical and physical processes, and a typical example is steam pretreatment, with or without explosion [89]. In steam explosion

(autohydrolysis), the lignocellulosic material is subjected to high-pressure steam for a few seconds up to a few minutes; addition of chemicals can also be employed to improve the process. The overall effect of the process is degradation of hemicellulose and lignin [84]. Other physico-chemical pretreatments include ammonia fibre explosion, CO_2 explosion, liquid hot water pretreatment and microwave-chemical pretreatment [85].

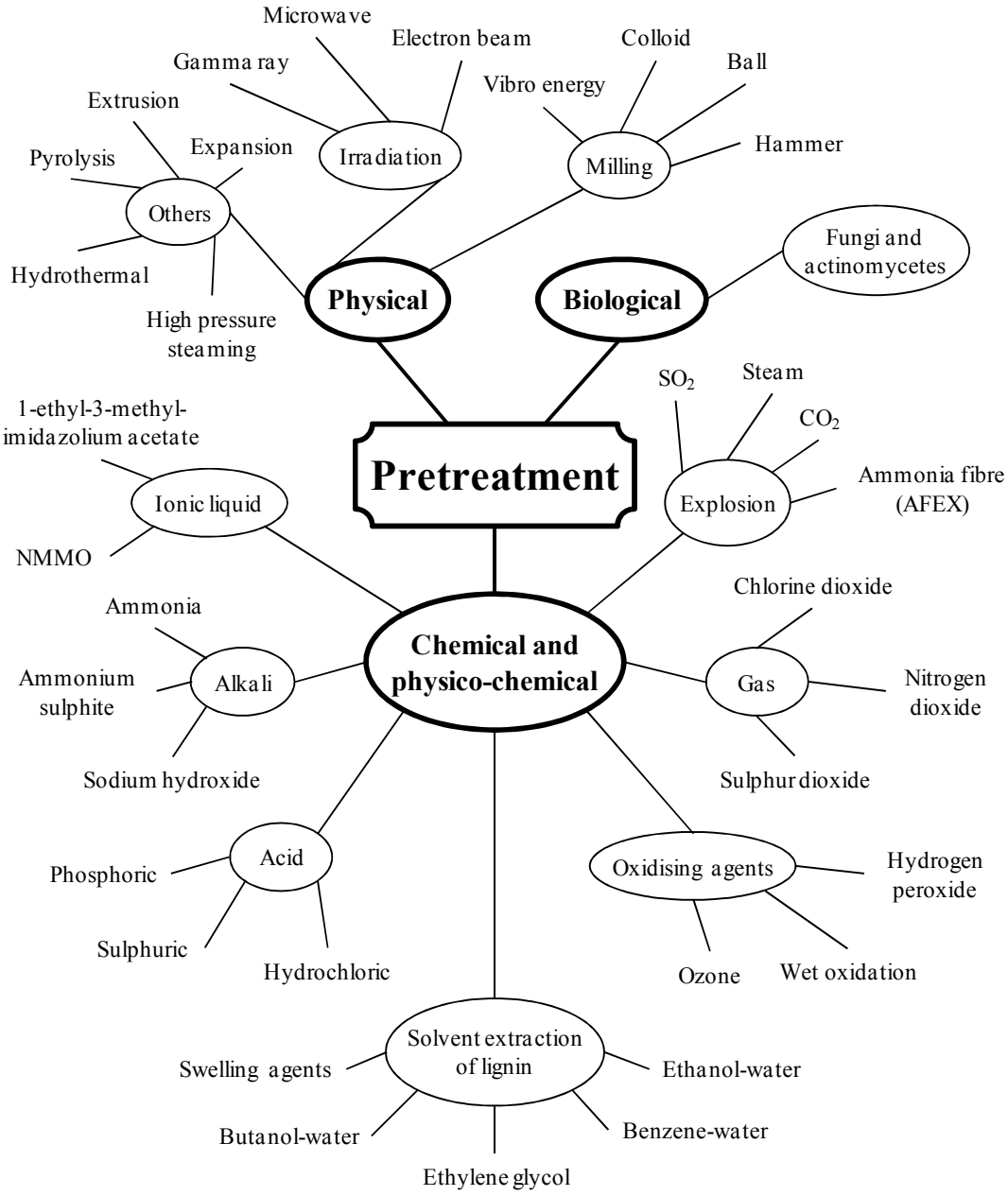

Figure 3.1: Pretreatment methods for lignocelluloses, divided into physical, biological, chemical, and physico-chemical procedures [85, 90].

The most common physical pretreatments are milling and irradiation. However, irradiation faces difficulties in industrial applications and is considered expensive, while milling procedures, such as ball milling, involves significant energy costs [85]. Nonetheless, some form of size reduction is required prior to most of the other pretreatments [87].

Recently, a pretreatment utilising N-methylmorpholine-N-oxide (NMMO) was discovered and consequently, several investigations on different materials were carried out. These include bagasse [91], cotton [92], softwood (e.g. spruce), and hardwood (e.g. birch) [93] (Paper II, see also Section 3.2). Furthermore, the effect of different NMMO concentrations on cotton has been investigated [94]. NMMO dissolves cellulose and is currently used industrially for production of Lyocell [95, 96]. The dissolution, in theory a purely physical process, is strongly dependent on the NMMO / water content. At the highest NMMO concentrations, above 83 % (w/w), the cellulose fibres are fully dissolved, but can afterwards be precipitated by addition of water. The pretreatment notably decreases the crystallinity of the cellulose [97].

Pretreatment with NMMO has two disadvantages: it is comparatively expensive and it is based on petroleum. Thus, to make pretreatment of NMMO economically and environmentally feasible, an effective and efficient recycling process is crucial. Furthermore, while NMMO is biodegradable [98], excessive concentrations left in the medium may cause a change in the metabolic flux, resulting in some of the sugar being shunted from the ethanol pathway into the glycerol pathway (Fig 3.2). The relatively strong oxidative property of NMMO [99] is probably the underlying reason (Paper II).

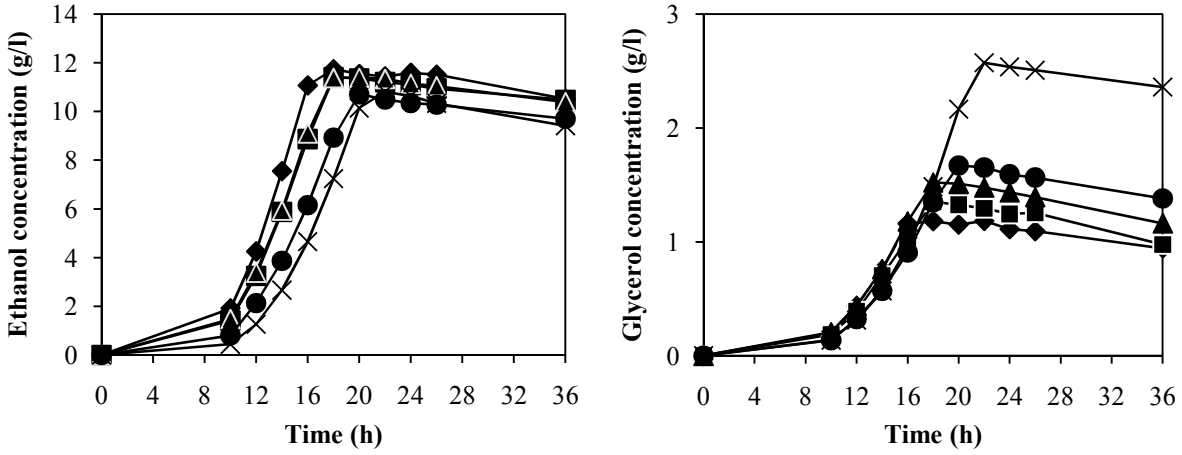

Figure 3.2: The production of ethanol and glycerol from 30 g/l glucose by aerobically cultivated *M. indicus* in the presence of 0 % (♦) 1 % (■) 2 % (▲) 4 % (●) 8 % (×) NMMO. The symbols represent single values from the different batches (Paper II).

3.1.3. Hydrolysis

Hydrolysis can be carried out either chemically, where acid hydrolysis dominates, or enzymatically [86]. Regardless of the method, the desired effect of the hydrolytic reaction is always release of sugar monomers from the cellulose and the hemicellulose.

Acid hydrolysis, normally catalysed by sulphuric acid, can be performed either as concentrated or dilute acid hydrolysis. In concentrated acid hydrolysis, acid concentrations of 30-70 % are used at temperatures around 40 °C, giving good yields (90 % of theoretical glucose). However, there are drawbacks. Recycling of the acid is required and that process is energy demanding, corrosive resistant reactors are needed, and large amounts of gypsum are produced [100]. Conversely, dilute acid hydrolysis requires low acid concentrations and temperatures in the range of 200 °C, resulting in lower glucose yields (50-60 % of the theoretical estimation). Nevertheless, the yields of hemicellulose sugars are still relatively high, with recoveries up to 80-95 %. The main disadvantage of the dilute acid process is the formation of inhibitors, decreasing the yield as well as severely inhibiting the subsequent fermentation [86, 100].

Enzymatic hydrolysis is facilitated by a multitude of enzymes, i.e. cellulases and hemicellulases [101]. Cellulases further comprise endocellulases (hydrolysing the glycoside bonds inside the cellulose chain) and exocellulases (hydrolysing the glycoside bonds at the ends of the cellulose chain). The cellulase reaction results in the release of the glucose dimer cellobiose, which is further hydrolysed by β-glycosidase into glucose monomers [102]. There are significantly many more different hemicellulases due to the wide variety of compounds normally referred to as hemicellulose. Nevertheless, hemicellulases comprise depolymerases (hydrolysing the hemicellulosic backbone) and debranching enzymes (accessory enzymes), hydrolysing the hemicellulosic branches [103].

Enzymatic hydrolysis holds three major advantages over acid hydrolysis: it is carried out under relatively mild conditions, the achievable hydrolysis yield is close to 100 %, and only insignificant amounts of inhibitors are formed. However, there are disadvantages: enzymes are relatively expensive, enzymatic hydrolysis requires days compared to minutes for acid hydrolysis, the reaction is inhibited by the released sugars, and pretreatment (Section 3.1.2.) is

mandatory [104]. Nonetheless, enzymatic hydrolysis is considered to be the most promising method for future industrial applications [105].

3.2. NMMO-pretreatment and hydrolysis of birch and spruce

Birch and spruce are two common trees in Sweden and excellent examples of hardwood and softwood, respectively. Theoretically, both wood materials could yield approximately 70 % of their dry weight as hexoses and pentoses (Table 3.1), but recalcitrant biomass causes considerable difficulties. To overcome the recalcitrance, pretreatment with NMMO at 130 °C was investigated, with two factors being particularly investigated: the effect of wood chip size (Fig 3.3) and the effect of pretreatment time (Fig 3.4) (Paper II).

Table 3.1: Sugar composition of birch and spruce, measured as mg hydrated sugars per g dry wood. The intervals represent 95 % confidence intervals (Paper II).

	Composition mg/g	
Monomer	**Birch**	**Spruce**
Glucose	408 ± 16	463 ± 15
Mannose	21 ± 1	129 ± 4
Galactose	18 ± 1	24 ± 3
Xylose	261 ± 11	79 ± 3
Arabinose	7.9 ± 0.8	13.0 ± 0.8
Total	715 ± 29	710 ± 23

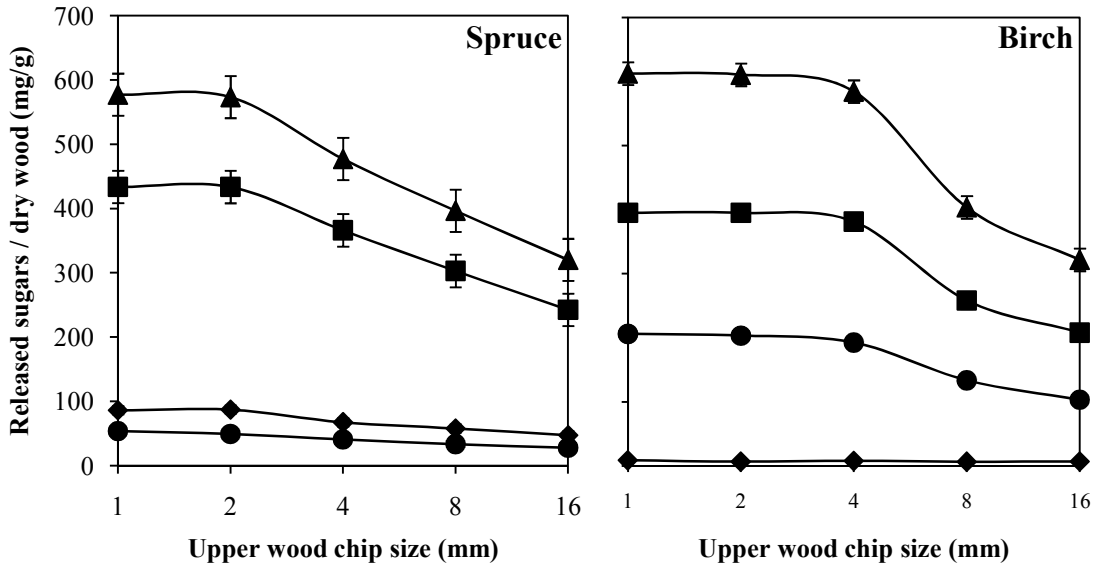

Figure 3.3: Influence on wood chip size on hydrolysis yield from spruce and birch treated with NMMO at 130 °C for 5 h. The symbols represent total sugars (▲), glucose (■), mannose (♦) and xylose (●). The error bars correspond to 95 % confidence intervals for pooled standard deviations.

