

Ethanol and Biomass Production from Spent Sulfite Liquor by Filamentous Fungi

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Abstract—Since filamentous fungi are capable of assimilating several types of sugars (hexoses and pentoses), they are potential candidates for bioconversion of spent sulfite liquor (SSL). Three filamentous fungi such as *Aspergillus oryzae*, *Mucor indicus*, and *Rhizopus oryzae* were investigated in this work. The SSL was diluted in order to obtain concentrations of 50, 60, 70, 80, and 90% and supplemented with two types of nutrients. The results from cultivations in shake flask showed that *A. oryzae* and *M. indicus* were not able to grow in pure SSL and SSL90% while *R. oryzae* could grow only in SSL50% and SSL60%. Cultivation with *A. oryzae* resulted in the highest yield of produced fungal biomass, while *R. oryzae* cultivation resulted in the lowest fungal biomass yield. Although, the mediums containing yeast extract, $(\text{NH}_4)_2\text{SO}_4$, KH_2PO_4 , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ as nutrients supplementations produced higher fungal biomass compared to the mediums containing $\text{NH}_4\text{H}_2\text{PO}_4$ and ammonia, but there was no significant difference between two types of nutrients in terms of sugars and acetic acid consumption rate. The sugars consumption in *M. indicus* cultivation was faster than *A. oryzae* and *R. oryzae* cultivation. Acetic acid present in SSL was completely consumed during cultivation of all fungi. *M. indicus* was the best and fastest ethanol producer from SSL among the fungi examined, when yeast extract and salts were used as nutrients supplementations. Furthermore, no further improvement in ethanol concentration and rate of sugars consumption was obtained in medium supplemented with $\text{NH}_4\text{H}_2\text{PO}_4$ and ammonia compared to medium containing yeast extract, $(\text{NH}_4)_2\text{SO}_4$, KH_2PO_4 , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. On the other hand, the higher dilution of SSL resulted in a better fermentability, and better consumption of sugars and acetic acid.

Keywords—Ethanol, filamentous fungi, fungal biomass, spent sulfite liquor.

I. INTRODUCTION

THE fossil fuels resources, petroleum, natural gas, and charcoal are decreasing in world while energy requirements are progressively growing up. Moreover, the environmental concerns and the future risks of global warming from fossil sources utilization are increasing. Accordingly, the search for sustainable alternatives to produce fuels and chemicals from non-fossil feedstocks, especially biomass, is

attracting a considerable interest worldwide [1]-[3]. In this context, the development of “second generation biofuels” from lignocellulosic biomass has called attention from public, politics, and research all over the world, since the use of crops and food resources for the production of the so called “first generation biofuels” contributed for the rise of prices of food worldwide, resulting in social disturbance in many countries [1], [4]-[6]. The production of bioethanol from lignocellulosic biomass serves many advantages from both energy and environmental point of views in the area because it can be easily produced in large scale for blending with gasoline or to be used as pure “green” fuel [4], [7]. Among the lignocellulosic biomasses, residues from pulp and paper industry with high organic load and being abundantly available and low-priced play an important role [2], [6]. SSL is side products from acidic sulfite pulping of wood, and is normally burned to recover energy and the inorganic base [3], [4]. Several sulfite pulp mills in the world have utilized SSL for ethanol production.

The major components of SSL are lignosulfonates (LS) and sugars, which are recognized as valuable by-products for the production of added value products [4]. In addition to sugar, SSL also contains 5-hydroxymethylfurfural (HMF) and furfural, organic acids, wood extractives, dissolved solids, and residues from the cooking process such as sulfite ions, and is therefore a highly inhibitory fermentation medium which negatively affects fermentation efficiency. In addition, low sugar content and variations in hexose and pentose composition may limit cell growth and reduce final ethanol yield [2], [8]-[10]. While, Baker’s yeast (*Saccharomyces cerevisiae*) is the most commonly used microorganism for the fermentative conversion of hexoses to ethanol, it may be severely inhibited under these conditions, which will result in poor productivity and incomplete fermentation [10], [11]. The selection of a suitable ethanol-producing microorganism which is tolerant of all the inhibitors in the hydrolysates (e.g. SSL) and able to ferment all the sugars, including pentoses, into ethanol, is a key solution to attack this problem [5], [12].

