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

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

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

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INTRODUCTION

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

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

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

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

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

EXPERIMENTAL

Fungal Strain

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

Cultivation in Shake Flasks

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

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

Cultivation in Airlift Bioreactor

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

Determination of Protein Composition

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

Total Nitrogen

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

Determination of Gross Lipids

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

Cell Wall Composition

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

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

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

Chromatography

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

Statistical Analysis

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

RESULTS AND DISCUSSION

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

Cultivation in Shake Flasks

Fungal growth

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

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

Protein content

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

AIM concentration

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

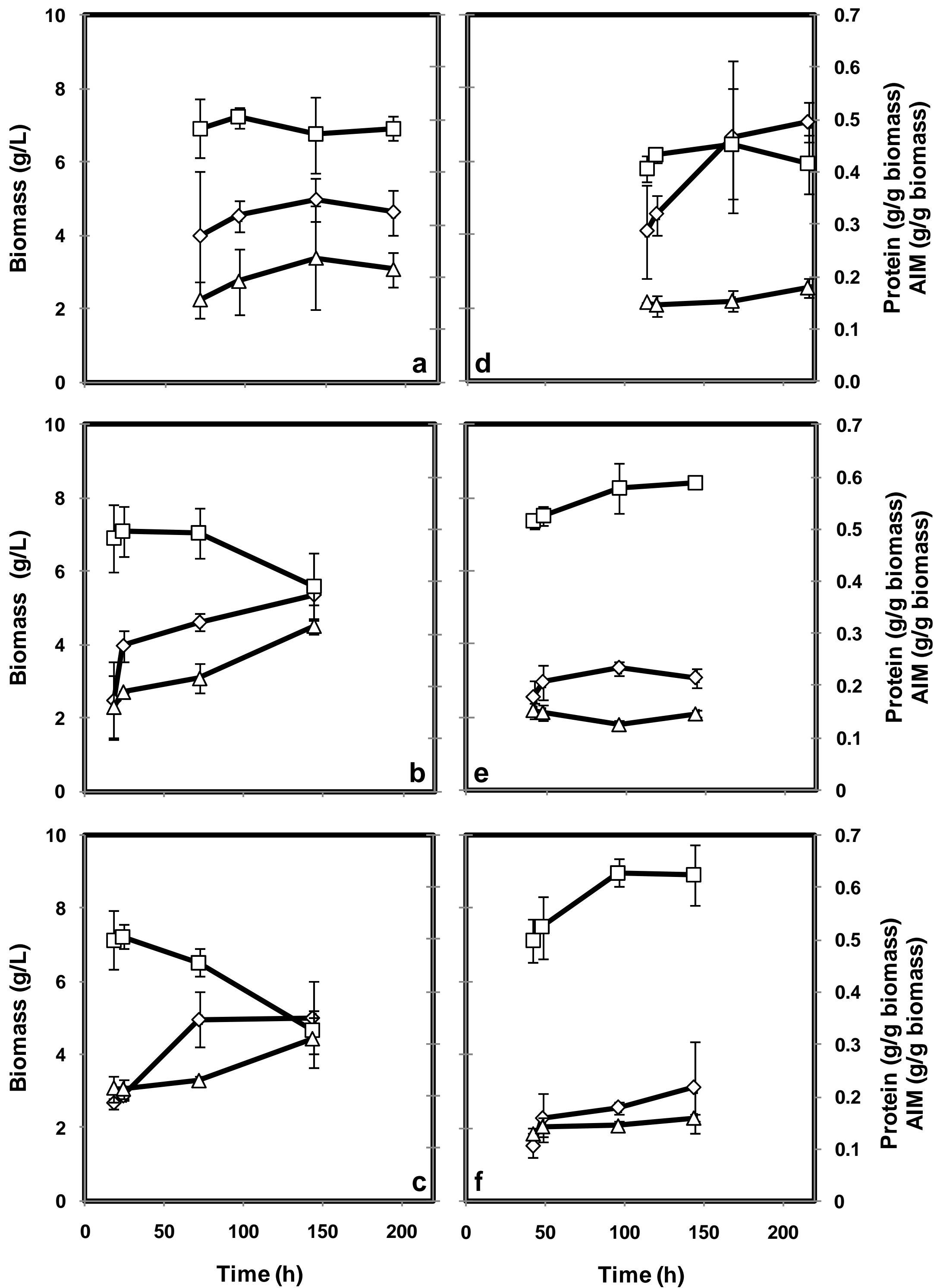

Figure 1. Biomass concentration (◊), protein (□) and AIM (Δ) profiles obtained from *Rhizopus* sp. cultivation in GYV (a – c) and SSL50% (d – f) at 20 (a, d), 32 (b, e) and 40°C (c, f)