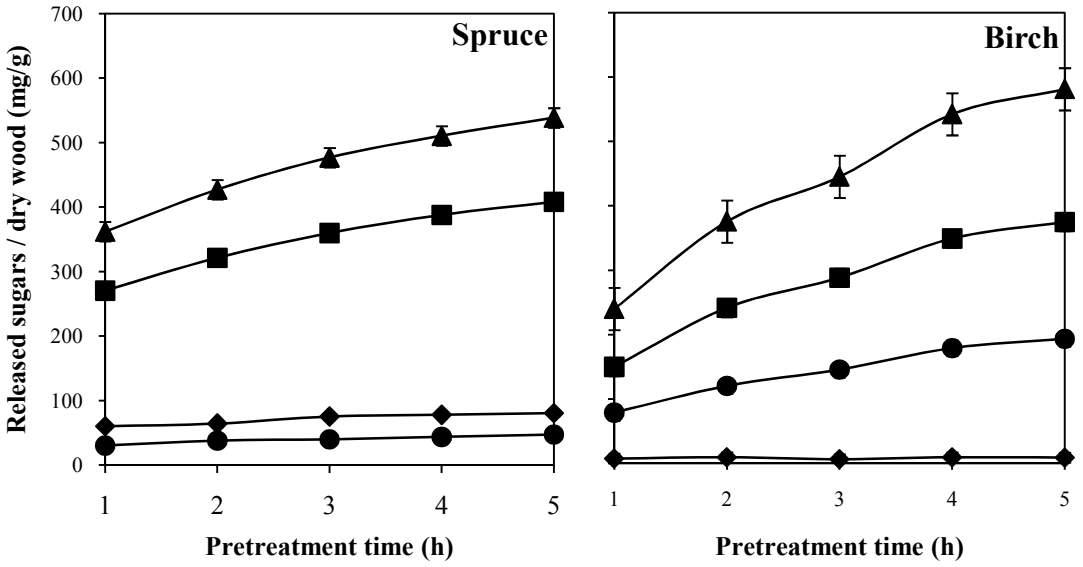

Figure 3.4: Influence of NMMO pretreatment time on hydrolysis yield of 1-2 mm spruce and birch chips. The symbols represent total sugars (▲), glucose (■), mannose (♦) and xylose (●). The error bars correspond to 95 % confidence intervals for pooled standard deviations.

Clearly, wood chip size as well as pretreatment time had significant impact upon the final yield (Fig. 3.3, 3.4). Regarding wood chip size, the maximum yield was reached when the size was reduced to 2 mm, at which point further reduction did not influence the yield. In terms of pretreatment time, the yield increased with increased pretreatment time up to the maximum time (5 h) tested. This trend implies that it may be possible to further increase the yield, by prolonging the pretreatment even more. Under the most optimal conditions investigated for spruce and birch, the glucose yields were 88 % and 92 %, while the hemicellulose sugars yielded 53 % and 67 %, respectively (Paper II).

When using pretreatment with NMMO, the interdependence between optimal pretreatment time and optimal wood chip size should be taken into consideration. This statement is supported by three different observations. (1) Using a similar pretreatment, Shafie et al. [93] acquired higher glucose yields during shorter pretreatments in a study utilising wood particles less than 0.8 mm. (2) Increased enzymatic yield after NMMO pretreatment showed a linear correlation to increased enzyme accessibility and an enrichment of cellulose on the surface of the wood particles [106]. (3) With smaller particles follows larger surface to volume ratios, further improving the accessibility for the NMMO. Thus, a prolonged pretreatment time can most likely compensate for the negative effect of increased wood chip size, and vice versa. Pretreatment with NMMO has also been shown to increase the hydrolysis reaction rate [107].

3.3. Orange waste

As one of the major citrus fruits, the orange has experienced a continuous increase in world production (Fig 3.5). However, after industrial juice production, ca 50-60 % of the orange remains as peels, membrane sections and seeds [108], requiring processing prior to waste treatment. Citrus juice manufacturers normally attempt to dry and sell the waste as animal feed or as a source for pectin extraction. However, this is often carried out at a net loss for the producer [109, 110]. Furthermore, only part of the waste is composed of pectin (Table 3.2). Therefore, a considerable amount is transported to waste disposal sites [111].

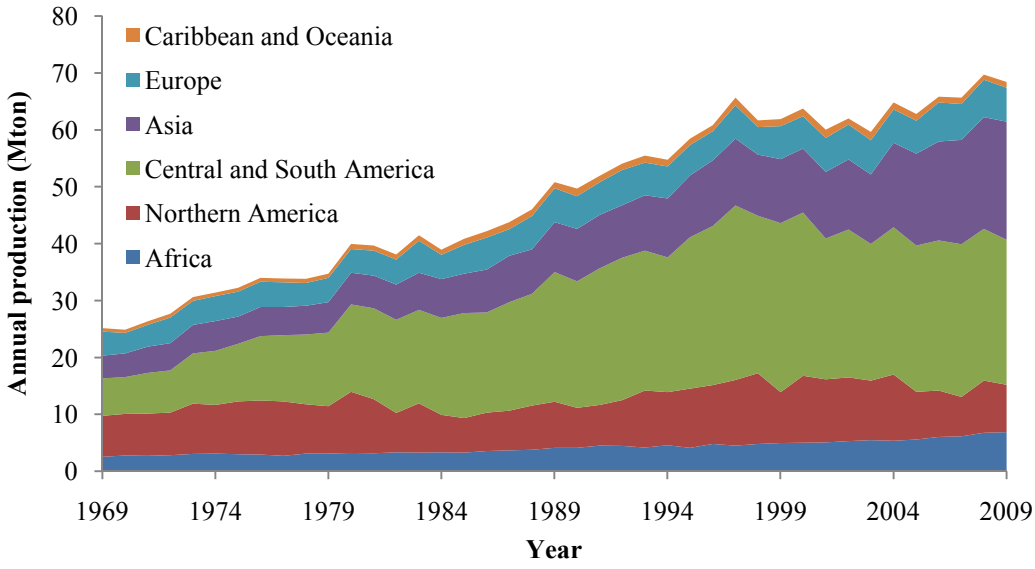

Figure 3.5: Annual production of oranges 1969-2009 in megaton. Data gathered from the FAO[1] online statistic database.

Table 3.2: Composition of peels and pulp fractions of orange waste from the juice industry, based on dry weight. The intervals represent one standard error [112].

	Composition (mg/g)	
Compound	**Peels**	**Pulp**
Ash	26 ± 1	26 ± 1
Sugar	96 ± 2	60 ± 4
Fat	40 ± 2	15 ± 1
Protein	91 ± 4	66 ± 3
Flavonoid	45 ± 2	110 ± 5
Pectin	230 ± 21	121 ± 11
Lignin	75 ± 6	75 ± 6
Cellulose	371 ± 31	245 ± 20
Hemicellulose	110 ± 11	76 ± 7

[1] Food and Agriculture Organisation of the United Nations http://faostat.fao.org

The problem with orange peel waste is twofold, together making orange peel waste quite a challenge. The moisture content of approximately 80 % (Paper III) makes drying [111] and combustion difficult. As an obstacle to biological treatment, the peel oils, primarily limonene (95 % D-enantiomer), hold considerable antimicrobial activity [113]. The ability of the hydrophobic compounds to target membranes, primarily mitochondria, is generally considered to cause the antimicrobial effect [114-116]. The concentrations of limonene required for antimicrobial effect are generally low; less than 0.05 % (v/v) is sufficient to inhibit *S. cerevisiae* [117]. Most orange peel hydrolysates contain more than 0.5 % limonene, and consequently, several attempts have been made to overcome, or circumvent, the problems associated with limonene. In general, the limonene has either been removed prior to fermentation [118-121] or the yeast has been kept separated from the limonene [122].

Incidentally, zygomycetes, such as *M. indicus*, have been observed to possess the ability to tolerate the limonene in orange peel hydrolysate (Paper III). For further information, see Section 4.3.4.

3.4. Spent sulphite liquor

Spent sulphite liquor (SSL) is a byproduct formed during the sulphite cooking process in the paper pulp production (Fig 3.6). Various chemicals are utilised, e.g. bisulphite, hydrogen ions, and one more cation, e.g. magnesium, calcium, sodium, or ammonium. Sulphurous acid and dissolved sulphur dioxide also appear in the process. The process itself can be divided into three main steps [123, 124]. In the first step, the cooking liquor is allowed to impregnate the wood chips at relatively low temperatures. In the second step, as the temperature rises to 50-60 °C, the first chemical reactions set in. In the end, at temperatures not exceeding 130-150 °C, the final cook generally takes place for 6 to 14 h, depending on the wood and the desired pulp characteristics. The three major compounds of the lignocellulosic material react very differently. Cellulose is left relatively untouched, forming the final pulp, while hemicellulose is hydrolysed, and lignin is solubilised by sulphonation. Thus, after sieving the cellulose, the major compounds of the spent sulphite liquor are hemicellulosic sugars (Table 3.3) and sulphonated lignin [123, 124].

In the sulphite process, ca 50 % of the wood is converted to paper pulp cellulose [124] and correspondingly, ca 50 % is left in the SSL [125]. Extremely high pollution loading is

expected if the SSL is discharged into the environment, since the sugars in the SSL cause high biological oxygen demands (5,000-25,000 mg/l [126]) and the lignosulphonates contribute to high chemical oxygen demands and discolouring of the effluent [125]. Consequently, considerable treatments are required before it can be safely released.

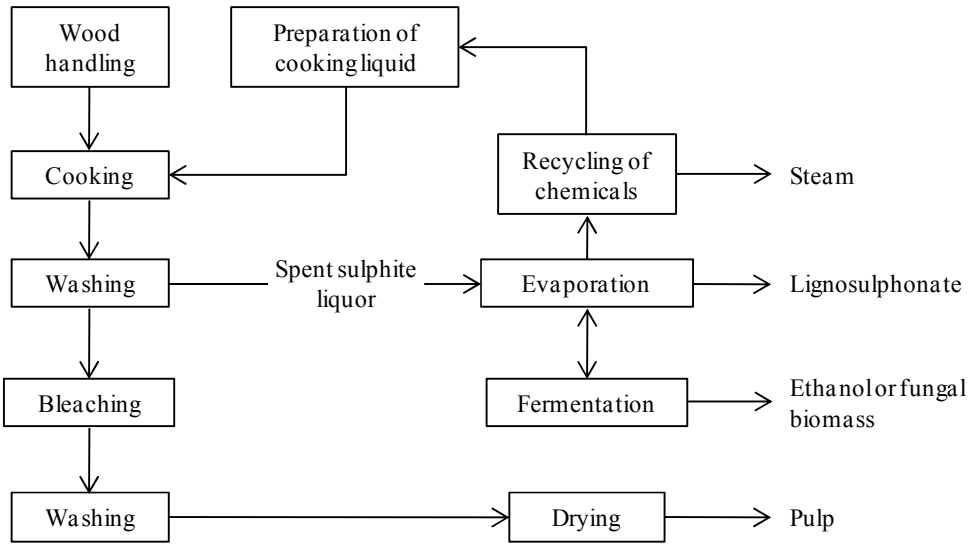

Figure 3.6: The sulphite cooking process in the paper pulp production.

Table 3.3: Sugar composition of the spent sulphite liquor (received from Nordic Paper Seffle AB, Sweden) used in Paper IV.

Carbohydrate	Concentration (g/l)
Glucose	6.2
Mannose	19.1
Galactose	6.5
Xylose	8.0
Arabinose	3.0
Total	42.8

It has been known since 1907 that hexose sugars in SSL can be fermented to ethanol, while lignosulphonates were left untreated. By 1970, several treatments had been investigated. The only method known to be truly effective is evaporation of the liquid and combustion of the remaining solids, recovering the energy [125]. Even though many more applications were tested, e.g. production of yeasts [127], fungi [128], xylanase [129], biogas [130], and vanillin, not much has happened since then, and combustion is still a necessary step [124]. In the present thesis, utilisation of carbohydrates in SSL was tested for cultivation of the edible *Rhizopus* sp., to be used e.g. in the animal feed industry (Chapter 5) (Paper IV).

