Production of Fungal Biomass Protein by Filamentous Fungi Cultivation on Liquid Waste Streams from Pulping Process

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The aim of this study was to convert the spent liquors obtained from acidic sulfite and neutral sulfite semi-chemical (NSSC) pulping processes into protein-rich fungal biomass. Three filamentous fungi, *Aspergillus oryzae*, *Mucor indicus*, and *Rhizopus oryzae*, were cultivated on the diluted spent liquors in an airlift bioreactor with airflow of 0.85 vvm at 35 °C and pH 5.5. Maximum values of 10.17 g, 6.14 g, and 5.47 g of biomass per liter of spent liquor were achieved in the cultivation of *A. oryzae*, *M. indicus*, and *R. oryzae* on the spent sulfite liquor (SSL) diluted to 60%, respectively, while *A. oryzae* cultivation on the spent NSSC liquor (SNL) diluted to 50% resulted in the production of 3.27 g biomass per liter SNL. The fungal biomasses contained 407 g to 477 g of protein, 31 g to 114 g of fat, 56 g to 89 g of ash, and 297 g to 384 g of alkali-insoluble material (AIM) per kg of dry biomass. The amino acids, fatty acids, and mineral elements composition of the fungal biomasses corresponded to the composition of commercial protein sources especially soybean meal. Among the fungi examined, *A. oryzae* showed better performance to produce protein-rich fungal biomass during cultivation in the spent liquors.

Keywords: Fungal biomass; Protein; Aspergillus oryzae; Mucor indicus; Rhizopus oryzae; Spent sulfite liquor; Spent NSSC liquor

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INTRODUCTION

Continued population growth in the world corresponds with a demand for a higher supply of human food and animal feed, particularly for protein sources. In addition, global environmental concerns encourage researchers to develop new climate-smart proteins, where insects and microbial biomass protein (MBP) is of special interest. The production of MBP from organic wastes is one solution to the protein shortage and essential for cheaper production cost, while also reducing global environmental and industrial waste challenges (Alriksson *et al*. 2014; Ahmed *et al*. 2017). The MBP is used as a substitute for protein-rich foods, especially in animal feeds or as dietary supplements. One of the main advantages of the MBP in comparison with plant and animal proteins is that its requirement for growth are neither seasonal nor climate-dependent; therefore it can be produced throughout the year (Ukaegbu-Obi 2016). A wide variety of microorganisms, such as bacteria, yeast, and fungi, are known to produce the MBP (Azam *et al*. 2014; Ahmed *et al*. 2017). Among these microorganisms are fungi that can be grown in large quantities in

inexpensive media, such as mushrooms, Quorn®, or Indonesian tempeh, which are in the global market. Moreover, their pellet or filamentous morphology leads to cost-effective isolation of fungal biomass from culture media (Moore and Chiu 2001; Zhang *et al*. 2008; Ahmed *et al*. 2017).

Generally, filamentous fungi have played a vital role in the industrial production of biological products and in the fermentation industry due to their ability to secrete proteins and enzymes, high growth rates, ease of handling in large-scale production, and low-cost requirements for production in comparison to other microorganisms (Nevalainen *et al*. 2005; Wang *et al*. 2005; Tarshan 2016). The output of the filamentous fungi cultivation is a high-quality biomass (high protein and fat levels) that can be used as an alternative added to the main diet instead of more expensive sources such as soybean and fish (Ward 2012; Nitayavardhana *et al*. 2013; Mahboubi *et al*. 2017).

Various organic waste streams, such as rice hull (bran) hydrolysate (Rudravaram *et al*. 2003; Pogaku *et al*. 2009), rice polishing (Ahmed *et al*. 2017), winery wastes and wastewater (Zhang *et al*. 2008; Zepf and Jin 2013; Jin *et al*. 2016), starch processing wastewater (Jin *et al*. 2001, 2002, 2010; Li *et al.* 2015; Ying *et al*. 2015; Filho *et al*. 2017), agricultural wastes (Rao *et al*. 2010), fruit wastes (Yousufi 2012; Hamdy 2013; Azam *et al*. 2014), vinasse (Nitayavardhana and Khanal 2010; Nitayavardhana *et al*. 2013; Nair and Taherzadeh 2016), thin and whole stillage (Rasmussen *et al*. 2014; Bátori *et al*. 2015; Ferreira *et al*. 2015), and residual streams from wood-based biorefineries (Alriksson *et al*. 2014), have previously been used for fungal biomass protein (FBP) production through filamentous fungi.

The pulp and paper industry is among the major industries in the world that generates vast amounts of organic/lignocellulosic wastes over the course of several processes. Liquid waste from the cooking (pulping) process in a pulp mill, namely spent (black) liquor, contains a significant amount of the dissolved organic compounds derived from wood such as lignin and hemicelluloses (Ferreira *et al*. 2012; Koutinas *et al*. 2014; Rueda *et al*. 2015). The biorefinery concept, which is based on the use of this spent liquor, can produce various value-added products through a biotechnological route, instead of burning it for energy production or treating it in the costly wastewater treatment systems of mills (Koutinas *et al*. 2014). Hemicelluloses and lignin sulfonate present in the spent liquor of acidic sulfite and neutral sulfite semi-chemical (NSSC) pulping processes, namely SSL and SNL respectively, may be safely used as food additives in animal feed (Code of Federal Regulations (CFR) 2017). The SSL also has the approval of the U. S. Food and Drug Administration (FDA) for use as a binding agent in feed due to its non-detrimental properties at low concentrations and lack of stable toxic and/or accumulating chemicals (Ferreira *et al*. 2012). Several recent studies were conducted to use filamentous fungi *Rhizopus oryzae* and *Fusarium venenatum* for protein-rich fungal biomass production from SSL for feed purposes (Edebo 2009; Ferreira *et al*. 2012; Alriksson *et al*. 2014). However, further investigation on the cultivation of other filamentous fungi in the spent liquor is needed to define the suitability and possibility of various filamentous fungi towards maximum production and high quality of the FBP. Moreover, to the best of the authors' knowledge, there has been no publication that reports the use of the SNL for the production of FBP.

Three edible filamentous fungi used in this study, ascomycete *Aspergillus oryzae*, zygomycetes *Mucor indicus*, and *Rhizopus oryzae*, are qualified with GRAS (Generally Regarded As Safe) status so that metabolites and biomass produced by these strains can be safely used for food-chain products. All the fungi are traditionally used for production of

fermented foods for human consumption, *e.g.* tempeh (Ferreira *et al.* 2012; Karimi and Zamani 2013; Mahboubi *et al.* 2017).