Cell wall composition

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

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

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

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

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

Growth in Airlift Bioreactor

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

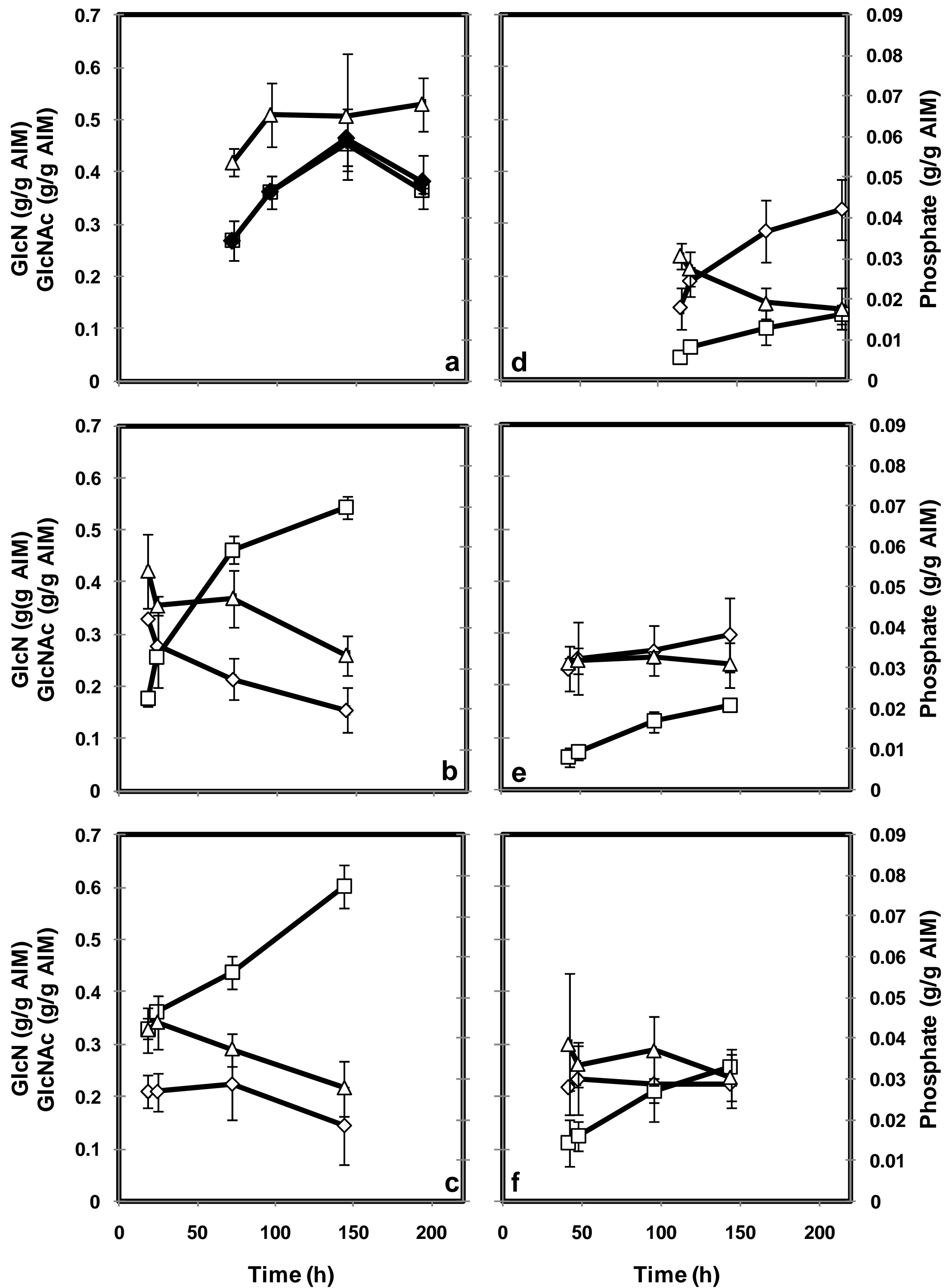

Figure 2. GlcN (◊), GlcNAc (□) and phosphate (Δ) content in acid-hydrolysed AIM derived from *Rhizopus* sp. cultivation in GYV (a–c) and SSL50% (d–f) at 20 (a, d), 32 (b, e) and 40°C (c, f)

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

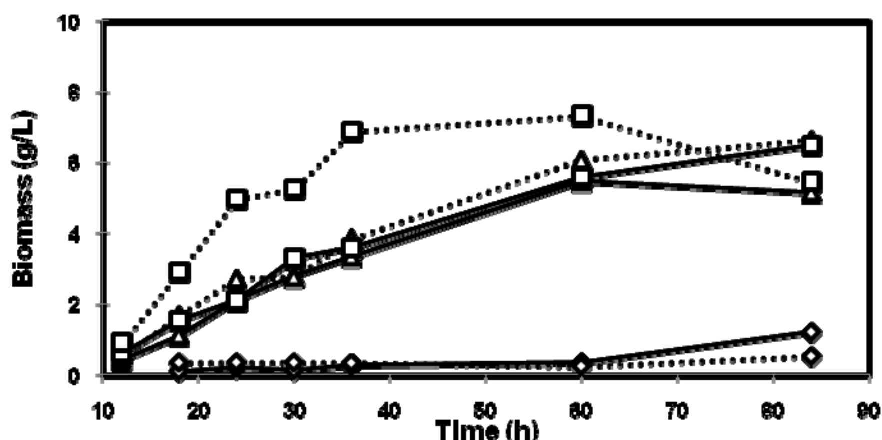

Figure 3. Biomass production of *Rhizopus* sp. in airlift at 0.15 (◊), 0.5 (Δ) and 1.0 vvm (□) on SSL50%. All experiments were done in duplicate, experiment 1 (—) and 2 (····), and their individual growth performance curves are presented