4. ETHANOL AND ZYGOMYCETES

Without an application, substrates and products are nothing but waste. However, reflecting on the current huge energy demand in the world (Section 4.1), this might not necessarily be true for certain substrates (Chapter 3), providing that fuel ethanol can be produced.

4.1. World fuel and energy supply

In 2008, the annual world production of crude oil was 3.6×10^9 ton [81], corresponding to 33 % of the total primary energy supply, making it the largest single contributor [131]. Together with the second and third largest contributors, coal and gas, fossil fuels made up over 80 % of the total primary energy supply in 2009 (Fig. 4.1). However, oil production is estimated to peak in the near future [132, 133], if it has not already happened [134]. Furthermore, combustion of fossil fuels is generating considerable environmental problems [135]. Thus, the use of fossil fuels cannot continue indefinitely, and new sustainable alternatives are required.

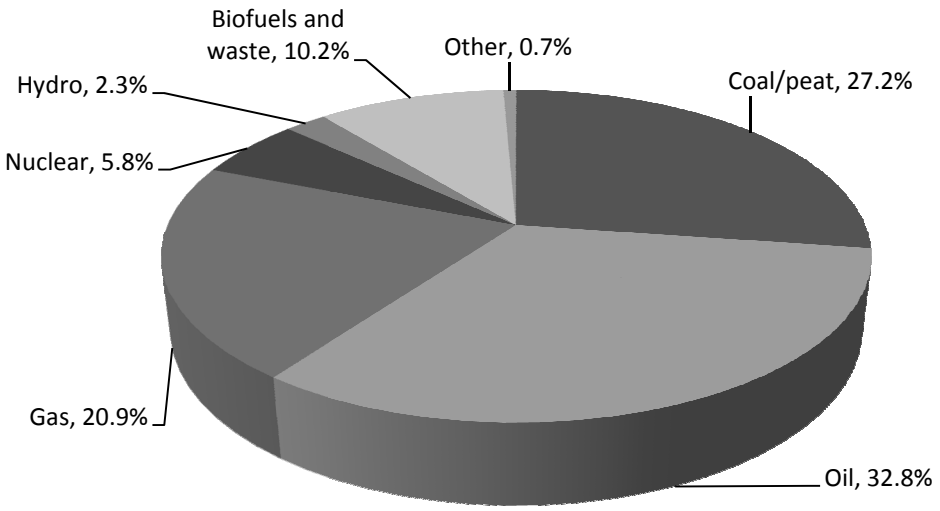

Figure 4.1: Total world energy supply in 2009 according to IEA, divided by fuel source [131].

The main consumer of oil is clearly the transport sector; in 2008, this sector was responsible for ca 60 % of the consumption by end users (Fig. 4.2). Hence, in order to phase out the use of oil, new transportation fuels need to be deployed. Several alternative and more environmentally friendly fuels have been proposed and are currently under development. The more prominent alternatives include hydrogen, electricity, biodiesel and bioethanol. However,

unless an unexpected breakthrough occurs in the near future, no single alternative fuel can replace oil, and a combination of them are thus required [136].

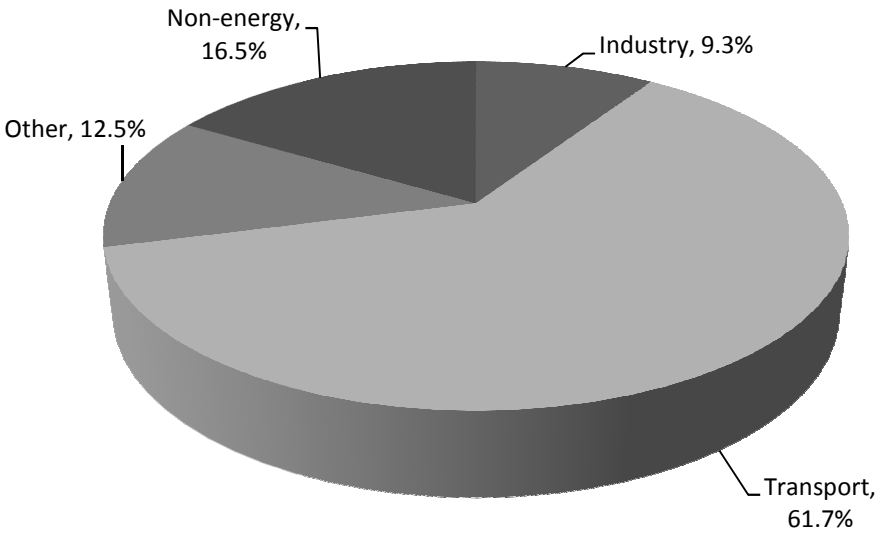

Figure 4.2: End users of oil in 2009, according to IEA [131].

4.2. Ethanol production

As mentioned in Section 4.1, ethanol is one of the renewable alternative fuels and its production has increased dramatically during recent years (Fig. 4.3). Approximately 95 % of the world production is performed by fermentation of agricultural products [137], while the remainder is produced via e.g. chemical synthesis. The fermentation can be carried out by a plethora of microorganisms, both prokaryotic and eukaryotic, though *S. cerevisiae* is the one most frequently used [138]. Other than being renewable, ethanol has the added benefit of boosting the octane number, and spills do not contaminate water sources. However, ethanol is more costly than petrol and has its own environmental drawbacks, although much more local than global in scale [138].

At present, ethanol is produced industrially from either sugar based or starch based crops, the first generation materials. Coincidentally, the two main ethanol producing countries reflect this. In the USA, corn starch is used while in Brazil, sugarcane sucrose is used; added together, this represents ca 70 % of the world production [138]. The production of ethanol has now been put under considerable scrutiny, and is expected to continue to be in the future [139, 140].

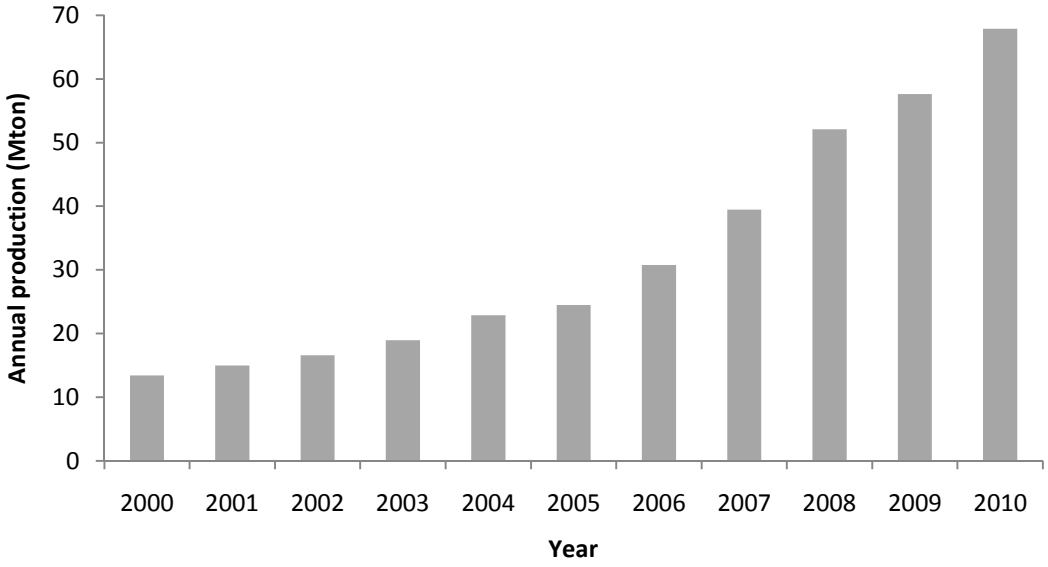

Figure 4.3: World production of ethanol 2000-2010, measured in megaton [141].

4.2.1. Ethanol metabolism from hexoses

Detailed information on ethanol metabolism in zygomycetes is lacking. However, information is plentiful for another species of *Fungi*, one of the most well studied organisms in the world, the yeast *S. cerevisiae* (Fig. 4.4).

The first step in the ethanol metabolism is the transport of monosaccharides through the plasma membrane into the cell. In *S. cerevisiae* this is facilitated by 20 different hexose (passive) transporters, tightly regulated both on the transcriptional and the posttranslational level. The most determining factor is the extracellular hexose concentration. However, *S. cerevisiae* is the microorganism with the highest amount of hexose transporters. Others, such as *Pichia stipitis* is known to express only three different hexose transporters [142].

Within the cell, hexose enters the glycolytic pathway, in which one mol hexose is converted into two mol pyruvate:

$$\text{Hexose} + 2\text{ADP} + 2\text{NAD}^+ \rightarrow 2\text{Pyruvate} + 2\text{ATP} + 2\text{NADH} \qquad (4.1)$$

Thus, the reaction(s) also entail a net production of two mol ATP, the energy currency of the cell, and two mol NADH, with reductive power for other reactions [143].

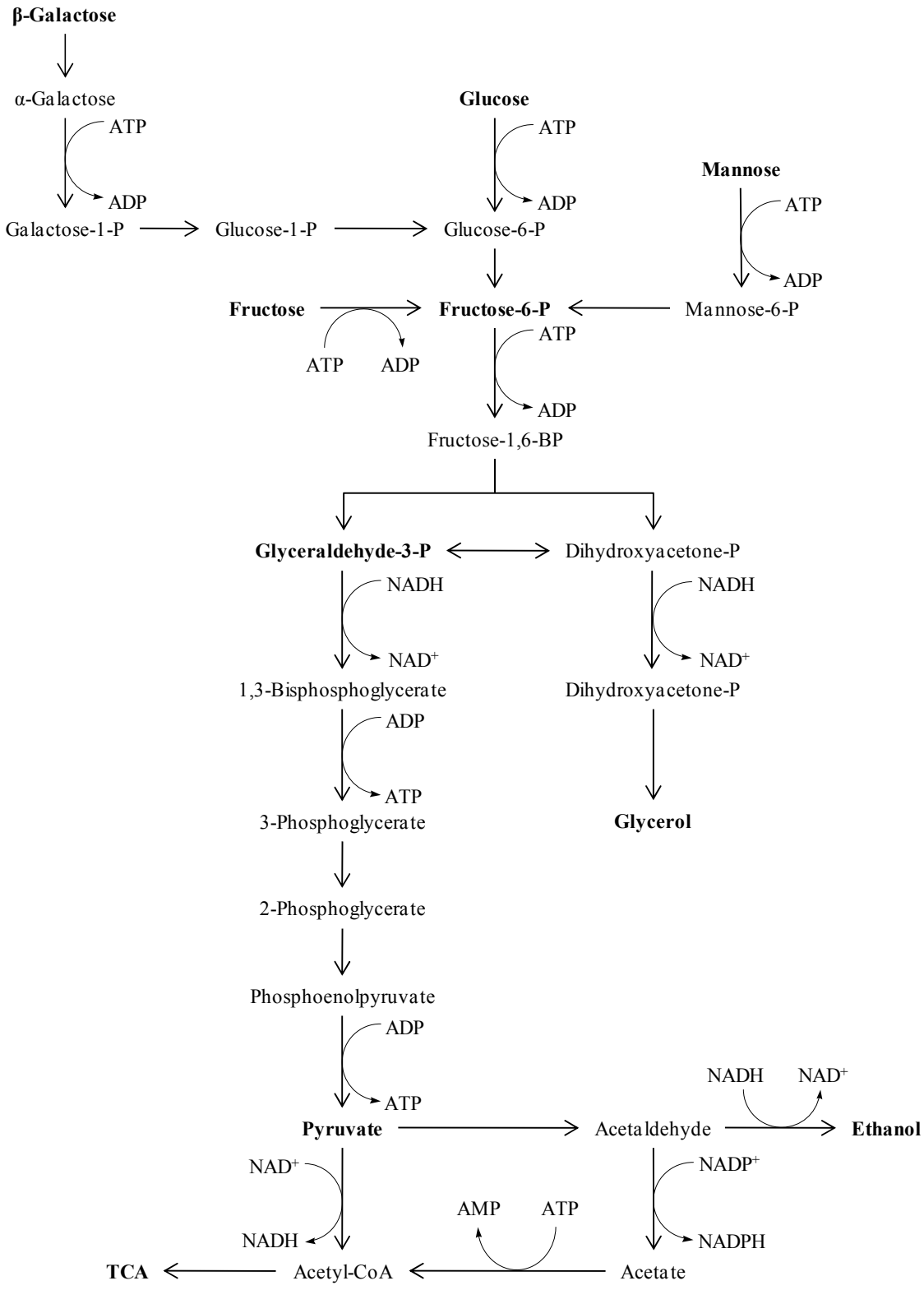

Figure 4.4: The metabolic pathway in *Fungi* of hexoses into ethanol, glycerol or the tricarboxylic acid cycle (TCA). Pathway modelled from *S. cerevisiae* [144, 145].