This study investigated the potential for fermentative production of the FBP in two different lignocellulosic wastes from pulp mills, namely SSL and SNL. The performance of *A. oryzae*, *M. indicus*, and *R. oryzae*, on the cultivation of spent liquors in an airlift bioreactor was compared in terms of sugar and acetic acid consumption, fungal biomass concentration, and composition defined by crude protein, total fat, ash, amino acids, fatty acids, mineral elements, and cell wall contents in the form of alkali-insoluble material (AIM).

EXPERIMENTAL

Materials

The fungal strains *Aspergillus oryzae* var. *oryzae* CBS 819.72 (Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands), *Mucor indicus* CCUG 22424, and *Rhizopus oryzae* CCUG 28958 (Culture Collection, University of Gothenburg, Gothenburg, Sweden) were used in the current study. The strains were maintained on potato dextrose agar (PDA) plates containing 20 g/L glucose, 15 g/L agar, and 4 g/L potato extract. The prepared PDA plates were inoculated with the specific fungal strain and then incubated at 30 ºC for 4 days followed by storage at 4 ºC until use for cultivation. For the spore suspension preparation, the plates containing fungal mycelia were flooded with 20 mL sterile distilled water and then the spores were extracted by a disposable plastic spreader. Among the strains used, *A. oryzae* and *R. oryzae* were edible strains from Asian food sources.

Spent liquors

The softwood SSL and mixed hardwood SNL originated from an acidic sulfite pulping plant (Stora Enso Nymölla Mill, Nymölla, Sweden) and a neutral sulfite semichemical (NSSC) pulping plant (Mazandaran Wood and Paper Industry, Sari, Iran), respectively. The spent liquors were collected before they went through the evaporators and stored at 4 °C before use. The properties of spent liquors received from the industries are listed in Table 1.

Cultivation conditions

The experimental investigations for producing fungal biomass were conducted in a laboratory-scale batch system using a 4-L airlift bioreactor (Belach Bioteknik, Stockholm, Sweden) with a nominal working volume of 3.5 L. The riser and downward pattern was applied for the reactor flow and circulation. The inlet air passed through a 0.2-µm pore sterile polytetrafluoroethylene filter and entered the bottom of the reactor. The SSL diluted to 60%, 70%, and 80% with distilled water (SSL60%, SSL70%, and SSL80%, respectively) was supplemented with 2 mL/L 1 M ammonium dihydrogen phosphate ($NH_4H_2PO_4$) and 6.5 mL/L 25% ammonia (NH_3) in order to provide extra sources of nitrogen and phosphate. Next, it was inoculated by *A. oryzae*, *M. indicus*, and *R. oryzae*, while the SNL diluted to 50% (SNL50%) was inoculated by *A. oryzae* in the medium containing ammonium sulfate ($(NH_4)_2SO_4$) 7.5 g/L, potassium dihydrogen phosphate (KH_2PO_4) 3.5 g/L, calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$) 1.0 g/L, and magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$) 0.75 g/L as the nutrient supplementations. The used nutrient supplementations in the SSL and SNL cultures were chosen according to Ferreira

et al. 2012 and Taherzadeh *et al.* 2003, respectively. A total of 3 mL antifoam (after sterilization) and 2% (v/v) fungal spore suspension were added to 3.5 L of the sterile medium through the inlet top of the running reactor at the beginning of cultivation. The antifoam was gradually used to control foam throughout the cultivation. The airflow, temperature, and pH were set to 0.85 vvm (volume of air per volume of culture per minute), 35 ºC, and 5.5 ± 0.02 (adjusted by sodium hydroxide (NaOH) or phosphoric acid (H_3PO_4)), respectively. All media were sterilized in an autoclave at 121 °C for 20 min.

Liquid samples were taken from the fermentation broth at regular time intervals during cultivation and centrifuged at 10,000 ×*g* for 10 min. The supernatant was kept at minus 20 °C until analysis. The supernatants from the last liquid samples were analyzed in terms of total soluble sugars, chemical oxygen demand (COD), and dissolved trace elements content. The fungal biomass was harvested by pouring out the cultivation medium through a fine mesh (1 mm^2 pore area) stainless steel sieve after 48 h and 72 h cultivation on SSL and SNL, respectively. The biomass was washed with tap water until a clear filtrate was observed, then it was freeze-dried and stored at minus 20 ºC for further analysis of crude protein, total fat, amino acids, fatty acids, ash, and cell wall contents in the form of AIM. The biomass amount was gravimetrically measured and reported as grams of dried biomass per liter of the spent liquor.

Analytical Methods

Analyses of the spent liquors and culture samples

The amount of total dissolved solids and ash in the spent liquors were measured according to NREL/TP-510-42621 (Sluiter *et al.* 2008) and NREL/TP-510-42622 (Sluiter *et al.* 2005), respectively. The lignosulfonate (LS) was determined following the procedure described by Llano (2016) using an ultraviolet-visible (UV-Vis) spectrophotometer (UNICO SQ-4802; Unico, New York, USA). A colorimetric closed reflux method with a thermo-reactor system (ECO 16; VELP Scientifica, Usmate Velate, Italy) was used to measure the COD (APHA 5220D 1990).

The sugars and acetic acid content in the spent liquors and culture supernatants were quantified by high-performance liquid chromatography (HPLC; Waters, Milford, USA), equipped with UV–vis and RI detectors (Waters 2695, Waters, Milford, USA) with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) at 60 °C with 0.6 mL/min of 5 mM H_2SO_4 as eluent. A Pb-based ion-exchange column (Aminex HPX-87P, Bio-Rad, Hercules, CA, USA) operated at 85 °C with 0.6 mL/min ultrapure was used for the monomeric sugars analysis. A dilute acid hydrolysis prior to the HPLC analysis was applied to quantify total dissolved sugars (oligosaccharides) present in the spent liquors and culture supernatants (Sluiter *et al.* 2006).

Analysis of the fungal biomass

The crude protein content of fungal biomass was determined according to the Kjeldahl method using a Kjeltec 2300 analyzer unit (FOSS, Höganäs, Sweden), and expressed as N × 6.25. The total fat content was measured by soxhlet extraction system (Soxtec Avanti 2050 device, FOSS, Höganäs, Sweden) using chloroform as solvent. The ash content in fungal biomass was obtained as the residue after ignition of the biomass at 600 °C for 6 h. For measuring AIM, the fungal biomass was treated with 1 M NaOH (30:1, v/w) at 121 °C for 20 min. The material was collected by centrifugation at 3857 ×g for 15 min, washed with distilled water to approximately pH 7, freeze-dried, and weighed (Suntornsuk *et al*. 2002).

Amino acids were analyzed with an HPLC device (Knauer D14136; Knauer, Berlin, Germany) equipped with a C18 (ODS 2; Knauer, Berlin, Germany) column at a flow rate of 1 mL/min as mobile phase with a fluorescence detector (RF-530; Shimadzu, Kyoto, Japan). Before analysis, the fungal biomass was hydrolyzed with 6 M HCl for 24 h at 110 °C and then derivatized with o-phthaldialdehyde (OPA; Sigma–Aldrich, St. Louis, MO, USA).