Filamentous fungi have played a vital role in the industrial production of biotechnological products due to the metabolic versatility of this group of microorganisms. Filamentous fungi are able to grow under both anaerobic and aerobic conditions and to assimilate various carbon sources including hexose and pentose sugars. In addition, fungal cells are characterized by an extraordinary ability to secrete large amounts of proteins, metabolites, and organic acids into their growth medium [13], [14].

Various filamentous fungi have been used on wide variety

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of lignocellulosic materials, providing cellulosic and hemicellulosic feedstocks, for ethanol production. Millati et al. [11] examined nine zygomycetes strains including three strains of *Rhizopus oryzae*, *Mucor corticolous*, *M. hiemalis*, *M. indicus*, *Rhizomucor pusillus*, *R. miehei*, and zygomycete IT for ethanol production from glucose, xylose, and wood hydrolyzates. The results of their research indicated that all strains were capable of growing on glucose or xylose as single carbon source while all *Mucor* and two *R. oryzae* strains could grow on dilute-acid hydrolyzate from wood. Moreover, two *Mucor* species, *M. hiemalis* and *M. indicus* showed greater ethanol production than the other strains. Ethanol production from dilute-acid pretreated rice straw with *Mucor indicus*, *Rhizopus oryzae*, and *Saccharomyces cerevisiae* was investigated by Karimi et al. [15], whereby *R. oryzae* had the best ethanol yield as 74% from rice straw followed by *M. indicus* with an overall yield of 68% with 15 FPU/g dry matter (DM) of cellulose. The evaluation of ethanol and protein production from the stillage using filamentous fungi *Neurospora intermedia*, *Aspergillus oryzae*, *Fusarium venenatum*, *Monascus purpureus*, and *Rhizopus sp.* indicated that all filamentous fungi were able to successfully grow in the medium. *N. intermedia* was also shown to be superior to *S. cerevisiae* regarding ethanol production from whole stillage [16], [17]. Fermentation of SSL has been studied for the production of different metabolites. Taherzadeh et al. [18] investigated the cultivation conditions for *Rhizopus oryzae* grown in synthetic medium and SSL to achieve high biomass and ethanol yields using shake flasks and bioreactors. All the sugars present in SSL were assimilated with the fungus and the medium composition and cultivation conditions had strong influence on the time course and yields of biomass as well as the metabolites from SSL and also other carbon sources.

The present work was aimed at studying the performance of three filamentous fungi *Aspergillus oryzae*, *Mucor indicus*, and *Rhizopus oryzae*, and compare them in terms of sugar and acetic acid consumption and ethanol production from SSL. In addition, the effect of dilution of SSL and different nutrient supplementations on the fungi growth and ethanol production were examined.

II. MATERIALS AND METHODS

A. The Fungal Strains

Aspergillus oryzae var. *oryzae* CBS 819.72 (Centraalbureau voor Schimmelcultures, The Netherlands), *Mucor indicus* CCUG 22424, and *Rhizopus oryzae* CCUG 28958 (Culture Collection, University of Gothenburg, Gothenburg, Sweden) were used in all fermentations. The strains were cultivated on agar plates with medium composition of 20 g/L glucose, 15 g/L agar, and 4 g/L potato extract. The inoculated medium was incubated at 30 °C for 4 days to form cotton-like mycelium and spores. The plates were maintained at 4 °C until use. The plates were then flooded with 20 mL sterile distilled water and the spores were extracted by a disposable plastic spreader.

B. Spent Sulfite Liquor

The softwood SSL was originated from a sulfite pulp plant (Stora Enso Nymölla Mill, Sweden). The liquor was collected before evaporators. The SSL was stored at 4 °C before use. The chemical composition of softwood SSL with total dissolved solids of 11.5 % was approximately as follows: mannose 4.8 g/L, glucose 2.3 g/L, xylose 1.7 g/L, galactose 0.8 g/L, arabinose 0.3 g/L, acetic acid 3.9 g/L, lignosulfonate 68 g/L, and ash content 12.2 g/L. The pH of this SSL was 3.2.