Depending on the conditions, pyruvate can take two different pathways. When oxygen is limited, pyruvate is reduced to ethanol:

$$\text{Pyruvate} + \text{NADH} \rightarrow \text{CO}_2 + \text{Ethanol} + \text{NAD}^+ \quad (4.2)$$

Since each pyruvate to ethanol reaction corresponds to the reoxidation of one NADH, the entire hexose to ethanol reaction can be summarized as:

$$\text{Hexose} + 2\text{ADP} \rightarrow 2\text{Ethanol} + 2\text{CO}_2 + 2\text{ATP} \quad (4.3)$$

Therefore, for each mol of ethanol formed, the cells produce one mol of ATP without shifting the internal redox potential [143]. The reaction also produces the theoretical ethanol yield of 0.51 g ethanol/g hexose.

However, the main goal of the cells is not ethanol production; it is growth and production of new biomass. These processes, in conjunction with drainage of intermediary compounds of the glycolysis, result in a net gain of NADH. Thus, in order to restore the internal redox potential, the cells are forced to produce glycerol:

$$\text{Hexose} + 2\text{NADH} + 2\text{ATP} \rightarrow 2\text{Glycerol} + 2\text{NAD}^+ + 2\text{ADP} \quad (4.4)$$

This process consumes ATP [144], which explains why the presence of some inhibitors, forcing the cells to expend energy, may lead to an increase in ethanol yield, since less carbon can be used for growth and less glycerol needs to be produced to restore the redox potential [146].

When oxygen is provided the metabolism changes dramatically and production of ethanol is no longer required to reoxidise the NADH. Instead, the pyruvate enters the TCA (tricarboxylic acid) cycle, were it is further oxidised to CO_2:

$$\text{Pyruvate} + \text{FAD} + 4\text{NAD}^+ + \text{GDP} \rightarrow 3\text{CO}_2 + 4\text{NADH} + \text{FADH}_2 + \text{GTP} \quad (4.5)$$

The NADH and $FADH_2$ are then reoxidised in the respiratory chain, ending with the reduction of O_2 to H_2O and production of more ATP. If all NADH from the glycolysis is accounted for, oxidation of one mol hexose results in 36 mol ATP. The TCA cycle also functions as a source of precursors needed in the anabolism of amino acids [143].

However, several yeasts, including *S. cerevisiae*, are known to be Crabtree positive; they continue to produce ethanol even under aerobic conditions in the presence of high glucose

concentrations [147]. This energy inefficiency might be due to an evolutionary response of ethanol tolerant yeasts to competing microorganisms [148], manifested as inhibition of the latter.

4.2.2. Pentose fermentation and assimilation

Several microorganisms are able to utilise not only hexoses, but pentoses as well. However, the pathways are not identical and focus is placed on the fungal pathways. Of the two most common hemicellulose pentoses, L-arabinose is the hardest to ferment, while D-xylose is considerably easier. This difference can best be explained by comparing the corresponding metabolic pathways (Fig 4.5).

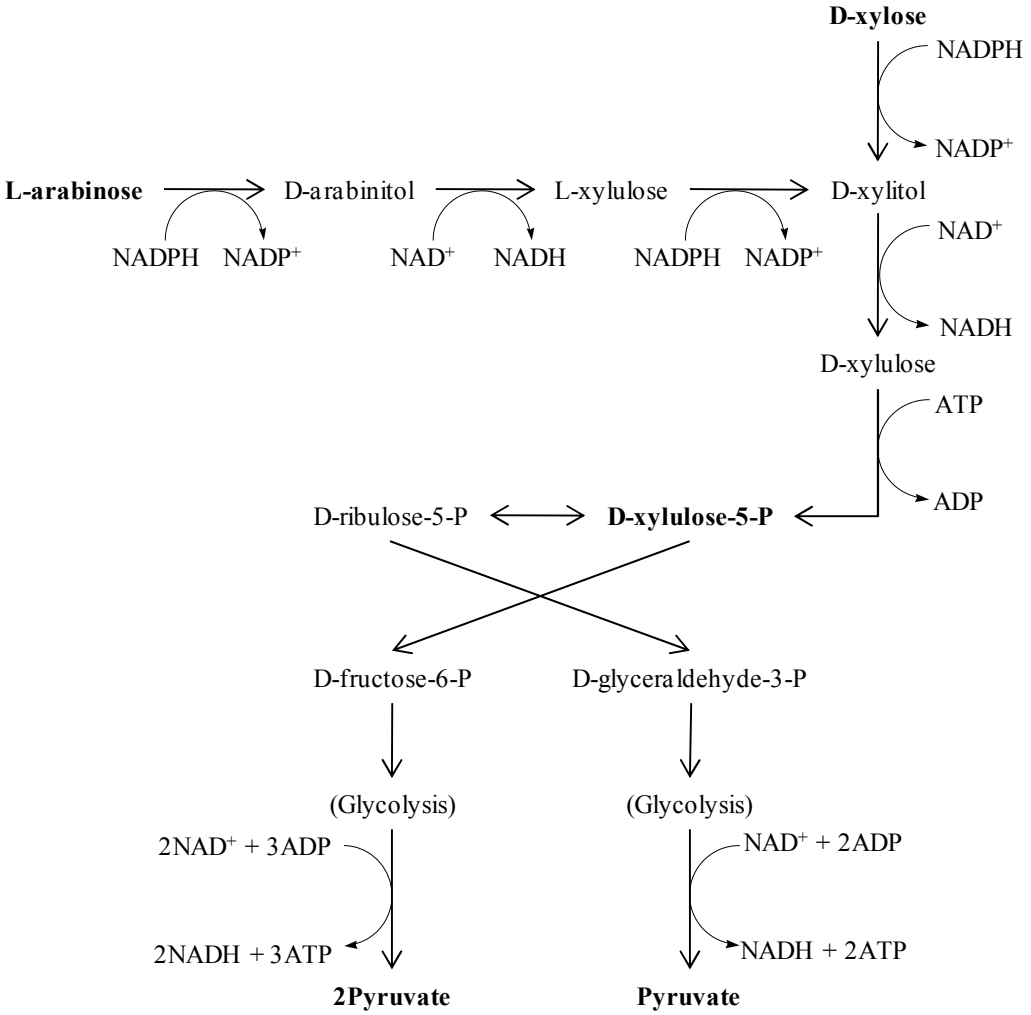

Figure 4.5: The most common metabolic pathway of L-arabinose and D-xylose to D-xylulose-5-P in *Fungi*, and a subsequent simplified pathway into the glycolysis and formation of pyruvate [143, 149-152].

The reaction of D-xylose to ethanol can be summarised as:

$$2\text{Xylose} + 2\text{NADPH} + 2\text{NAD}^+ + 3\text{ADP} \rightarrow 3\text{Ethanol} + 2\text{NADP}^+ + 2\text{NADH} + 3\text{ATP} \quad (4.7)$$

Furthermore, the reaction of L-arabinose into ethanol can be summarised as:

$$2\text{Arabinose} + 4\text{NADPH} + 4\text{NAD}^+ + 3\text{ADP} \rightarrow 3\text{Ethanol} + 4\text{NADP}^+ + 4\text{NADH} + 3\text{ATP} \quad (4.8)$$

In both cases, 1 mol pentose yields 1.5 mol ATP and 1.5 mol ethanol (1.67 mol ATP and ethanol is theoretically attainable, with a slightly different pathway). However, the net result also includes conversion of 1 and 2 mol NADPH into NADH for the xylose and arabinose pathways, respectively.

Attempts to rebalance the NADPH/$NADP^+$ pool by allowing part of the produced fructose-6-P to react in the hexose monophosphate pathway, result in an excesses of NADH. This excess is large enough for attempts at reoxidising it back to NAD^+ via glycerol production, to result in a net loss of ATP [153]. These imbalances are most likely the reason for the inability of the vast majority of fungi to grow anaerobically on xylose [153, 154]. However, fungi capable of anaerobic growth do exist; the xylose reductase in the yeast *Pichia stipitis* is able to use either NADH or NADPH for the reduction of D-xylose to D-xylitol, allowing the fungus to ferment xylose to ethanol anaerobically [155, 156]. Nevertheless, in order to avoid formation of xylitol limited aeration is required [157], and the yeast is only able to assimilate, not to ferment, L-arabinose [158]. Interestingly, *Kluyveromyces lactis* has a different approach; in this species a dehydrogenase incorporated in the glycolysis is capable of producing NADPH as well as NADH [159].

Another example of anaerobic utilisation of xylose is *Piromyces* sp., which involves a xylose isomerase similar to those found in bacteria. This allows conversion of D-xylose to D-xylulose-5-P without involving NADPH or NAD^+ [160, 161]. Accordingly, due to differences in their metabolic pathway, several bacteria are known to be capable of fermenting D-xylose and L-arabinose to ethanol, with significant yields [158].

No naturally occurring member of the fungal kingdom has thus far been positively identified as being able to produce ethanol from arabinose, and only half of the investigated yeasts able

to grow on xylose under aerobic conditions, were able to grow on arabinose at all [158]. The group of L-arabinose assimilators includes species of the genus *Mucor* [158, 162].

4.3. Ethanol production by zygomycetes

4.3.1. Ethanol from single sugars

Various zygomycetes have been evaluated regarding their ability to produce ethanol from single sugars. Taherzadeh et al. [76] worked with *R. oryzae* cultivated on glucose, with ethanol yields reaching 200-374 mg/g depending on the medium composition. However, *R. oryzae* also produced lactic acid, which severely limited the possible ethanol yield.

Millati et al. [39] compared different *Zygomycetes* species belonging to *Rhizopus*, *Rhizomucor*, and *Mucor*, evaluating their production of metabolites and biomass. On glucose, most of them produced ethanol with a yield very similar to *S. cerevisiae*; *Rhizomucor pusillus* and *Rhizomucor miehei*, however, produced no ethanol. Furthermore, all ethanol producing zygomycetes were able to produce ethanol from xylose under limited aerobic conditions, with varying yields (100-280 mg/g). Further studies on *M. indicus* [75], regarding the medium composition, resulted in ethanol yields of up to 460 mg/g under anaerobic conditions, although aerobic cultivations were almost twice as fast, yielding 400 mg/g ethanol. For further information regarding sugar utilisation by *M. indicus*, see Table 4.1. This pattern of sugar utilisation is an approximate description, applicable also on other ethanol producing zygomycetes.

Glucose tolerance of *M. indicus* was also assessed [163] with positive results. Even the highest concentration tested (350 g/l) was tolerated, although the highest ethanol concentration attained under those conditions was ca 70 g/l, after which glucose consumption ceased.

Table 4.1: Ethanol and biomass production from hexoses, pentoses, and dimers, by *M. indicus*.

Sugar	Ethanol	Biomass	Aerobicity	Reaction rate	Reference
Glucose	++	+++	Aerobic	+++	[39]
Glucose	+++	++	Anaerobic	++	[75]
Mannose	++	+++	Aerobic	+++	[75]
Mannose	+++	++	Anaerobic	++	[164]
Fructose	++	+++	Aerobic	+++	[77]
Fructose	+++	++	Anaerobic	++	[77]
Galactose	++	+++	Aerobic	++	[75]
Galactose	+++	++	Anaerobic	++	[164]
Xylose	+	++	Aerobic	+	[39]
Xylose	-	-	Anaerobic	-	[75]
Arabinose	-	+	Aerobic	+	[75]
Sucrose	-	-	Aerobic	-	[77]
Sucrose	-	-	Anaerobic	-	[77]
Cellobiose	+	++	Aerobic	+	NP*
Cellobiose	++	+	Anaerobic	+	NP*

* NP = not published, but supporting information is available [165]

4.3.2. Ethanol from multiple sugars

Several zygomycetes have had their ethanol production evaluated, using many different sources related to lignocelluloses. Taherzadeh et al. [76] initiated the current research by using *R. oryzae* for ethanol production from spent sulphite liquor. However, the yield left room for improvement. Only 160 mg/g was produced in a bioreactor.

Millati et al. [39] significantly improved the yield in their comparison of different zygomycetes (4.3.1), using dilute-acid hydrolysate mainly from spruce. The two *Mucor* strains *M. indicus* and *M. hiemalis* reached ethanol yields of 440 mg/g. Furthermore, investigating the influence of the aeration rate on *M. indicus* more thoroughly, Millati et al. [164] suggested that the best ethanol yield (440 mg/g) was obtained from xylose containing wood hydrolysate under limited aeration conditions (0.067 vvm).