For fatty acids analysis, lipid was extracted from the freeze-dried biomass with a mixture of chloroform:methanol (2:1, v/v) for 1 h according to the procedure described by Zhu *et al*. (2002). The fatty acids profile of the extracted lipid was determined by saponifying followed by methylation to fatty acid methyl ester (FAME) using methanolic sodium hydroxide and boron trifluoride (BF_3; Merck, Darmstadt, Germany). The FAME samples were analyzed using a Varian CP-3800 GC (Varian, Houten, Netherlands) equipped with a fused silica capillary column Stabilwax 10626 (60 m × 0.25 mm, film thickness 0.25 μm) (Restek, Bellefonte, DE, USA) and a flame ionization detector (FID) detector. The carrier gas was nitrogen. The run method involved a temperature gradient from 180 °C to 240 °C with an increase rate of 8 °C/min. Fatty acid identification was accomplished by comparison of the sample peak retention times with those of FAME's external and internal standards.

The concentrations of mineral elements (Ca, Na, K, and Mg) were determined after digestion in HNO_3/ $HClO_4$ by atomic absorption spectrophotometry (SavantAA; GBC Scientific Equipment, Braeside, Australia). Phosphorus (P) was measured according to the photometric Molybdovanad phosphate method by a spectrophotometer Jenway 6300 (Jenway, Staffordshire, UK). The results were expressed as absorbance at 440 nm.

The gross energy of the fungal biomass samples was measured by combustion of the samples in an excess of oxygen in a bomb calorimeter Parr 1261 (Parr Instrument Company, Moline, IL, USA) under standardized conditions. Analysis for all items was conducted in duplicate on a dry matter basis.

RESULTS AND DISCUSSION

Pulp and paper industry facilities can be upgraded to advanced biorefineries *via* bioconversion of liquid wastes produced from pulping (cooking) processes into various value-added products. The current study aimed to exploit three filamentous fungi *A. oryzae*, *M. indicus*, and *R. oryzae* for FBP production from the liquid waste streams received by acidic sulfite and neutral sulfite semichemical pulp mills. A proximate analysis of the obtained fungal biomass, such as crude protein, total fat, ash, and AIM, along with the determination of amino acids, fatty acids, and mineral elements composition and gross energy, was performed to investigate its suitability for feed or human consumption applications. Moreover, the fungal biomass was compared with fish meal and soybean meal (as commercial protein sources for feed) in terms of its protein, fat, ash, amino acids, fatty acids, mineral elements, and gross energy contents.

The sulfite pulping process can be performed at a pH ranging from 1 to 2 in acidic sulfite pulping to 7 to 9 in neutral sulfite pulping. The acidic sulfite process is based on the digestion of wood chips by means of sulfurous acid (H_2SO_3) and bisulfite ions ($H_2SO_3^-$), while NSSC is based on the treatment of wood chips with sodium sulfite (Na_2SO_3) and sodium bicarbonate ($NaHCO_3$) followed by mechanical disintegration (Sixta 2006; Sitter *et al*. 2014). In these processes, a considerable amount of lignocellulosic materials, such as

lignin and hemicelluloses, are dissolved in the spent liquors and removed from the pulp (solid residue) (Guo and Olsson 2014; Rueda *et al*. 2015). Bioconversion of the spent liquors through filamentous fungi cultivation can produce useful final products, such as readily harvestable fungal biomass, while the still bottoms and the residual wastes left over from cultivation are also delivered to a recovery boiler to produce energy and cooking chemicals.

The typical compositions and characteristics of the spent liquors received from SSL and SNL pulp mills are listed in Table 1.

Table 1. Characteristics of the Spent Liquors Used in the Study

Parameter	Value	
	SNL*	SSL**
pH	5.80 ± 0.10	3.20 ± 0.09
Total dissolved solids (g/L)	61.10 ± 0.01	115.00 ± 0.18
Ash (g/L)	24.35 ± 0.30	12.20 ± 0.25
Total monomeric sugars (g/L)	2.92 ± 0.26	9.95 ± 0.17
Total dissolved sugars (g/L)	9.47 ± 0.15	17.20 ± 0.20
Arabinose	0.44 ± 0.18	0.50 ± 0.01
Galactose	1.55 ± 0.07	2.09 ± 0.09
Glucose	1.43 ± 0.09	3.96 ± 0.06
Mannose	ND	7.96 ± 0.27
Xylose	6.05 ± 0.21	2.81 ± 0.24
Acetic acid (g/L)	9.58 ± 0.40	3.90 ± 0.06
Lignosulfonate (g/L)	17.60 ± 0.19	81.00 ± 0.65
COD (g/L)	70.50 ± 1.18	234.00 ± 2.36

Data are mean ± SD and n = 3; * SNL- spent NSSC liquor; ** SSL- spent sulfite liquor

The SSL from the acidic sulfite process was richer in lignocellulosic/organic materials than the SNL from neutral sulfite process due to more severe pulping conditions. The composition of spent liquors depended strongly on the type of wood and chemicals used in the pulping process as well as the pulping conditions (Sixta 2006; Pereira *et al*. 2013). As shown in Table 1, both spent liquors were composed of three major groups of nonvolatile components: ash, lignosulphonates, and sugars, while acetic acid was the most abundant volatile compound. The composition of sugars in the spent liquors depended on the composition of wood processed in the pulping stage. Because hexosans are mainly predominant hemicelluloses of softwoods, and pentosans are essentially dominant hemicelluloses of hardwoods, the corresponding spent liquors left over from the pulping processes contain mainly hexose and pentose sugars, respectively (Pereira *et al*. 2013; Weissgram *et al*. 2015). Therefore, mannose and xylose were the dominant sugars in the softwood SSL and mixed hardwoods SNL, respectively. Because oligosaccharides are present in the spent liquors, further processing into their monomeric units is required prior to HPLC analysis. Therefore, the sugars concentration in the original spent liquors (as received) was measured after diluted acid hydrolysis and expressed as total dissolved sugars. However, the measurement of sugars concentration in the liquid fermentation samples was performed without acid hydrolysis and expressed as total monomeric sugars. As shown in Table 1, the concentration of total monomeric sugars in the SSL and SNL was 9.95 g/L and 2.92 g/L, respectively. There was a considerable amount of oligomeric sugars in the spent liquors. Acetic acid was released during the early stages of the pulping (cooking). Thus, the concentration of acetic acid in the spent liquor appeared to be somewhat independent of the cooking conditions, and is directly attributed to the acetyl

content in the wood species processed in pulping stage (Sixta 2006). The concentration of acetic acid was much higher in the SNL than in the SSL due to the high acetyl groups of hardwood hemicelluloses. A higher dissolution of lignin in the acidic sulfite pulping resulted in increased lignosulfonate content in the SSL.