C. Cultivation in Shake Flask

The series of cultivations were carried out in 250 mL cotton-plugged flasks containing 100 mL of medium. The SSL dilution was performed by distilled water in order to achieve SSL concentration levels of 50, 60, 70, 80, and 90 %. The pure SSL (SSL 100%) was examined as well. The performance of each strain was assayed in two mediums supplemented with different nutrients supplementations (see Table I). The mediums were supplemented with two different types of nutrients. Some mediums used yeast extract 4 g/L, (NH₄)₂SO₄ 7.5 g/L, KH₂PO₄ 3.5 g/L, CaCl₂·2H₂O 1.0 g/L, and MgSO₄·7H₂O 0.75 g/L as nutrients supplementations and the other ones were supplemented with 2 mL/L 1 M NH₄H₂PO₄ and 6.5 mL/L 25 % solution NH₃. The medium pH was adjusted up to 5.5±0.1 before cultivation with 10 M NaOH. After spore suspension addition into the flasks containing the mediums under sterilized conditions, the incubations were carried out by a shaker bath with shaker speed of 125 rpm at 35 °C for 4 days. Samples were taken from the fermentation broth at predestined times and centrifuged at 10,000 ×g for 10 minutes. The supernatant was kept at -20 °C until analysis. All mediums were sterilized in an autoclave at 121 °C for 20 min. All the experiments were triplicated.

D. Analytical Methods

Harvested biomass at the end of the experiment (cultivation) was dried to achieve constant weight in an oven for 24h at 70 °C and reported as biomass production in g/L.

The liquid samples of the cultivations were analyzed by high-performance liquid chromatography (HPLC), equipped with UV-vis and RI detectors (Waters 2695, Waters, Milford, USA). For quantifying the sugars, a Pb-based ion-exchange column (Aminex HPX-87P, Bio-Rad, USA) at 85 °C and 0.6 mL.min⁻¹ ultrapure was used while acetic acid, ethanol and glycerol were analyzed with a hydrogen-ion based ion-exchange column (Aminex HPX-87H, Bio-Rad) at 60 °C with 0.6 mL.min⁻¹ of 5 mM H₂SO₄ as eluent.

III. RESULTS AND DISCUSSION

A. Fungi Growth and Biomass Production

The effect of SSL dilution rates and nutrients supplementations on fungi growth and biomass production was investigated by cultivating *A. oryzae*, *M. indicus*, and *R. oryzae* in shake flasks, and the results are summarized in Table I. The biomass concentrations were measured after 4 days of cultivation as g/L medium and g/L SSL. The mediums containing yeast extract, (NH₄)₂SO₄, KH₂PO₄, CaCl₂·2H₂O,

and $MgSO_4 \cdot 7H_2O$ as nutrients supplementations resulted in higher fungal biomass compared to the mediums containing $NH_4H_2PO_4$ and ammonia (Table I). Yeast extract present in the first medium might be the reason for this increase because it provides the nutrients such as protein, free amino acids, vitamins, and minerals for optimal growth rates, but is too expensive to be used on an industrial scale [9]. Therefore, finding cheaper options for nitrogen source is inevitable. This experiment showed that ammonium phosphate and ammonia were sufficient as nutrients supplementations for fungi growing in SSL. The results of cultivation in diluted SSL and pure SSL indicated that no growth was obtained in SSL90% and SSL100% (pure SSL) when *A. oryzae* and *M. indicus* were cultivated in both of mediums. Although SSL concentrations of 50, 60, 70, and 80% supported the growth of *A. oryzae* and *M. indicus*, but *R. oryzae* could grow only in SSL50% and SSL60%. When yeast extract, $(NH_4)_2SO_4$, KH_2PO_4 , $CaCl_2 \cdot 2H_2O$, and $MgSO_4 \cdot 7H_2O$ were used as nutrients supplementations in the cultivations, the biomass