Karimi et al. [46] found in their study of ethanol production by *M. indicus* in a dilute-acid lignocellulosic hydrolysate medium, that a fed-batch approach was sufficient for the zygomycete to overcome the inhibitory effect of the liquid medium, and an ethanol yield of 430 mg/g was reached. Karimi et al. [166] furthermore compared *M. indicus* with *S. cerevisiae* in a simultaneous saccharification and fermentation (SSF) process, using rice straw. The process resulted in more than 10 % higher ethanol yields for *M. indicus*, based on

the initial sugars. In conjunction with this, the hemicellulose fraction of the rice straw [167], which is very rich in xylose, was also investigated as a possible source for ethanol production by *M. indicus*. This resulted in yields of 240 mg ethanol and 370 mg fungal biomass, per g sugars. Furthermore, Karimi et al. [168] employed *M. indicus* as a living biofilter for *S. cerevisiae*. The yeast was successfully retained during continuous fermentation of a lignocellulosic hydrolysate, and an ethanol yield of 460 mg/g was obtained at the dilution rate 0.3 h^{-1}. By comparison, at the dilution rate 0.1 h^{-1} and no retention, washout occurred.

4.3.3. Ethanol from birch and spruce

After pretreatment with NMMO, spruce and birch chips were enzymatically hydrolysed (Section 3.3) and used for cultivation of *M. indicus*, mainly for ethanol production (Paper II). The cultivations were carried out in cotton-plugged shake flasks and in an airlift reactor.

As presented in Figure 3 (Paper II), rapid consumption of hexoses and production of ethanol occurred during the initial 16 h of cultivation in the cotton-plugged shake flasks. Later during the cultivation, as the hexose concentration approached zero, consumption of xylose set in with significantly lower and slower production of ethanol. When the cultivation was concluded, 36 h after start, nearly all the xylose had been consumed and high yields of ethanol and biomass had been acquired (Tables 4.2-4.3).

To scale-up the process, an airlift reactor of the internal-loop concentric tube type was utilised. According to Merchuk and Gluz [169], all airlift reactors are "characterised by fluid circulation in a defined cyclic pattern built specifically for this purpose". The fluid circulation is brought about by injection of air (or other gases) at the bottom of the riser. Thus, no impellers are required in this type of reactors. Compared with traditional stirred tank reactors, airlift reactors have the advantage of no focal input of mechanical energy from impeller(s). Instead, the nature of the shear forces in the airlift is much milder, which is particularly advantageous to filamentous organisms. Additionally, mixing in airlift reactors generally requires little energy [169]. Added together, this has made airlift reactors advantageous to cultivation of fungi.

Airlift cultivations of spruce and birch hydrolysates, however, showed significantly lower ethanol yields, considerably larger variations, as well as somewhat slower reaction rates (Fig

4 Paper II; Tables 4.2-4.3), compared to the shake flask experiments. Since streaming and mixing in an airlift reactor is dependent on differences in density of the gas/liquid mixture in the riser and in the downcomer [169], the low aeration was probably the major cause of these results.

Table 4.2: Ethanol, glycerol and fungal biomass yield acquired in cotton-plugged shake flasks and in a 0.15 vvm airlift reactor, after enzymatic hydrolysis of NMMO treated birch and spruce (Paper II).

Wood	Bioreactor	Ethanol yield [a)] (mg/g)	Glycerol yield [a)] (mg/g)	Biomass yield [b)] (mg/g)
Spruce	Shake flask	438 ± 13	52 ± 8	214 ± 8
Birch	Shake flask	463 ± 13	37 ± 8	177 ± 8
Spruce	Airlift	335 ±71	47 ± 18	166 ± 41
Birch	Airlift	400 ± 71	50 ± 18	167 ± 41

[a] Ethanol and glycerol yields, based on consumed hexoses.

[b] Biomass yield at the end of cultivation, based on total sugars consumed.

Table 4.3: Yield of ethanol, glycerol and fungal biomass, based on dry wood, acquired in cotton-plugged shake flasks and in a 0.15 vvm airlift reactor, after enzymatic hydrolysis of NMMO treated birch and spruce (Paper II).

Wood	Bioreactor	Ethanol yield (mg/g)	Glycerol yield (mg/g)	Biomass yield (mg/g)
Spruce	Shake flask	195 ± 7	22 ± 3	103 ± 4
Birch	Shake flask	175 ± 7	13 ± 3	86 ± 4
Spruce	Airlift	128 ± 37	18 ± 5	70 ± 12
Birch	Airlift	136 ± 37	17 ± 5	66 ± 12

4.3.4. Orange peel hydrolysate and the influence of limonene

The challenge associated with orange peels is not the recalcitrance of the biomass, but rather the presence of the highly potent inhibitor limonene, present in the material (Section 3.3.). In order to use orange peel as substrate, limonene, able to inhibit *S. cerevisiae* at 0.05 % concentration [117], and generally present at a concentration of >0.5 % (v/v) in the hydrolysate, must be tolerated by the zygomycetes. Thus, the tolerance of *M. indicus* and *Rhizopus* sp. to limonene was evaluated in Paper III.

Not only *S. cerevisiae* is known to be strongly inhibited by limonene; gram-positive bacteria (including *Bacillus subtilis*, *Lactobacillus plantarum*, *Streptococcus faecalis*, and *Micrococcus* sp.*)*, gram-negative bacteria (including *Salmonella schottmülleri*, *Klebsiella*

(Aerobacter) aerogenes, and *Serratia marcescens*), the yeasts *S. cerevisiae, Zygosaccharomyces mellis*, and *Candida (Torula) utilis*, as well as some *Aspergillus* species (*A. niger*, *A. awamorii*, and *A. flavus*) were (with the exception of *S. marcescens*) inhibited by peel oil at a concentration of 0.2 % or less [113]. Since all microorganisms but one of the microorganisms in that study were inhibited, the conclusion that limonene causes inhibition across all types of microorganism, would not be far-fetched. *M. indicus*, however, was able to germinate and grow in the presence of 2 % limonene (Fig 4.6), although the rate and amount of ethanol and glycerol production differed between cultures.

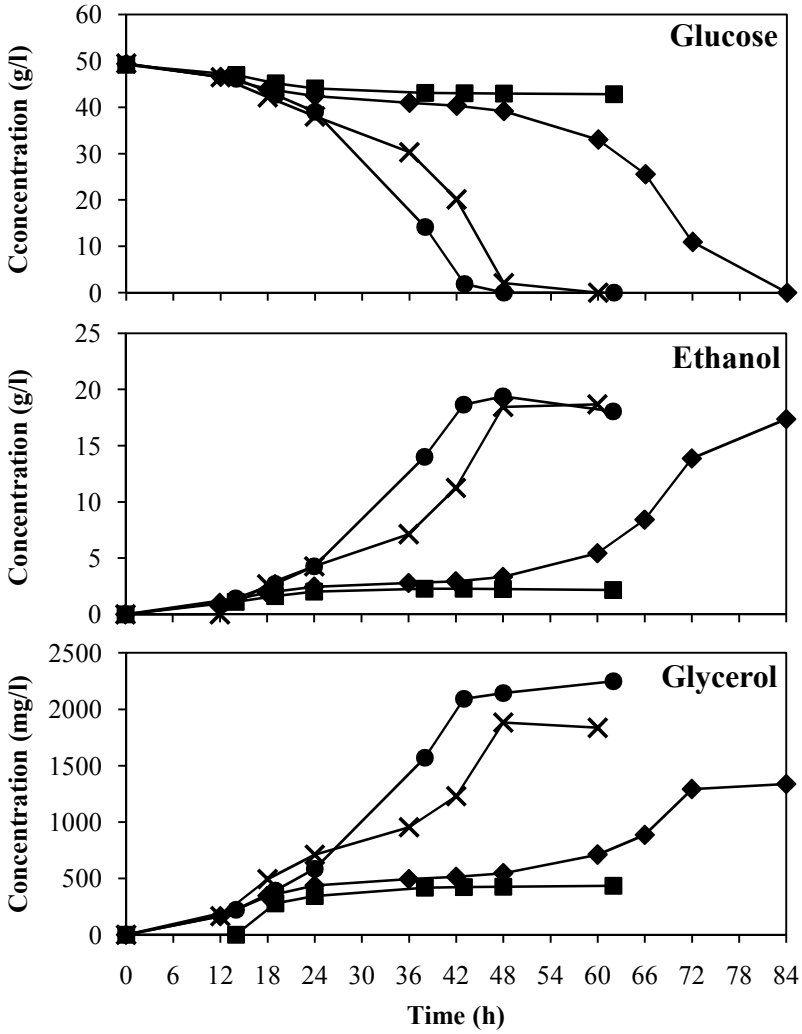

Figure 4.6: Aerobic cultivation of *M. indicus* in a bioreactor on GYV (Glucose Yeast extract Vitamin media), in the presence of 2 % limonene. The symbols represent four different cultures, 1 (●), 2 (■), 3 (×) and 4 (♦), of which 3 and 4 started from the same inoculum. Glucose, ethanol and glycerol were the major compounds studied during the cultivation (Paper III).

The divergence between the different cultures was considerable, and one culture even ceased to grow before entering a true exponential phase (Fig. 4.6). Similar behaviour was also observed in the presence of 1 % limonene, since two replicates finished growing after 70 h, while a preliminary experiment resulted in complete glucose assimilation after 36 h. The differences, however, had little effect on the ethanol yield with an average of 380 ± 17 mg/g, which is slightly less than reported previously for cultures without limonene (Sections 4.3.1-4.3.3). The lower ethanol yield may have been caused by evaporation during the relatively long cultivation, increased aerobic conditions due to slow growth, or limonene generating a metabolic shift.

The inhibitory effect of limonene on cultivation of *Rhizopus* sp. was also assessed in baffled shake flasks under aerobic as well as anaerobic conditions (Table 4.4), with results comparable to those of *M. indicus*. It was evident though, that *M. indicus* was considerably less sensitive to the presence of limonene than *Rhizopus* sp., which was growing significantly slower, with lower ethanol yields (Table 4.4).

Table 4.4: Effect of 0-2 % limonene in aerobic and anaerobic cultivations of *M. indicus* and *Rhizopus* sp. (Paper III).

		M. indicus		***Rhizopus* sp.**	
Limonene conc. (%)	**Condition**	**Yield ethanol (mg/g)**	**Consumed glucose (%)**	**Yield ethanol (mg/g)**	**Consumed glucose (%)**
0	Aerobic	416	100	373	100
0.25	Aerobic	400	100	343	65
0.50	Aerobic	428	99	293	11
1.0	Aerobic	393	94	285	16
2.0	Aerobic	396	99	317	29
0	Anaerobic	436	100	390	86
0.25	Anaerobic	423	100	310	26
0.50	Anaerobic	405	100	325	21
1.0	Anaerobic	404	100	198	11
2.0	Anaerobic	359	63*	247	15
Confidence interval		± 29	± 3	± 71	± 38

*Due to high residuals, this point was excluded from the calculation of the confidence interval.

Presence of limonene causing the same type of extremely large differences has been reported previously. Wilkins et al. [108] used *S. cerevisiae* and *Kluyveromyces marxianus* for ethanol production in a medium containing 0.05-0.2 % limonene, and in the higher limonene concentration, extensive discrepancies occurred, suggested by the authors to be a result of irregular adaptation of the yeast to the limonene. However, since limonene toxicity increases

with increasing droplet size [114], small differences in the mixing procedure and a subsequent variation in the distribution of the limonene droplet size, may have attributed to the divergences as well.

The inhibitory effect of orange peel hydrolysate, containing 0.601 ± 0.064 % limonene, turned out to be even greater than the 2 % limonene in GYV media. *M. indicus* was the only zygomycete which continuously succeeded to grow, and only during aerobic conditions. Successful anaerobic growth seemed to happen at random. The other zygomycete tested, *Rhizopus* sp., failed to germinate in orange peel hydrolysate. The aerobic cultivation of *M. indicus*, however, made progress and produced ethanol with a yield of 400 ±13 mg/g hexoses, 66.4 ± 6.4 mg glycerol/g hexoses and 75.0 ± 9.2 mg fungal biomass/g sugars. This corresponds to 158 ± 6 mg ethanol, 18.2 ± 0.7 mg glycerol and 31.8 ± 3.1 mg fungal biomass per g dry peel biomass. The cultivation speed in orange peel hydrolysate (Fig. 4.7), was slightly lower than in wood hydrolysate (Fig 3-4 Paper II), indicating an influence of limonene.

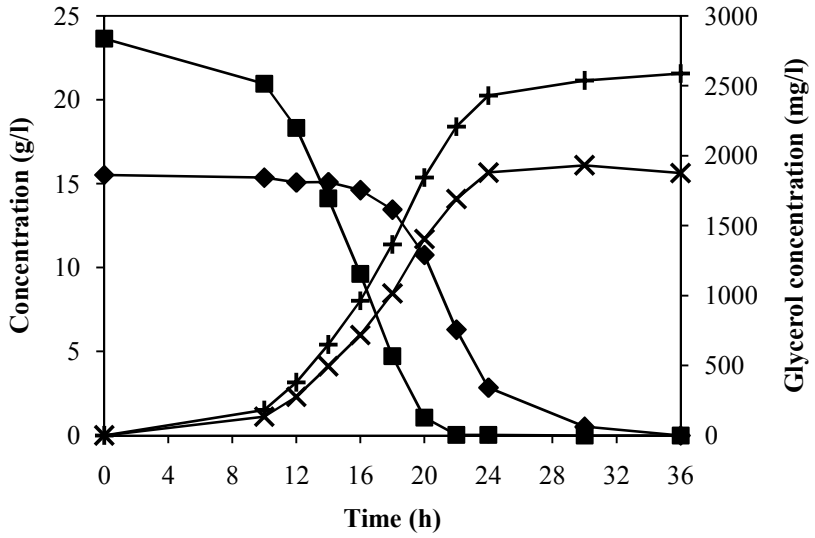

Figure 4.7: Glucose (■), fructose (♦), ethanol (×), and glycerol (+) concentrations during cultivation of *M. indicus* in orange peel hydrolysate, in a bioreactor. The glycerol concentration is reported as mg/l, with values on the right hand y-axis (Paper III).