Fungal Growth and Biomass Production

The first screening experiments for determination of possible growth conditions by shake flask showed that *A. oryzae* and *M. indicus* were able to grow in the SSL diluted to 80%, while *R. oryzae* only grew in the SSL diluted to 60%. Earlier it has been reported that fungi growth and biomass production depended to dilution rate of SSL (Taherzadeh *et al.* 2003; Alriksson *et al.* 2014). The results of *R. oryzae* cultivation in SSL diluted to 50%, 33%, 25%, and 20% showed that no growth was obtained within 152 to 173 h, when the SSL was diluted to 50% and 33% while the highest biomass yield (0.43 g/g) belonged to the SSL diluted to 25% (Taherzadeh *et al.* 2003). The lack of the fungi growth in the concentrated SSL is probably attributable to the osmotic activity, ionic strength, and/or inhibitory activity of the high concentration of dissolved materials in the SSL (Taherzadeh *et al.* 2003). In addition, *A. oryzae* was the only strain examined that could grow in the SNL diluted to 50%. The higher tolerance of *A. oryzae* to the inhibitors present in the medium as compared to the other fungi might be reason for this case. In contrast, the SNL50% supplemented with $NH_4H_2PO_4$ and ammonia was not able to support the growth of *A. oryzae*, while the medium (SNL50%) containing $(NH_4)_2SO_4$, KH_2PO_4, $CaCl_2{\cdot}2H_2O$, and $MgSO_4{\cdot}7H_2O$ as nutrient supplementations resulted in growth and fungal biomass production.

The cultivation conditions in the airlift bioreactor were set according to the results obtained from the first screening experiments by shake flask. The fungal biomass concentrations from *A. oryzae*, *M. indicus*, and *R. oryzae* cultivation on the SSL of different dilution rates (SSL60%, SSL70%, and SSL80%) and SNL diluted to 50% (SNL50%) in the airlift bioreactor are presented in Fig. 1. The highest and lowest yields of fungal biomass were produced by *A. oryzae* in SSL60% and SNL50%, respectively. Moreover, *A. oryzae* produced more fungal biomass at all tested dilution rates compared to the two other strains. Ferreira *et al.* (2014) observed similar results *via* cultivation of *Rhizopus* sp., *Aspergillus oryzae*, *Fusarium venenatum*, *Monascus purpureus*, and *Neurospora intermedia* on wheat-based thin stillage. They found that *A. oryzae* resulted in the highest amount of produced fungal biomass (19 g/L). Maximum biomass production of *A. oryzae* in SSL60%, SSL70%, and SSL80% were 10.17 g/L, 8.63 g/L, and 6.47 g/L of SSL, respectively. *M. indicus* showed the second-best performance for the fungal biomass production in all dilution rates. The maximum values of biomass produced by *M. indicus* in SSL60%, SSL70%, and SSL80% were 6.14 g/L, 5.99 g/L, and 5.86 g/L of SSL, respectively. It was noteworthy that *R. oryzae* had no activity or growth in SSL70% and SSL80% but up to 5.47 g of biomass per liter of SSL was obtained during cultivation of *R. oryzae* in SSL60%. *Rhizopus* sp. cultivation on SSL50% supplemented with $NH_4H_2PO_4$ and ammonia resulted in the biomass concentration of 1.23, 6.64, and 7.33 g/L at 0.15, 0.5, and 1 vvm, respectively (Ferreira *et al.* 2012). In *A. oryzae* cultivation on SSL, the dilution rate had a remarkable effect on fungal biomass production. The fungal biomass concentration increased with further increase in the SSL dilution rate. There was no obvious effect on fungal biomass concentration from *M. indicus* cultivation under various dilution rates of the SSL.

The cultivation of *A. oryzae* on SNL50% presented a longer lag phase for the growth and fungal biomass production and 3.27 g biomass per liter SNL was reached after 72 h cultivation. The higher dilution needed for the SNL was possibly attributed to inhibitory activity caused by the high concentration of some dissolved materials in SNL, such as acetic acid, in comparison to the SSL.

The high viscosity of the cultivation broth, caused by the filamentous nature of the fungal growth, can negatively affect the air circulation, aeration flow pattern, and consequently the mixing of the culture, which can lead to a decrease in production efficiency and bioreactor performance (Nair and Taherzadeh 2016). In this study with the cultivation of filamentous fungi in SSL, the authors made similar observations. As mycelial clumps formed, considerable amounts of fungal mycelia were wrapped around the sparger ring (air inlet) and accumulated in the top head-space of the bioreactor after 48 h of cultivation. This was why all of the cultivation experiments on the SSL were conducted up to 48 h.

However, there was a distinct morphological difference in the growth of *A. oryzae* in the SSL as compared to the SNL. In the SSL, *A. oryzae* grew as mycelial clumps, while in the SNL it grew as compact pellets. This difference was probably related to the different medium composition between the two cultures.

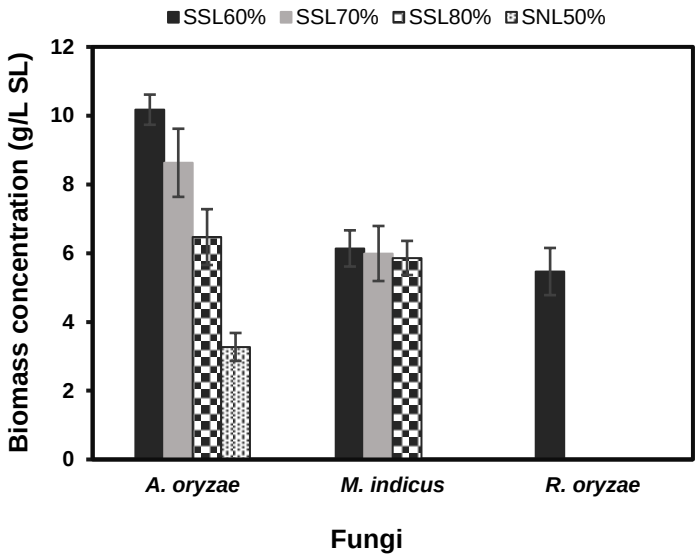


Fig. 1. Biomass concentration (g biomass per liter spent liquor) from *A. oryzae*, *M. indicus*, and *R. oryzae* cultivation on SSL60%, SSL70%, SSL80%, and SNL50%; data are averages of two replicates ± SD.