concentration for all the strains decreased by increasing the SSL concentrations while using ammonium phosphate and ammonia in the mediums, biomass concentration increased until SSL70% and SSL60% at the end of *A. oryzae*, *M. indicus*, and *R. oryzae* cultivation, respectively. Cultivation with *A. oryzae* resulted in the highest amount of produced fungal biomass while *R. oryzae* cultivation produced the lowest fungal biomass (Table I). The fungi did not grow in pure and concentrated SSL, probably due to osmotic activity, ionic strength, and/or inhibitory activity of the high concentration of dissolved materials. The liquor had to be diluted with distilled water in order to support the growth of the fungi [18].

B. Assimilation of Sugars and Ethanol Formation

The sugars consumption profiles during the cultivation *A. oryzae*, *M. indicus*, and *R. oryzae* on several SSL concentrations with two types of nutrients are presented in Fig. 1.

TABLE I
BIOMASS CONCENTRATION DURING CULTIVATION OF *A. ORYZAE*, *M. INDICUS*, AND *R. ORYZAE* IN DIFFERENT SSL CONCENTRATIONS AND NUTRIENTS SUPPLEMENTATIONS

Fungi	Nutrient supplementations	SSL concentrations (%)	Biomass concentration (g/L medium)	
			(g/L medium)	(g/L SSL)
<i>Aspergillus oryzae</i>	Yeast extract, 4 g/L	50	5.6	10.065
	$(NH_4)_2SO_4$, 7.5 g/L	60	5.06	7.577
	KH_2PO_4 , 3.5 g/L	70	5.004	6.43
	$CaCl_2 \cdot 2H_2O$, 1.0 g/L	80	4.74	5.318
	$MgSO_4 \cdot 7H_2O$, 0.75 g/L	50	3.92	6.97
	1 M $NH_4H_2PO_4$, 2 mL/L	60	4.21	6.275
	25 % solution NH_3 , 6.5 mL/L	70	4.37	5.557
		80	2.79	3.106
	Yeast extract, 4 g/L	50	4.58	8.3
	$(NH_4)_2SO_4$, 7.5 g/L	60	3.81	5.64
<i>Mucor indicus</i>	KH_2PO_4 , 3.5 g/L	70	3.49	4.45
	$CaCl_2 \cdot 2H_2O$, 1.0 g/L	80	2.95	3.275
	$MgSO_4 \cdot 7H_2O$, 0.75 g/L	50	2.15	3.88
	1 M $NH_4H_2PO_4$, 2 mL/L	60	2.72	4.06
	25 % solution NH_3 , 6.5 mL/L	70	2.99	3.84
		80	2.47	2.78
	Yeast extract, 4 g/L	50	2.98	5.35
	$(NH_4)_2SO_4$, 7.5 g/L	60	1.96	2.94
	KH_2PO_4 , 3.5 g/L	70	1.96	2.94
	$CaCl_2 \cdot 2H_2O$, 1.0 g/L	80	1.96	2.94
<i>Rhizopus oryzae</i>	$MgSO_4 \cdot 7H_2O$, 0.75 g/L	50	1.88	3.35
	1 M $NH_4H_2PO_4$, 2 mL/L	60	1.99	2.92
	25 % solution NH_3 , 6.5 mL/L	60	1.99	2.92
		60	1.99	2.92

In this study, the sugar consumption was analyzed as total monomeric sugars consumption. The concentration of total monomeric sugars decreased during *A. oryzae* cultivation in all SSL dilutions but they have still remained in the medium at the end of cultivation. On the other hand, higher SSL concentrations such as 70% and 80% achieved longer time for sugar assimilating (Fig. 1 (a)). The sugars consumption was faster in *M. indicus* cultivation and there were no sugars in the medium at the end of cultivation (Fig. 1 (b)). *R. oryzae* assimilated monomeric sugars completely but SSL60% spent more time for this purpose (Fig. 1 (c)). The lag phase for *A.*

oryzae was longer in comparison with *M. indicus* and *R. oryzae*. There was no significant difference between two types of nutrients in terms of sugars consumption rate. All the fungi could assimilate sugars in the medium containing $NH_4H_2PO_4$ and ammonia the same as the medium containing yeast extract, $(NH_4)_2SO_4$, KH_2PO_4 , $CaCl_2 \cdot 2H_2O$, and $MgSO_4 \cdot 7H_2O$.