The reason for *M. indicus* (and *Rhizopus* sp.) being able to tolerate ten times higher limonene concentrations than *S. cerevisiae* is yet to be explained, and since limonene is primarily targeting mitochondria (Section 3.3), the positive effect of aeration needs to be explained as well. It suggests that the methods zygomycete fungi utilise, require aerobic conditions, allowing production of compounds possible only under aerobic conditions or through an

energy demanding process. This may explain the low biomass production. Overall, two different types of methods may explain the mechanism behind the tolerance to limonene. In the first method, limonene is kept outside the cell by the cell envelope, which in *M. indicus* is known to be different from that of *S. cerevisiae* (Section 2.4). The second method renders the limonene entering the cell harmless, which may be transpired by active transport of the limonene out of the cell, or by chemical modification of the limonene.

Since zygomycete fungi were considered well adopted for growth on orange peels, D-galacturonic acid, the main monomer of pectin, was investigated as a carbon source (Paper III). Since orange peels contain ca 20 % pectin [170, 171], sizable consumption of D-galacturonic acid should be advantageous. However, neither *M. indicus* nor *Rhizopus sp.* was able to utilise the galacturonic acid under anaerobic conditions. Aerobic growth, on the other hand, was successful but slow, requiring 9.2 ± 0.8 and 11.4 ± 1.4 days for complete utilisation in *M. indicus* and *Rhizopus sp.*, respectively. No metabolites were detected in the media of either of them, and the only product was fungal biomass. Thus, both zygomycetes probably follow the common fungal catabolic pathway for D-galacturonic acid (Fig. 4.8).

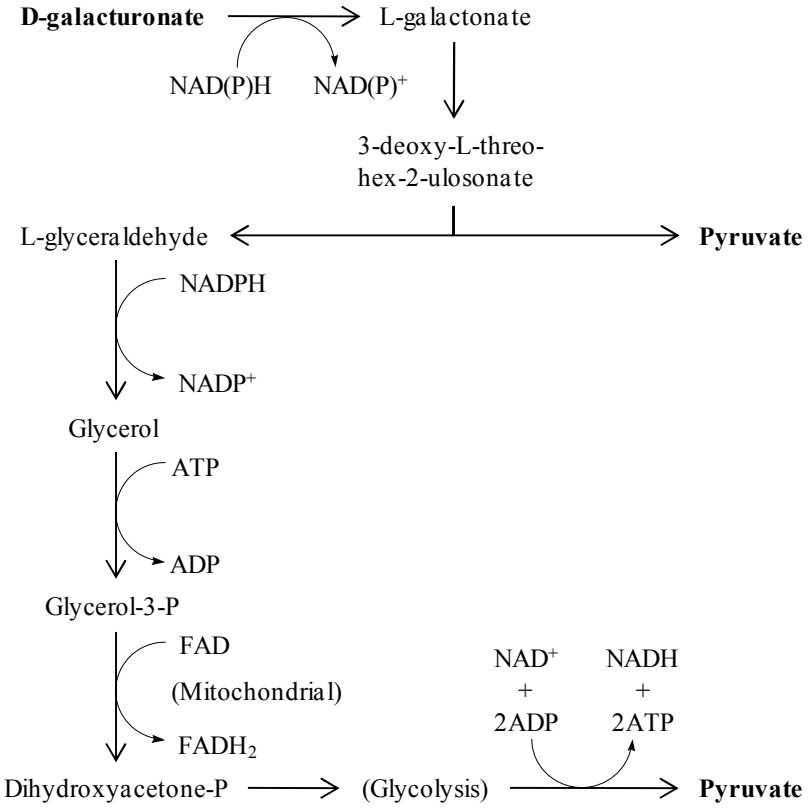

Figure 4.8: The most common fungal pathway for catabolism of D-galacturonate to pyruvate. Depending on the organism, the first step can be either strictly NADPH-dependent or dependent on either NADPH or NADH [172-174].

4.3.5. Effect of Mucor dimorphism on ethanol production

M. indicus is dimorphic and can grow either as yeast-like or filamentous cells (Section 2.3-2.4). However, information on possible influence of this characteristic on ethanol production, is lacking in the literature. Hence, the influence of dimorphism on ethanol production and inhibitor tolerance was investigated (Paper I). Four different characterisations of microscopic morphology were used, *viz.* purely filamentous, mostly filamentous, mostly yeast-like and purely yeast-like, as defined by Bartnicki-Garcia and Nickerson [69]. Initial spore concentrations of 1-2×10^5, 6-18×10^5, and 6-8×10^6 spores/ml kept under aerobic conditions, were used to produce purely filamentous, mostly filamentous, and mostly yeast-like growth, respectively. Purely yeast-like growth was induced by anaerobic conditions, using an initial spore concentration of 6-8×10^6 spores/ml.

When *M. indicus* was grown on GYV media, no obvious differences in production were detected. In all cases, ethanol was the main metabolite with yields of ca 400 mg/g, while glycerol was the main secondary metabolite. However, the time required for complete consumption of the glucose differed, the purely yeast-like cells being slightly slower than the others (Table 1 Paper 1). More profound effects were encountered when 10 g/l acetic acid, an inhibitory compound released during hydrolysis of wood [86], was added to exponentially growing cells, kept in GYV media under anaerobic conditions (Table 4.5). In its presence, morphologies dominated by filamentous growth were significantly more dependent on aeration for rapid glucose consumption and ethanol production, than those where yeast-like cells dominated. Concurrently, significantly more biomass was produced by the cultivations containing purely filamentous morphology, than in the other categories; biomass production was also dependent on air supply.

Table 4.5: The effect of acetic acid (10 g/l) on the different growth morphologies of *M. indicus*, (Paper I). The error (± 2 s.d.) was estimated as 10.4 % of the reported averages.

□□□□p□□□□□□F	Fermentation condition	Time needed for total glucose consumption (h)	Ethanol yield (mg/g)	Biomass yield (mg/g)
Purely filamentous	Aerobic	3.5	380	93
Purely filamentous	Anaerobic	18	400	58
Mostly filamentous	Aerobic	3.5	410	27
Mostly filamentous	Anaerobic	16	430	21
Mostly yeast-like	Aerobic	4	420	27
Mostly yeast-like	Anaerobic	13	430	20
Purely yeast-like	Aerobic	4	420	24
Purely yeast-like	Anaerobic	5	420	20

Addition of furfural, another inhibitor produced during acid hydrolysis [86], under identical conditions had the opposite effect. The filamentous growth morphologies tolerated the inhibitor significantly better than the morphologies with yeast-like growth (Table 4.6). Generally, aeration during cultivation had little or no effect on the time required for glucose consumption and furfural conversion.

Table 4.6: The effect of furfural (4.6 g/l) on the different growth morphologies of *M. indicus* (Paper I). The error (± 2 s.d.) was estimated as 10.4 % of the reported averages.

Morphology	Fermentation condition	Time needed for total glucose consumption (h)	Time needed for total furfural conversion (h)	Ethanol yield (mg/g)	Biomass yield (mg/g)
Purely filamentous	Aerobic	5	3	390	94
Purely filamentous	Anaerobic	5	3	410	81
Mostly filamentous	Aerobic	6	2	420	53
Mostly filamentous	Anaerobic	6.5	2	430	25
Mostly yeast-like	Aerobic	5.5	4	420	55
Mostly yeast-like	Anaerobic	5	4	440	37
Purely yeast-like	Aerobic	18	7	400	60
Purely yeast-like	Anaerobic	19	10	410	34

The different growth forms were furthermore subjected to dilute-acid hydrolysate (Table 4.7). All morphological categories were able to overcome the inhibitors, and they all converted the furfural, whether aeration was provided or not. Consumption of acetic acid, on the other hand, required aerobic conditions, regardless of growth morphology. The anaerobic cultivations had a slightly higher ethanol yield than the aerobic cultivations.

The general lack of difference in metabolite production by the different growth morphologies is an advantage when cultivating *M. indicus*; any desired growth form can be chosen with no adverse effects on the yields.

Table 4.7: Effect of dilute-acid hydrolysate on the different growth morphologies of *M. indicus* (Paper I). The error (± 2 s.d.) was estimated as 10.4 % of the calculated averages.

Morphology	Fermentation condition	Ethanol yield (mg/g)	Biomass yield (mg/g)	Acetate consumption after 25 h (%)	Furfural conversion (%)
Purely filamentous	Aerobic	420	67	95	100
Purely filamentous	Anaerobic	430	70	2.5	100
Mostly filamentous	Aerobic	430	56	73	100
Mostly filamentous	Anaerobic	440	31	1.1	100
Mostly yeast-like	Aerobic	420	41	79	100
Mostly yeast-like	Anaerobic	440	27	0	100
Purely yeast-like	Aerobic	420	33	89	100
Purely yeast-like	Anaerobic	440	30	1.2	100

5. APPLICATIONS FOR THE ZYGOMYCETES BIOMASS

5.1. Importance of zygomycetes biomass

Cellulosic materials (Chapter 3) have been studied for ethanol production for several decades, but with the exception of some special cases of waste products, such as spent sulphite liquor, no industrial ethanol production has been initiated. This stems from difficulties in building an economically feasible process [175]. A second valuable product, or a new main product, might improve the process economy, and in the case of zygomycetes, biomass is such a product.

5.2. Animal feed

5.2.1. Current market and concerns

The large interest in feed production is logical, as animal production, specifically fish production in aquaculture, has increased dramatically since 1980 (Fig. 5.1-5.2). Nevertheless, as the aquaculture production continues to expand, the need for fish feed increases correspondingly. With reference to carnivorous fish, such as salmon, this relates to an increase in the demand of fishmeal and oil. This production is limited, since wild fish constitutes the raw material, but is stable on an annual basis of ca 6.2-7.4 Mton fishmeal and 1.0-1.7 Mton oil, and is expected to remain relatively unchanged [176].

Fishmeal production has a negative side in that salmon rearing causes substantial depletion of the wild fish populations [177]. Furthermore, concerning carnivorous species, more fish is used as feed than is produced; on average, the ratio fish used/fish produced is approximately 1.4 [178]. Fortunately, the production of carnivorous fish is only a small part of the world aquaculture production. Unfortunately, the aquaculture sector is still responsible for the consumption of ca 70 % and 90 % of the world fishmeal and fish oil production, respectively [179]. Hence, focus should be on reducing the pressure on wild species [180], particularly in the light of high probability of aquacultures replacing fisheries, similar to hunting being replaced by animal husbandry thousands of years ago.

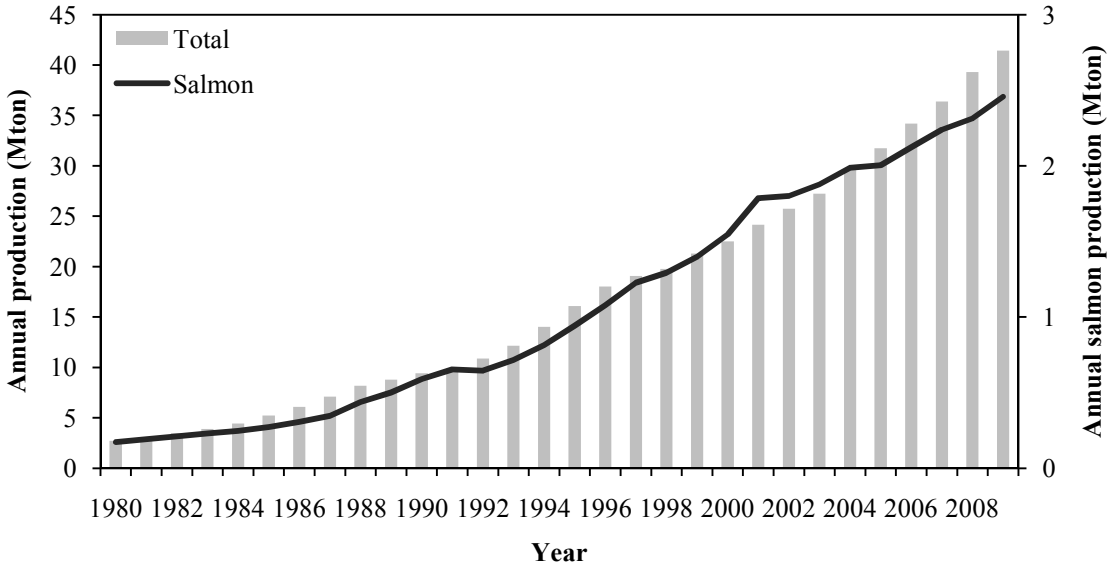

Figure 5.1: Global annual production from aquacultures 1980-2009, including crustaceans, diadromous, freshwater, and marine fish. Annual salmon production is accounted for on the right y-axis. Data gathered from FAO[2].