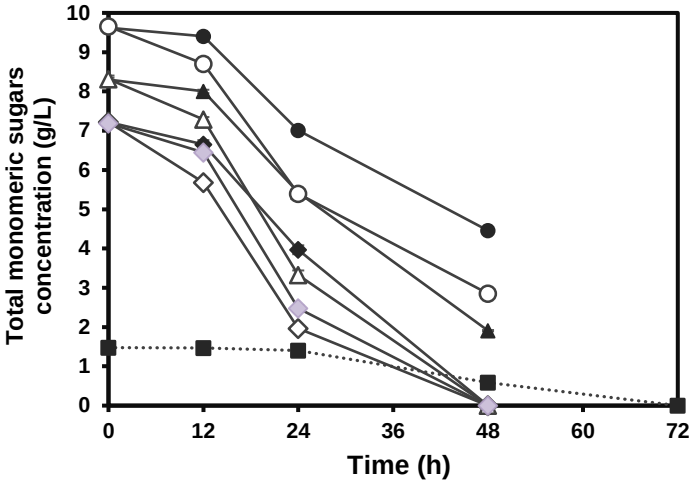
Assimilation of Sugars and Acetic Acid

The profile of the total monomeric sugars and acetic acid concentration during the cultivation of *A. oryzae*, *M. indicus*, and *R. oryzae* on the SSL and SNL is illustrated in Fig. 2. The culture broth samples collected during cultivation showed a gradual decrease in these concentrations (Fig. 2). As expected from the results of the experiments, simple (monomeric) sugars and acetic acid were consumed as sole carbon sources to allow fungal growth. The rate of monomeric consumption of sugars was different between the strains tested and media containing various dilution rates of the spent liquors. The consumption of sugars in *M. indicus* cultivation was faster than *A. oryzae* and *R. oryzae* cultivation in all of the experiments. *A. oryzae* showed a longer lag phase for the consumption of sugars

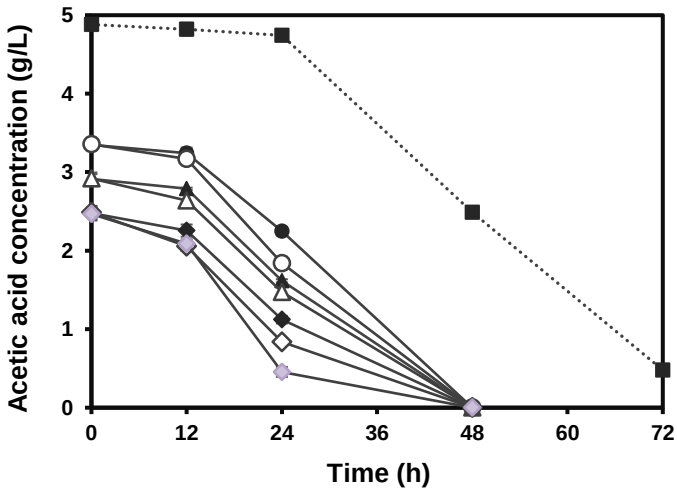
in comparison with *M. indicus* and *R. oryzae*. Although *A. oryzae* consumed all monomeric sugars during cultivation on SNL50%, it spent more time for this purpose (72 h).

Dilution of the spent liquors not only enhanced fungal growth and biomass production, it also increased the consumption rate of sugars and acetic acid. As a result, higher consumption was achieved in the more diluted SSL samples, such as SSL60%. There was no monomeric sugar in SSL60% after 48 h when it was cultivated with all three strains. The final consumption of monomeric sugars by *A. oryzae* and *M. indicus* decreased by increasing the concentration of original SSL in the medium. The final consumption of monomeric sugars by *M. indicus* was remarkably higher during cultivation in SSL70% and SSL80% than that achieved during *A. oryzae* cultivation in SSL70% and SSL80%.

Complete assimilation of acetic acid was achieved during the cultivation of filamentous fungi on the SSL60%, SSL70%, and SSL80% after 48 h, while up to 90% acetic acid was consumed during *A. oryzae* cultivation in the SNL50% after 72 h.



(a)



(b)

Fig. 2. Concentration profiles of total monomeric sugars (a) and acetic acid (b) during cultivation of *A. oryzae* (black symbols), *M. indicus* (white symbols), and *R. oryzae* (grey symbol) in SSL60% (♦ ◊ ⧫), SSL70% (▲ △), SSL80% (● ○), and SNL50% (■); data are averages of two replicates ± SD

Chemical Composition and Nutritive Value of Fungal Biomass

The nutritional value and organoleptic properties of fungal biomass can be attributed to its chemical composition. The main contents of the fungal biomass produced from the SSL and SNL by the filamentous fungi, per kg of dry matter, are shown in Table 2. As shown, protein was the major nutrient in all of the fungal biomass. It contained approximately 40% to 50% of the dry fungal biomass produced by all strains. The protein content of fungal biomass produced by *A. oryzae* grown in the medium containing SSL and SNL was higher than the protein content of fungal biomass generated by *M. indicus* and *R. oryzae*. The highest protein content was in the biomass produced by *A. oryzae* grown in SSL80% (476.6 g/kg biomass), followed by *A. oryzae* grown in SNL50% (462.3 g/kg biomass). Lower protein contents were achieved in the biomasses produced by *M. indicus* in all of the dilution rates. The fungal biomass produced by *M. indicus* grown in SSL60%, SSL70%, and SSL80% had almost the same protein content of 414.3 g/kg, 422.9 g/kg, and 407.0 g/kg biomass, respectively. It seems that there was a link between fungal growth (or fungal biomass concentration) and its protein content. In *A. oryzae* cultivations on the SSL, the crude protein content was slightly increased by decreasing the fungal biomass concentration. Higher initial fungal cell activity and consequently higher protein synthesis in the young cell mass might explain this observed inverse proportionality (Ferreira *et al.* 2012). On the other hand, *M. indicus* cultivations that presented similar concentrations of the fungal biomass (Fig. 1) contained almost the same protein contents. The protein content of *R. oryzae* biomass was 449.0 g/kg biomass, which was similar to the protein content in the biomass produced by *A. oryzae* in SSL60%.

Besides high protein content (approximately 40% to 50% of dry biomass weight), the fungal biomass also contained fat, ash, and cell wall fraction (*i.e.*, AIM). The total fat content varied from 31 g/kg to 114 g/kg biomass (3% to 11% of the dry fungal biomass) between the fungal biomass samples. The biomass produced by *A. oryzae* in SSL60% and SNL50% had higher fat content compared to the other fungal biomasses. In *A. oryzae* cultivations on the SSL, there was a direct correlation between fungal growth and its total fat content. In fact, the cultivations that included higher biomass concentration (in more diluted SSL) had also higher total fat contents. The biomass produced by *M. indicus*, unlike *A. oryzae* biomass, presented a negligible increase in total fat contents through an increase in SSL concentration in the medium, from 60% to 80%. The total fat content in *R. oryzae* biomass was 57.3 g/kg biomass, which was higher and lower than the total fat content achieved in *M. indicus* and *A. oryzae* biomass, respectively.