Fungal growth

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

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

Protein content

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

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

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

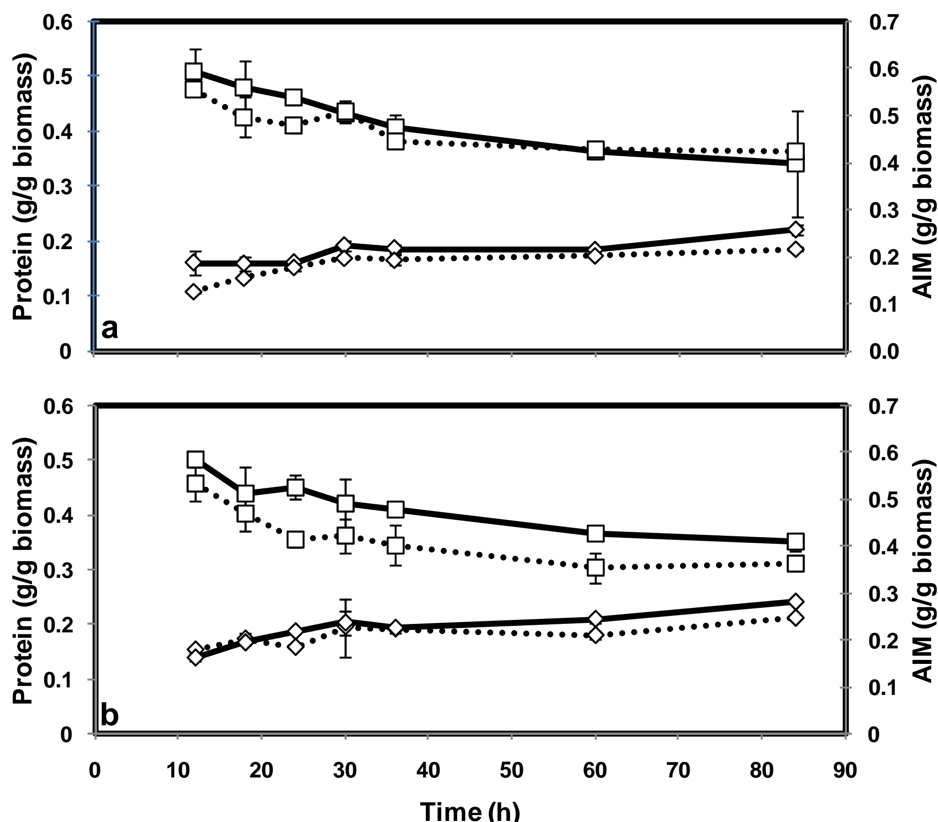

Figure 4. Protein (□) and AIM (◊) profiles during *Rhizopus* sp. cultivation in SSL50% using an airlift reactor at 0.5 (a) and 1.0 vvm (b). The individual performance of experiment 1 (—) and 2 (····) are presented.

AIM content

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

Lipid content

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

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

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

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

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