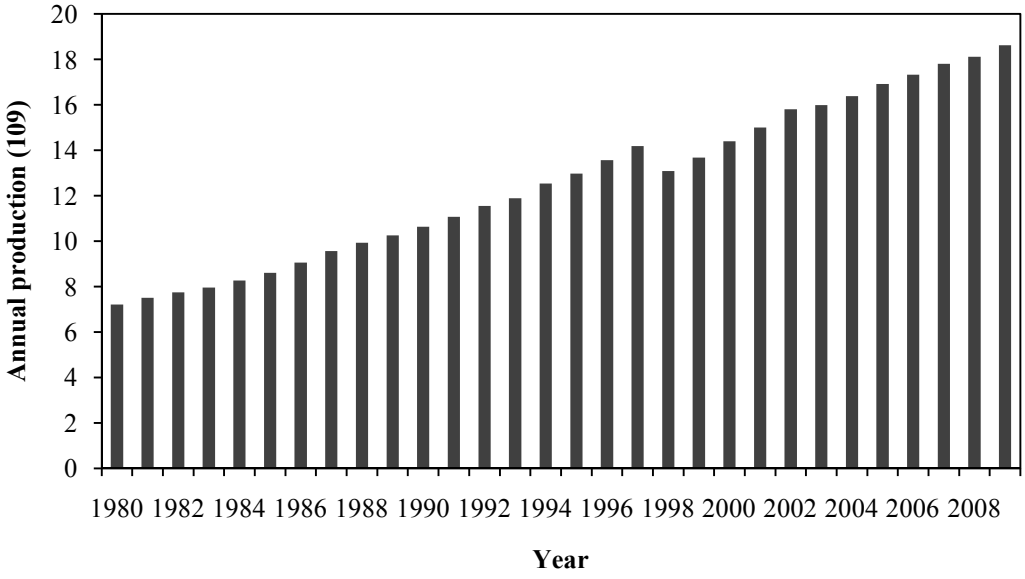

Figure 5.2: Global annual production of chicken 1980-2009. Data gathered from FAO[3].

To improve the aquaculture fish production, considerable effort has been made to find alternative feed sources for carnivorous and omnivorous fish. The sources investigated are generally based on plants (e.g. soy) or terrestrial animals, but since the feed substrate may have major impact on the growth and health of the fish, and thus the final product aimed for

[2]Food and Agriculture Organisation of the United Nations, Fisheries and Aquaculture Department http://www.fao.org
[3]Food and Agriculture Organisation of the United Nations, statistical database http://www.fao.org

human consumption, precautions are required. On the other hand, a successful replacement of fishmeal would alleviate much of the current concerns regarding the sustainability of aquacultures with carnivorous fish [181].

Feed for animals, such as the chicken example (Fig. 5.2) also has special requirements, mainly the levels of specific proteins, to assure satisfactory growth of the animals. However, the protein source for chicken is already from plant sources, mainly in the form of soy [182]. Thus, an alternative feed source for chicken is not as crucial as for aquacultures, and would compete solely from an economical point of view.

Another group of animals is the pet group, i.e. cats and dogs. Food production for this group has generated an entire industry, focusing on high value nutritional products. This industry has grown exponentially since 2000, and is expected to continue to grow at a high rate [183]. Among the more popular products are those for prevention and treatment of osteoarthritis, GlcN being one of the most common in that regard. Even though scientific investigations on osteoarthritis are limited, the existing ones appear to support administration of GlcN to alleviate the disease, and as a preventive measure [183]. Thus, a new source of GlcN in the feed production could prove very interesting in economical terms.

5.2.2. Zygomycetes and the feed industry

Zygomycetes and other (micro)-fungi have several advantages making them interesting for feed production. A major advantage entails employing fungi already in use for human consumption (Section 2.5), as they can be classified as GRAS (Generally Regarded As Safe), significantly reducing the amount of testing required, prior to a full-scale process. Further advantages include generally high contents of protein, comparably low levels of nucleic acids, ease of biomass separation from the media, and the taste and smell of the produced fungal biomass is generally pleasant [184].

In terms of feed production focused on protein, the first commercial process in the field should be mentioned; the full-scale Pekilo process 1975, in Finland. The fungus *Paecilomyces varioti* was in this process cultivated on spent sulphite liquor, which resulted in a crude protein content of 550 mg/g fungal biomass [185]. However, no Pekilo plants are in use today.

One of the current projects focuses on *Rhizopus* sp. cultivation on spent sulphite liquor, aimed towards fish feed production. The results thus far are promising; the biomass contains a remarkably advantageous composition of amino acid residues, along with 18 unsaturated carbon fatty acids, and vitamins. Most importantly, the carnivorous fish, fed a diet containing zygomycetes biomass, ate it, grew well, and exhibited only very minor differences from fish fed a standard diet [186, 187].

5.2.3. Spent sulphite liquor to zygomycetes for animal feed

In papers I-III, the biomass yield was determined mainly at the end of cultivation, with results varying from 20 to 200 mg/g sugars, depending on the conditions. The aim was however not maximal biomass production in these studies, and no in-depth study was performed on the biomass composition.

Paper IV, however, reports on in-depth studies on *Rhizopus* sp., cultivated on spent sulphite liquor (SSL, diluted to 50 %) or GYV in shake flasks. Cultivation was also performed on SSL50 % in an airlift with varying aeration (0.15, 0.50, or 1.0 vvm) (Fig. 5.3). Growth performance of *Rhizopus sp.* was improved by aeration, and the largest effect was acquired when changing aeration from 0.15 to 0.50 vvm (Fig. 5.3). Changing from 0.50 to 1.0 vvm resulted in a smaller and more irregular increase of biomass production, and the increase was accompanied by a decrease in lactic acid and ethanol production, changing from 2.03 and 1.77 g/l to 0.5 and 1.13 g/l, respectively.

As the cultivations proceeded (Fig. 5.4), three effects became evident. Longer cultivation resulted in a higher content of AIM, which was correlated to a general thickening of the cell wall. This matches well with previous publications, as ageing cells are well known to possess thicker cell walls [63]. It was also evident that the protein content of the cells decreased as the total biomass increased, a situation also detected when comparing the faster growing culture, receiving 1.0 vvm aeration, with the cultures receiving 0.5 and 1.0 vvm. Similarly, the lipid content decreased with increasing growth rate (Table 5.1). Higher initial fungal activity and higher rates of synthesis may explain these observations. The possibility of nutrient limitation playing a role, could however not be excluded.

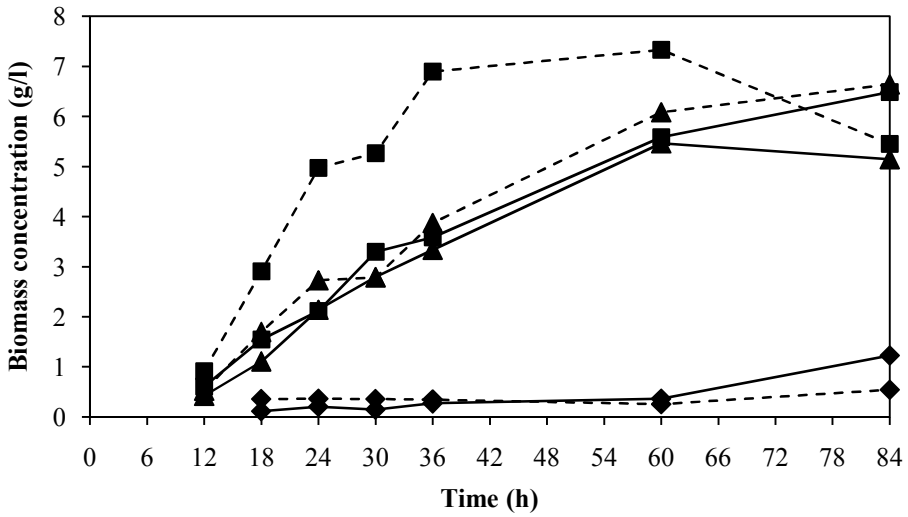

Figure 5.3: Biomass production of *Rhizopus* sp. on SSL 50 % in an airlift, at 0.15 (♦), 0.50 (▲), and 1.0 (■) vvm. All cultivations were performed in two replicates; 1 (—) and 2 (----), and their individual growth curves are presented (Paper IV).

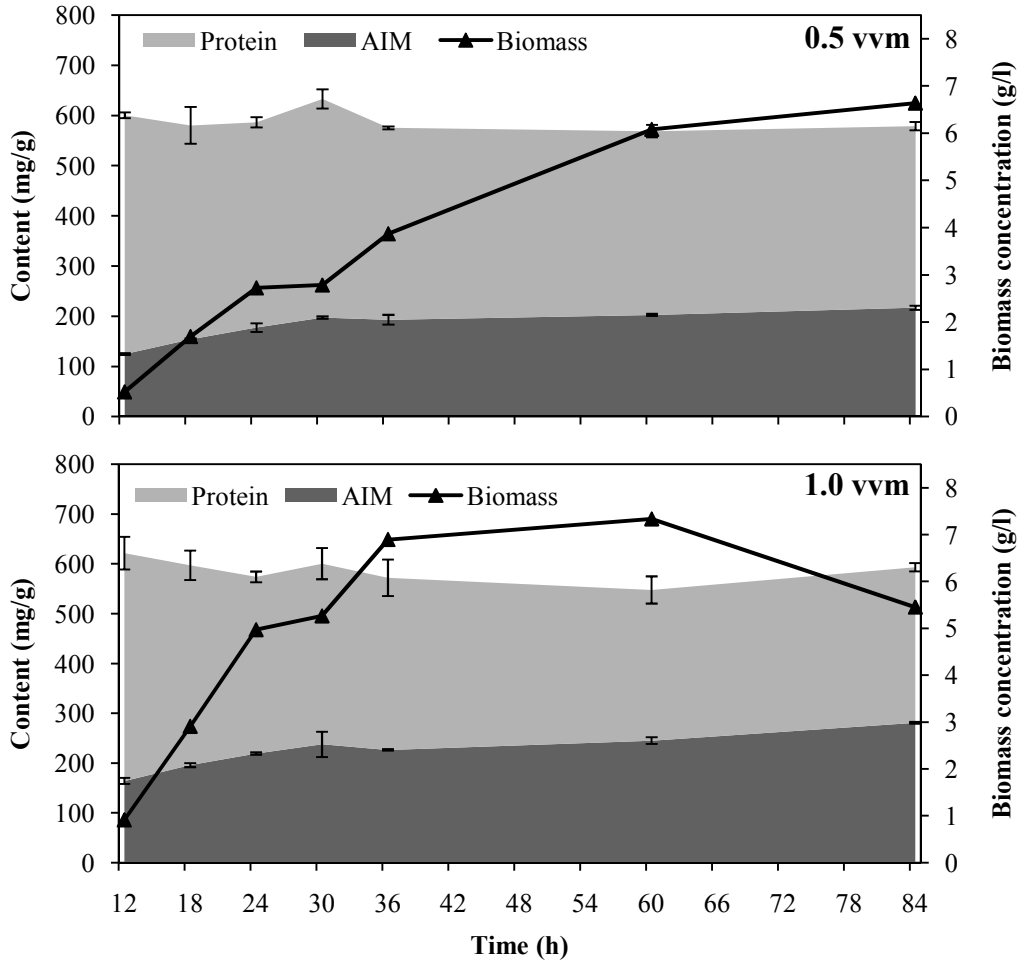

Figure 5.4: Protein and AIM content, and the biomass concentration of *Rhizopus* sp., replicate 2, in SSL 50 % at 0.50 and 1.0 vvm. The error bars represent 2 standard deviations (Paper IV).

Table 5.1: Lipid content of the biomass of *Rhizopus* sp. after 84 h cultivation in SSL 50 % at 0.15, 0.50, and 1.0 vvm. The error (± 2 s.d.) was estimated as 17 % of the reported values (Paper IV).