The biomass produced by *M. indicus* and *R. oryzae* had almost the same ash contents, which consisted of approximately 8% to 9% of the dry fungal biomass, while ash content in *A. oryzae* biomass was in the range of 56 g/kg to 70 g/kg biomass (approximately 6% to 7% of the dry fungal biomass).

The cell wall fraction was determined as AIM, which are mainly composed of glycoproteins and polysaccharides (mainly glucan and chitin). It is responsible for the shape of the cell wall and provides the fungal cell with mechanical resistance to endure the environmental changes in osmotic pressure (Edebo 2009; Tarshan 2016). Additionally, the nutritive value of the fungal biomass may enhance due to energy from polysaccharides and due to the antibacterial property of chitosan present in the cell wall of the filamentous fungi (Karimi and Zamani 2013). As shown in Table 2, the biomass produced by *M. indicus* in all dilution rates had the highest AIM content compared to the other fungal biomasses.

There was no remarkable difference in AIM content between *A. oryzae* and *R. oryzae* biomass. The AIM content in *A. oryzae* and *R. oryzae* biomass ranged from 300 g/kg to 335 g/kg biomass.

Table 2. General Chemical Composition (g/kg Dry Weight) of the Fungal Biomasses, Fish Meal, and Soybean Meal

Fungi	Dilution Rate of SL (%)	Crude Protein	Total Fat	Ash	AIM
A. oryzae	SSL60	443.84 ± 1.39	114.21 ± 0.80	56.24 ± 4.23	297.20 ± 2.83
	SSL70	455.95 ± 2.45	65.58 ± 1.22	70.14 ± 1.43	314.30 ± 3.53
	SSL80	476.63 ± 4.77	41.40 ± 1.44	62.60 ± 0.98	334.90 ± 1.27
	SNL50	462.28 ± 3.29	74.32 ± 1.49	65.22 ± 2.99	305.07 ± 2.93
M. indicus	SSL60	414.33 ± 1.81	31.09 ± 1.98	88.99 ± 2.72	371.24 ± 2.66
	SSL70	422.87 ± 2.74	39.46 ± 2.71	81.74 ± 2.53	357.00 ± 3.14
	SSL80	407.01 ± 2.47	51.87 ± 1.09	76.35 ± 1.04	383.59 ± 2.54
R. oryzae	SSL60	448.98 ± 1.70	57.28 ± 2.84	79.17 ± 1.21	324.22 ± 3.82
Commercial Protein Sources					
Fish meal*		600 to 720	40 to 200	170 to 250	-
Soybean meal**		440 to 560	5 to 30	50 to 90	-

* Based on Miles and Chapman 2012; ** Based on El-Shemy 2011; data related to the fungal biomasses are averages of two replicates ± SD

As fish meal and soybean meal are by far the most widely used protein sources in animal feed all over the world, the fungal biomasses obtained from this study were compared to these commercial protein-rich meals in terms of main components, amino acids, fatty acids, minerals contents, and gross energy. The comparative analysis in Table 2 shows that the fungal biomasses produced by all three strains were very close to soybean meal in relation to the content of crude protein and ash, while the fat content in the fungal biomasses was comparable with fish meal. As shown in Table 2, fish meal had much higher protein and ash content than the soybean meal and fungal biomasses.

An analysis of amino acids, fatty acids, mineral elements, and gross energy for the biomass produced by *A. oryzae* and *M. indicus* in different dilution rates of the SSL was accomplished when their biomass reached peak values. Therefore, the biomasses produced by *A. oryzae*, *M. indicus*, and *R. oryzae* in SSL60%, as well as the biomass produced by *A. oryzae* in SNL50%, were analyzed and compared with fish meal and soybean meal.

From a nutritional viewpoint, protein quality is distinguished *via* amino acids content (Tarshan 2016). Essential amino acids composition in the fungal biomasses, fish meal, and soybean meal are given in Table 3. As illustrated, the fungal biomasses obtained from the different experiments contained appreciable quantities of essential amino acids, and there was no remarkable difference in these quantities in various fungal biomasses.

The amounts of histidine, threonine, and valine in all of the fungal biomasses were higher than fish meal and soybean meal. All fungal biomasses contained less arginine, phenylalanine, and lysine than fish meal and soybean meal. The fungal biomasses and soybean meal had almost the same contents of methionine and leucine, but they contained less methionine and leucine compared to the fish meal. There was no obvious difference in isoleucine and tryptophan contents from the fungal biomasses, fish meal, and soybean meal. In general, the fungal biomass-derived amino acids were fairly well represented when compared to the fish meal and soybean meal.

Table 3. Essential Amino Acid Profiles (g/kg Dry Weight) of Fungal Biomasses, Fish Meal (Crude Protein, 639 g/kg DM), & Soybean Meal (Protein, 475 g/kg DM)

Amino Acid	*A. oryzae*		*M. indicus*	*R. oryzae*	Fish meal*	Soybean meal*
	SSL60%	SNL50%	SSL60%	SSL60%		
Histidine	33.55 ± 2.19	29.18 ± 1.07	32.94 ± 1.67	35.96 ± 1.25	17.8	12.8
Threonine	53.80 ± 0.44	57.44 ± 2.65	46.24 ± 1.22	50.19 ± 0.97	26.4	18.5
Arginine	23.12 ± 1.50	24.05 ± 0.85	20.47 ± 0.88	19.44 ± 1.35	36.6	34.8
Methionine	6.52 ± 0.10	8.97 ± 2.13	4.60 ± 0.75	13.10 ± 0.14	17.7	6.7
Valine	34.93 ± 2.16	35.98 ± 0.67	40.02 ± 1.77	34.39 ± 0.87	30.3	22.7
Phenylalanine	16.82 ± 0.15	16.23 ± 0.34	15.95 ± 0.10	16.48 ± 1.36	25.1	23.9
Isoleucine	25.25 ± 0.97	25.99 ± 0.91	23.86 ± 1.20	25.77 ± 0.89	25.7	21.6
Leucine	38.52 ± 1.08	38.52 ± 0.74	34.35 ± 0.96	36.50 ± 1.11	45.4	36.6
Lysine	12.65 ± 0.95	11.93 ± 1.82	9.07 ± 0.46	13.15 ± 0.93	48.1	30.2
Tryptophan	6.48 ± 0.13	5.78 ± 0.10	7.71 ± 0.87	6.78 ± 0.97	6.6	6.5

* Based on NRC 1998; data related to fungal biomasses are averages of two replicates ± SD

Table 4 shows the fatty acids composition of the fungal biomasses obtained from the different experiments, fish meal, and soybean meal. The main nutritional properties of lipids (or fats) come from fatty acids composition. In the food industry, supplementation or food enrichment with fatty acids of nutritional relevance produced by certain fungi species can minimize the risk factors related to, for example, cardiovascular or degenerative diseases (Francisco *et al*. 2017).