Cell wall composition

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

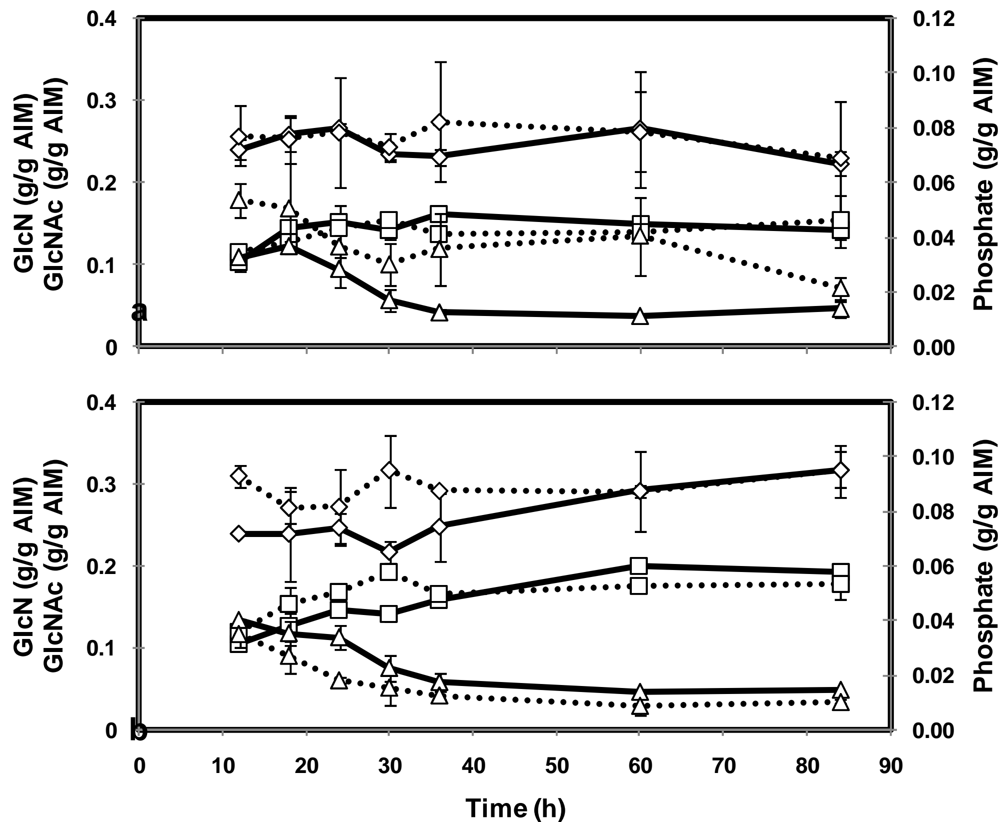

Figure 5. GlcN (◊), GlcNAc (□) and phosphate (Δ) content of AIM derived from *Rhizopus* sp. cultivation in SSL using an airlift at 0.5 (a) and 1.0 vvm (b). The individual performance of experiment 1 (—) and 2 (····) are presented.

Comparison of Protein Content with Crude Protein

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

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

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

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

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

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

General Discussion

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

CONCLUSIONS

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

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