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

5.3. Fungal chitosan and cell wall constituents

One of the hallmark characteristics of species belonging to *Zygomycetes* is presence of chitosan in the cell wall (Section 2.4). However, relatively little research has been carried out regarding its extraction and possible utilisation. Synowiecki and Al-Khateeb [188] evaluated the influence of cultivation time on chitin and chitosan content in *M. indicus*, and established that the chitosan content increased up to 48 h, after which it started to decrease. However, only one cultivation medium was tested, which was based on glucose, peptone, and yeast-extract. Chatterjee et al. [189] attempted to remedy this by using a potato dextrose broth and a molasses salt medium, in addition to the yeast peptone glucose medium. All things considered, the best medium was based on molasses, resulting in ca 7.8 g/l biomass, 60-77 mg chitosan/g fungal biomass, with a degree of deacetylation of 87%. Tan et al. [190] evaluated thirteen different strains of zygomycetes for extractable chitosan and optimal harvesting time. The extractable chitosan varied between 23 and 71 mg/g biomass, depending on the species, and large variations were observed even within the same genus. Thus, no general conclusions could be made.

Recently, Zamani [191] made several new advances in extracting and analysing fungal chitosan. A new method for analysing chitin and chitosan content was developed, based on a two-step acid hydrolysis: room temperature hydrolysis with 72 % (v/v) sulphuric acid, followed by hydrolysis at 120 °C with 2.48 % (v/v) sulphuric acid, and subsequent deamination. This method allowed measurement of GlcN and GlcNAc contents in the fungal cell walls, with a recovery of over 85 % [192]. The work with sulphuric acid continued with the development of a new method for extraction of chitosan, utilising the differences in

chitosan solubility in hot and cold dilute sulphuric acid [193]. The dilute sulphuric acid method was further developed and its hydrolysing effect utilised, to produce low molecular weight chitosan from chitosan with high molecular weight [194]. The method was also used to extract and purify chitosan from AIM (Alkali Insoluble Material) produced from the fungal cell wall of the zygomycete *Rhizomucor pusillus*. After optimisation, 340 mg chitosan/g AIM was recovered, with a purity higher than 83 % [195].

The content of GlcN, GlcNAc, and phosphate in AIM isolated from *Rhizopus* sp., grown on SSL 50 % in the airlift bioreactor, was studied in Paper IV. The GlcN content of the AIM ranged between 220-270 and 220-320 mg/g, while the GlcNAc content ranged between 100-160 and 100-200 mg/g, with aeration of 0.50 and 1.0 vvm, respectively (Fig 5.5). Calculated per liquid volume, the maximum values corresponded to 0.33 and 0.53 g/l GlcN and 0.22 and 0.31 g/l GlcNAc, with 0.50 and 1.0 vvm aeration, respectively.

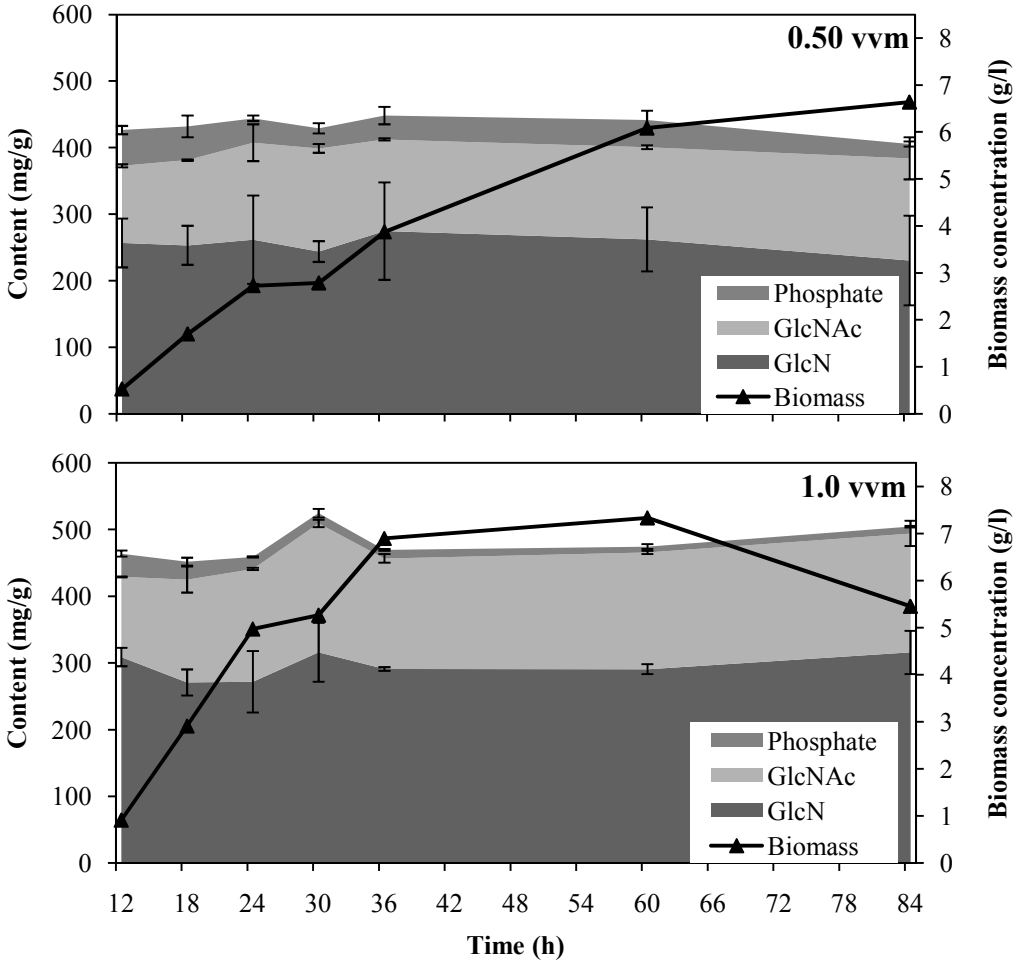

Figure 5.5: GlcN, GlcNAc and phosphate content of AIM, and the biomass concentration from *Rhizopus* sp. rep 2 in SSL50 % at 0.50 and 1.0 vvm. The error bars represent 2 standard deviations (Paper IV).

It has been suggested that the cationic chitosan in the cell wall of zygomycetes is accompanied by polyphosphate and other polymeric anions [196]. However, as was clearly demonstrated (Fig 5.5), GlcN content and phosphate content were not correlated. While the reason for the presence of polyphosphates is unclear, it is possible that they act as a phosphate reserve, which can be used when required.

5.4. Zygomycetes based superabsorbents

Liquid retaining polyelectrolytic polymers, better known as superabsorbents, is experiencing an increasing consumer demand in e.g. single-use medical and personal-care products. While the world market is dominated by the synthetic polyacrylates, polymers from natural sources, such as chitosan produced from shellfish waste chitin, are now being requested as alternatives [197]. Since the cell wall of zygomycetes is rich in chitosan (Section 5.3), one would expect it to be usable for the production of superabsorbents, and a production process was thus investigated and developed by Zamani [191]. As was demonstrated, producing a superabsorbent from the zygomycetes biomass is indeed possible, but requires several steps. Initially, AIM had to be isolated from the bulk of the biomass, and the phosphates removed by dilute sulphuric acid treatment. In the next step, the AIM was carboxymethylated to improve its hydrophilic properties, and then cross-linked to avoid dissolution of the material in the water phase. Finally, the material had to be frozen as well as freeze dried to remove the water and create the final product, holding a water binding capacity of 73 g/g.

A possible additional effect of a zygomycetes based superabsorbent also comes from the presence of chitosan, as it is known to have antimicrobial properties [198]. Should this characteristic remain after the treatment necessary for the production of the superabsorbent, it would be very attractive to use for medical applications and hygiene products.

6. CONCLUDING REMARKS

The main goal of the thesis was to investigate and develop the potential of zygomycetes fungi for two main purposes: production of ethanol, and utilisation of the resulting fungal biomass, using cellulosic materials as the starting point, and both have been accomplished. The ethanol yield acquired from hexoses rivals that of *S. cerevisiae* and is far superior when acquired from xylose, which *S. cerevisiae* is unable to ferment. The amount of biomass produced is sufficient to invoke further investigations, pending scale-up of the process.

The outcome of the different projects are concluded as follows:

- The different growth forms of *M. indicus* behaved in a similar fashion, making it possible to choose the desired growth form, expecting equivalent final ethanol yield.
- Birch and spruce were successfully pretreated with NMMO, and hydrolysed for ethanol and fungal biomass production.
- Orange peel hydrolysate, with an inhibitor concentration ca 10 times higher than that tolerated by *S. cerevisiae*, was successfully used as a substrate for *M. indicus*.
- Fungal biomass was successfully produced from spent sulphite liquor with useful contents of proteins, lipids, GlcN, and GlcNAc.
- Differential expressions of probably four genes were observed between the yeast-like and filamentous growth forms.

7. FUTURE WORK

A wise person once said, “for every question answered two more arise”, which proved to be true in this case as well. Thus, several new projects are possible:

- Developing a fungal biorefinery based on zygomycetes, either by exclusive use of zygomycetes, or by implementing them at specific points in an already existing biorefinery. Since zygomycetes are able to produce many highly interesting compounds, the end products would need to be evaluated and weighed against each other.

- Isolating new fungal strains from fermented food, and screen for advantageous properties. Possible properties could include biomass production and content, production of extracellular enzymes, lipid content and composition, and metabolite production.

- Evaluating other substrates for fungal cultivation, without limiting the search to low-value materials. Since several zygomycetes strains are already being used for human consumption, a direct possibility to improve nutritional quality of human food exists.

- Incorporating the use of modern DNA/RNA tools to reach a deeper understanding of the behaviour of zygomycetes. This might include sequencing whole genomes of many different zygomycetes, which with the current development should become feasible in the near future. Factors influencing dimorphism also need a considerably extended and thorough investigation, since it is most probable that more sequences than those currently identified, are involved.

NOMENCLATURE

AIM	Alkali insoluble material, mainly cell wall fraction
ATP	Adenosine triphosphate, main energy carrier in cells
BLAST	Basic Local Alignment Search Tool, software used for comparison of DNA and RNA sequences.
cDNA	Complimentary DNA, made from reverse transcription of RNA
GlcN	Glucosamine, main monomer of chitosan
GlcNAc	N-acetyl glucosamine, main monomer of chitin
FAD/$FADH_2$	Flavine adenine dinucleotide, cellular redox carrier
Fungi, higher	Fungi belonging to *Basidiomycota* and *Ascomycota*
Fungi, lower	Fungi not belonging to *Basidiomycota* or *Ascomycota*
GYV	Media containing glucose, yeast extract and vitamin.
NAD/NADH	Nicotinamide adenine dinucleotide, cellular redox carrier
NADP/NADPH	Nicotinamide adenine dinucleotide Phosphate, cellular redox carrier
NMMO	N-methylmorpholine-N-oxide
RACE-PCR	Rapid amplification of cDNA ends-PCR, a technique for mapping whole mRNA gene sequences
s.d.	Standard deviation
siRNA	Small interfering RNA, used for creating interference with the expression of a specific gene
SSH	Suppression subtractive hybridisation
SSL	Spent sulphite liquor
SSL 50 %	Spent sulphite liquor diluted to 50 %
TCA	Tricarboxylic acid cycle
VVM	Volume of air per volume of medium per minute

ACKNOWLEDGEMENTS

The road to the PhD is long, narrow, and filled with obstacles of various sizes. Fortunately though, it is one that I have had the pleasure of travelling in the company of others.

Mohammad, thank you for your support during the years and all the forward momentum you have provided. In some mysterious way, you always managed to find the time to help and read the manuscripts, no matter the circumstances and always so very soon. I have only now begun to understand how rare that actually is.

Lars, thank you for your guidance and all that you have taught me about scientific analysis, I would not have been the scientist I am today without your help. Even though our discussions for some reason always seemed to go on, I always enjoyed them.

Claes, thank you for keeping an eye on me during my studies and for our inspiring conversations.

Magnus, thank you for your course in experimental design and all the continued discussions we have had regarding statistical analysis. I think I am finally starting to get the hang of it now.

Jonas, thank you for your time in the lab, the constant help with all the chemicals we needed (preferably delivered yesterday), all the practical assistance, and all the fun stories about past mistakes. The lab has not been the same after you left.

I would also like to thank the people at the School of Engineering (University of Borås) for the good working environment. To name but a few, I would like to thank Hans and Peter for their support, Dag for the undergraduate courses and continued discussions about chemistry and different analyses, and Elisabeth for the introduction into the world of microbiology. I would also like to thank the (finished and current) PhD students I've come to know; Farid, Akram, Mohammad, Azam, Gergely, Anna, Päivi, Johan, Supansa, and Solmaz, thank you for all the good times.

At Chalmers, I would like to extend my gratitude to Marianne and Margareta for their assistance with the paperwork, Christer for his time as Director of Studies and for our discussions, Julie for her concerns about my studies during her time as Director of Studies, and Tom for his inspiring lectures in the general courses.

Finally, I would like to thank Jorge for our collaboration, and for providing the photograph.

Financial support was generously provided by the Swedish Energy Agency, the Swedish Knowledge Foundation (KK-stiftelsen), and the University of Borås.

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