Table 4. Fatty Acids Composition (g/kg Dry Weight) of the Fungal Biomasses, Fish Meal, and Soybean Meal

Fatty Acids	*A. oryzae*		*M. indicus*	*R. oryzae*	Fish Meal *	Soybean Meal *
	SSL60%	SNL50%	SSL60%	SSL60%		
14:0	0.25 ± 0.03	0.23 ± 0.07	0.38 ± 0.04	0.63 ± 0.07	2.99	0.16
15:0	-	-	-	-	0.23	-
16:0	18.06 ± 1.42	14.85 ± 0.92	6.27 ± 0.67	12.46 ± 1.66	18.36	2.5
17:0	-	-	-	-	0.39	-
18:0	6.05 ± 0.88	3.67 ± 0.42	1.42 ± 0.27	4.58 ± 0.23	4.86	0.77
20:0	0.51 ± 0.02	0.18 ± 0.01	0.14 ± 0.01	0.23 ± 0.03	-	-
24:0	1.85 ± 0.76	0.74 ± 0.11	0.26 ± 0.03	0.23 ± 0.01	-	-
16:1n-7	1.00 ± 0.24	0.42 ± 0.08	0.38 ± 0.10	0.37 ± 0.05	2.94	0.11
18:1n-7	0.21 ± 0.01	0.06 ± 0.00	0.50 ± 0.01	0.59 ± 0.04	3.25	0.28
18:1n-9	24.15 ± 1.35	16.50 ± 0.93	10.89 ± 1.22	22.32 ± 1.07	6.2	1.73
18:2n-6	50.60 ± 2.47	31.33 ± 2.90	3.43 ± 0.44	3.81 ± 0.35	0.61	4.33
18:3n-6	-	-	-	-	0.27	0.58
18:3n-3	0.42 ± 0.10	0.29 ± 0.07	2.31 ± 0.25	7.25 ± 0.09	0.7	0.08
20:1n-9	0.17 ± 0.01	0.08 ± 0.00	0.005 ± 0.00	0.01 ± 0.00	-	-
20:2n-6	0.04 ± 0.01	-	0.02 ± 0.00	0.03 ± 0.01	-	-
20:3n-3	6.73 ± 0.81	4.07 ± 0.96	2.13 ± 0.16	-	-	-
20:4n-6	0.31 ± 0.05	0.09 ± 0.00	1.04 ± 0.33	1.58 ± 0.29	0.89	-
20:5n-3	0.06 ± 0.00	0.01 ± 0.00	0.005 ± 0.00	-	10.7	-
22:1n-9	-	-	1.11 ± 0.07	1.90 ± 0.26	-	-
22:5n-3	-	-	-	-	0.63	-
22:6n-3	1.64 ± 0.21	1.16 ± 0.09	0.44 ± 0.03	0.60 ± 0.02	12.37	-

Note: The contents of crude protein and lipid (fat) in fish meal are 66.71% and 6.68% (based on wet weight) while soybean meal contains 46.17% and 1.08% (based on wet weight) crude protein and lipid (fat), respectively. * Modified from Gumus 2011; data related to fungal biomasses are averages of two replicates ± SD

Most of the lipids produced in the fungal biomasses are mainly represented by fatty acids C16, C18, C18:1n-9, C18:2n-6, C18:3n-3, C20:3n-3, and C20:4n-6. Nevertheless, there were differences in the content of each of these fatty acids in various fungal biomasses. Fatty acid C18:1n-9 was the dominant fatty acid in *M. indicus* and *R. oryzae* biomass, while the biomass produced by *A. oryzae* in SSL60% and SNL50% was richest in fatty acid C18:2n-6. Although *R. oryzae* biomass was richer in fatty acid 18:3n-3, it lacked fatty acid 20:3n-3. *A. oryzae* and *M. indicus* biomass had the highest and lowest total fatty acids content, respectively, which was attributed to higher and lower total fat content in these fungal biomasses, respectively.

Fatty acid compositions in all of the fungal biomasses presented more similarities with soybean meal as compared to fish meal. As shown in Table 4, approximately 60% of total fatty acids in the fungal biomasses and soybean meal belonged to unsaturated 18 carbon fatty acids. However, the fatty acids distribution in fish meal was quite different.

Energy and protein in any feedstuff are given the most attention in feed evaluation systems. They play the key roles for functions related to maintenance and production (Tarshan 2016). As reported in Table 5, the fungal biomasses obtained from different experiments had the same gross energy (approximately 20 MJ/kg), which was clearly superior to the gross energies of fish meal and soybean meal.

The analysis of macro mineral elements of the fungal biomasses indicated that potassium and phosphorus were the predominant minerals in all of the fungal biomasses. Aside from *R. oryzae* biomass, there was no remarkable difference in the mineral elements' contents in the biomasses produced by *A. oryzae* (in SSL60% and SNL50%) and *M. indicus*. The biomass produced by *R. oryzae* had higher phosphorus and lower calcium, potassium, magnesium, and sodium contents than the biomass produced by *A. oryzae* and *M. indicus*.

Calcium and phosphorus constituted the most minerals present in fishmeal. Their contents were much higher than in the fungal biomasses and soybean meal. A higher ash content in fish meal results from the higher minerals content, especially calcium and phosphorus. The contents of sodium and phosphorus in all three fungal biomasses were much higher than soybean meal, while potassium content in all three fungal biomasses were much lower than soybean meal. The fungal biomasses and soybean meal had almost the same calcium and magnesium contents. In general, the fungal biomasses were closer to soybean meal for total minerals content.

Table 5. Macro Mineral Elements Content (g/kg Dry Weight) and Gross Energy (MJ/kg) of Fungal Biomasses, Fish Meal, and Soybean Meal

Parameter	*A. oryzae*		*M. indicus*	*R. oryzae*	Fish Meal **	Soybean Meal **
	SSL60%	SNL50%	SSL60%	SSL60%		
GE *	20.08 ± 0.10	20.63 ± 0.10	20.32 ± 0.08	19.86 ± 0.17	18.5	17.8
Ca	2.93 ± 0.32	3.46 ± 0.62	4.43 ± 0.70	1.83 ± 0.64	52.1	3.4
K	11.25 ± 0.48	12.85 ± 0.97	14.45 ± 0.20	7.39 ± 0.66	7	21.4
Mg	3.59 ± 0.80	3.96 ± 0.60	3.92 ± 0.62	2.63 ± 0.70	1.6	3
Na	4.54 ± 0.06	4.87 ± 0.04	6.10 ± 0.20	3.19 ± 0.04	4	0.2
P	17.12 ± 0.90	16.75 ± 0.22	14.68 ± 0.68	20.50 ± 0.83	30.4	6.9

* GE means gross energy; ** Based on NRC 1998; data related to fungal biomasses are averages of two replicates ± SD

Visual observations of the fungal biomasses showed that *A. oryzae* biomass was lighter in color compared to the *M. indicus* and *R. oryzae* biomass. As shown in Fig. 3, the biomass produced by *A. oryzae* in SSL60% presented the brightest color among the fungal biomasses.

Fig. 3. The freeze-dried biomass obtained from *A. oryzae* in SSL60% (A), *A. oryzae* in SNL50% (B), *M. indicus* in SSL60% (C), and *R. oryzae* in SSL60% (D)

In contrast, after complete removal of the spent liquors from the fungal biomass by simple filtration and washing, the fresh wet fungal biomass had a pleasant odor. Therefore, if the wet fungal biomass were directly supplied to an animal farm, the costs for drying the fungal biomass would be avoided as well.

Properties of the Residues of Culture Medium

The chemical compositions and properties of unfermented culture medium and residues of culture medium obtained from the different cultivation experiments are presented in Table 6. As shown, total dissolved solids of the residues of culture medium obtained from the all fermentation experiments were higher than the corresponding unfermented culture media. This outcome may be attributed to slight reduction in water volume due to evaporation during fermentation and consequently the concentrating effect of the medium. As mentioned, some organic substances of the spent liquors, such as sugars and acetic acid, could be used by the fungi. Hence, total dissolved sugars of medium decreased during fungal fermentation, while lignosulfonate was not consumed by the fungi. The medium concentrating due to slight evaporation during fermentation is why lignosulfonate concentration of the medium residues increased. As most of the heating value of spent liquor comes from lignin, the same amount of lignosulfonate in the residues of culture medium support its usability in the recovery system. In addition, the COD values in different culture media were close to each other.

Considering the results obtained in the experimental section, the fungal process can be installed near the facilities responsible for recovery of energy and cooking chemicals from the spent liquors (before the evaporators) in order to valorize the waste streams. These installed processes would be focused on conversion of the spent liquors to mainly fungal protein for animal/fish or human consumption and therefore contribute to the income of the pulp mills. On the other hand, the components analysis of the residues of culture medium (Table 6) considered that the residual wastes left over from fungal processing can also be returned to the recovery plant.

Table 6. Properties of Unfermented Culture Medium and Residues of Culture Medium Obtained from Different Cultivation Experiments

Dilution rate of the SL (%)	Total dissolved solids (g/L)	Total dissolved sugars (g/L)	Lignosulfonate (g/L)	Ash (g/L)	COD (g/L)
Unfermented culture medium containing SSL					
80	95.54 ± 0.35	13.78 ± 0.02	66.42 ± 0.33	11.84 ± 0.08	199.17 ± 4.71
70	84.30 ± 0.26	12.16 ± 0.03	58.68 ± 0.17	10.67 ± 0.10	164.33 ± 9.43
60	70.33 ± 0.21	10.84 ± 0.15	46.37 ± 0.26	8.92 ± 0.23	141.67 ± 2.36
Unfermented culture medium containing SNL					
50	44.75 ± 0.06	4.58 ± 0.26	8.65 ± 0.41	25.32 ± 0.15	34.17 ± 3.55
Residue of culture medium obtained from *A. oryzae* cultivation on SSL					
80	122.86 ± 0.25	7.38 ± 0.32	88.23 ± 0.18	18.93 ± 0.06	195.83 ± 9.43
70	102.88 ± 0.21	4.89 ± 0.09	75.02 ± 0.29	17.01 ± 0.11	153.33 ± 11.78
60	89.38 ± 0.11	3.01 ± 0.10	63.86 ± 0.25	15.73 ± 0.35	133.33 ± 2.36
Residue of culture medium obtained from *A. oryzae* cultivation on SNL					
50	67.54 ± 0.37	2.91 ± 0.18	17.87 ± 0.30	41.68 ± 0.27	41.50 ± 2.07
Residue of culture medium obtained from *M. indicus* cultivation on SSL					
80	124.70 ± 0.06	9.30 ± 0.05	89.29 ± 0.45	19.46 ± 0.16	186.67 ± 4.71
70	105.86 ± 0.19	7.62 ± 0.11	77.22 ± 0.36	16.02 ± 0.29	151.67 ± 11.78
60	92.42 ± 0.22	5.64 ± 0.07	64.95 ± 0.09	14.57 ± 0.10	138.33 ± 7.07
Residue of culture medium obtained from *R. oryzae* cultivation on SSL					
60	91.77 ± 0.05	7.05 ± 0.24	65.79 ± 0.55	13.24 ± 0.26	144.83 ± 11.78

Data are mean ± SD and n = 3

CONCLUSIONS

1. The filamentous fungi *A. oryzae* and *M. indicus* grew well in the spent sulfite SSL diluted to 60%, 70%, and 80%, but *R. oryzae* could only grow in the SSL diluted to 60%. Furthermore, the SNL diluted to 50% could only support the growth of *A. oryzae*.
2. Maximum biomass production by *A. oryzae*, *M. indicus*, and *R. oryzae* after 48 h cultivation was obtained in the SSL60%, and the corresponding values were 10.2 g/L SSL, 6.14 g/L SSL, and 5.47 g/L SSL, respectively. Moreover, *A. oryzae* produced 3.27 g biomass per liter SNL after 72 h cultivation in the SNL50%.
3. The protein, fat, ash, and AIM contents in the fungal biomasses from the different experiments were in the range of 407 g/kg dry biomass to 477 g/kg dry biomass, 31 g/kg dry biomass to 114 g/kg dry biomass, 56 g/kg dry biomass to 89 g/kg dry biomass, and 297 g/kg dry biomass to 384 g/kg dry biomass, respectively. High protein and fat contents in the fungal biomasses, along with their edible character, may thus make an excellent animal feed.
4. The fungal biomasses produced by *A. oryzae*, *M. indicus*, and *R. oryzae* in the selected cultivations contained appreciable quantities of amino acids, fatty acids, and mineral elements that were comparable to that achieved in fish meal and soybean meal. A comparative analysis showed that the fungal biomasses were close to soybean meal in terms of protein, amino acids, fatty acids, and mineral contents.

5. *A. oryzae* was the best strain for the FBP production as animal feed from the spent liquors due to higher fungal biomass production, high protein and fat content, pleasant odor, and bright color of the obtained biomass.
6. The utilization of filamentous fungi, especially *A. oryzae*, for converting the dissolved organics present in the SSL and NSL into protein-rich fungal biomass can be a practical and promising alternative in industrial biotechnology (biorefinery) and generate additional revenue for the pulp and paper industry.

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