Fig. 2 shows acetic acid concentration during the cultivation *A. oryzae*, *M. indicus*, and *R. oryzae* on different SSL concentrations and nutrients. Acetic acid is a weak acid generated from the deacetylation of hemicellulose during pretreatment such as pulping process. Although acetic acid can

act as an inhibitor for some microorganisms and affect fermentation profiles but low concentrations of acetic acid may improve fermentation rates with increased ethanol yield. It can be utilized as a carbon resource and also leads the

variation of pH value during the fermentation [1], [19]-[21]. All of fungi were able to assimilate acetic acid present in SSL and it was completely consumed at the end of cultivation.

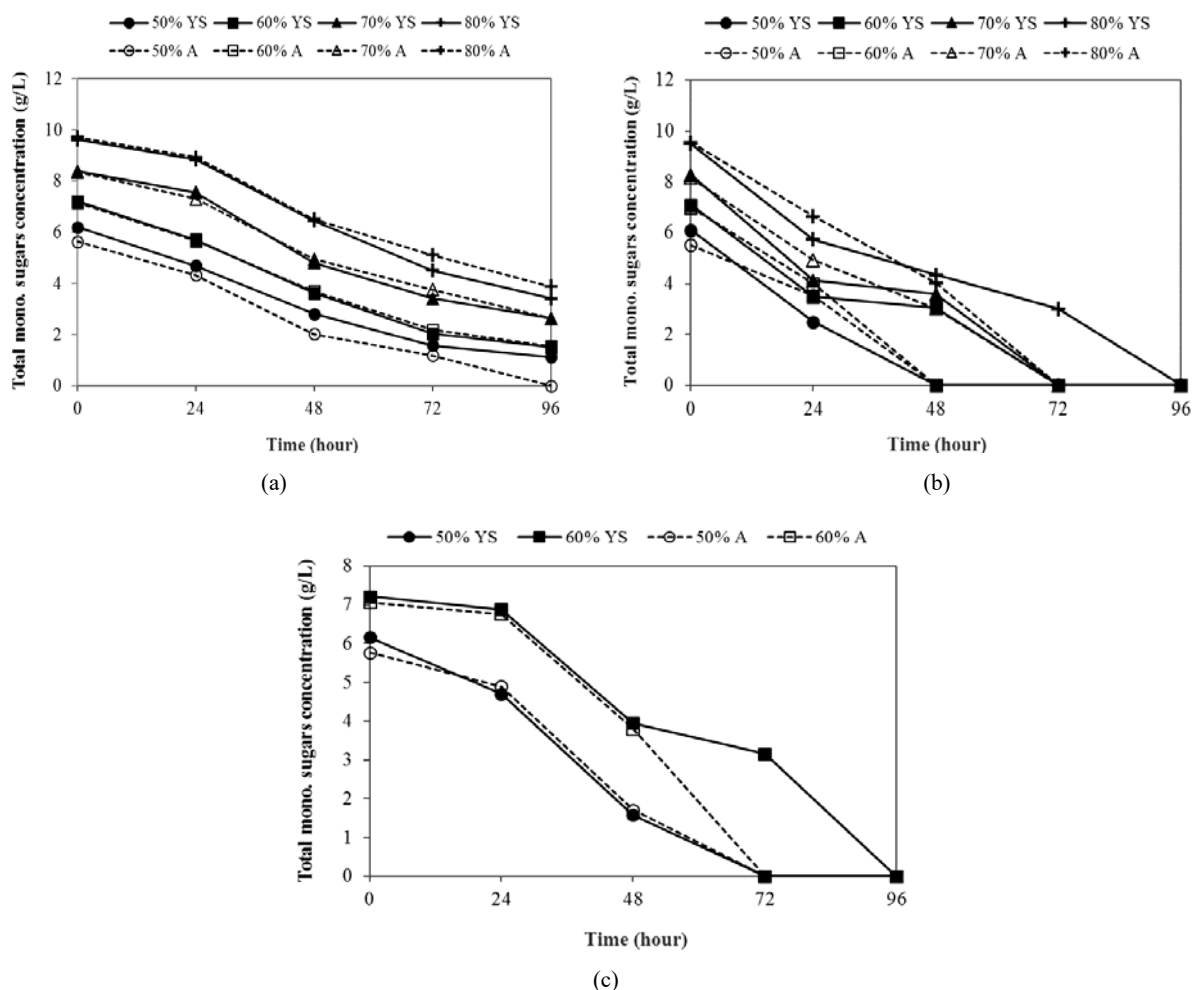
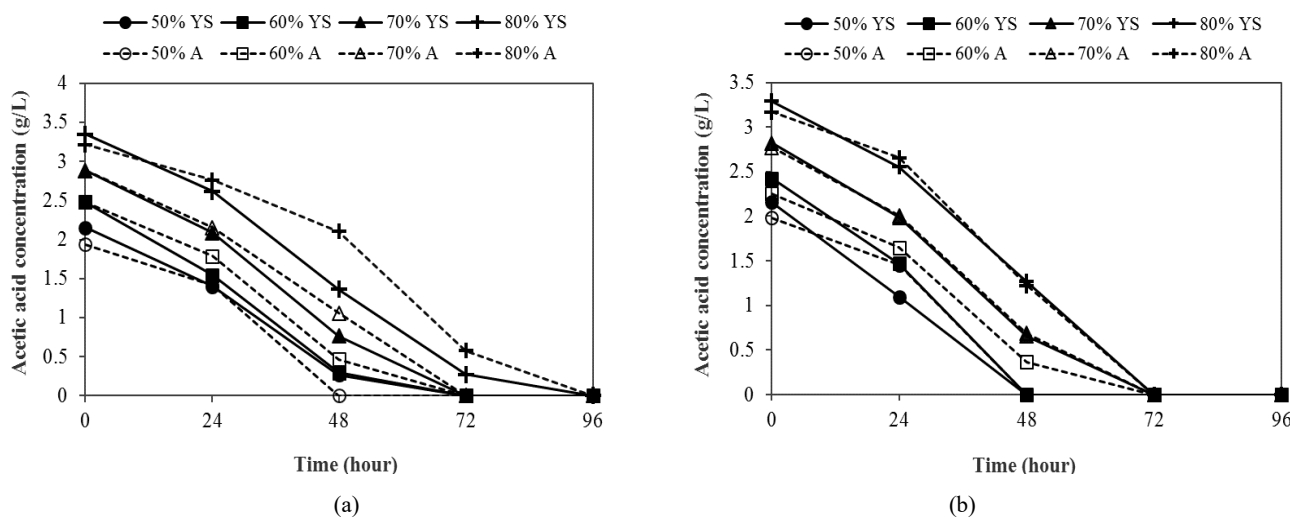
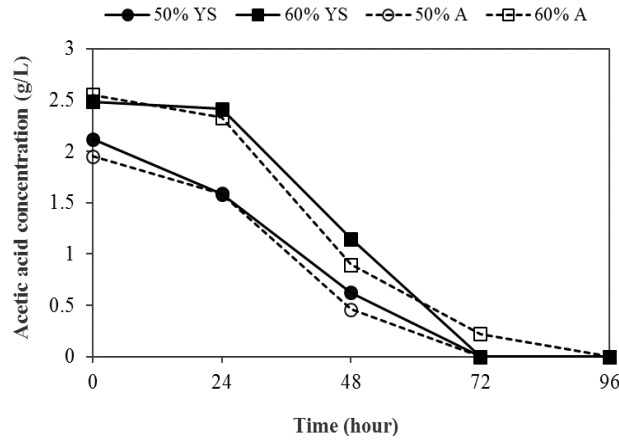


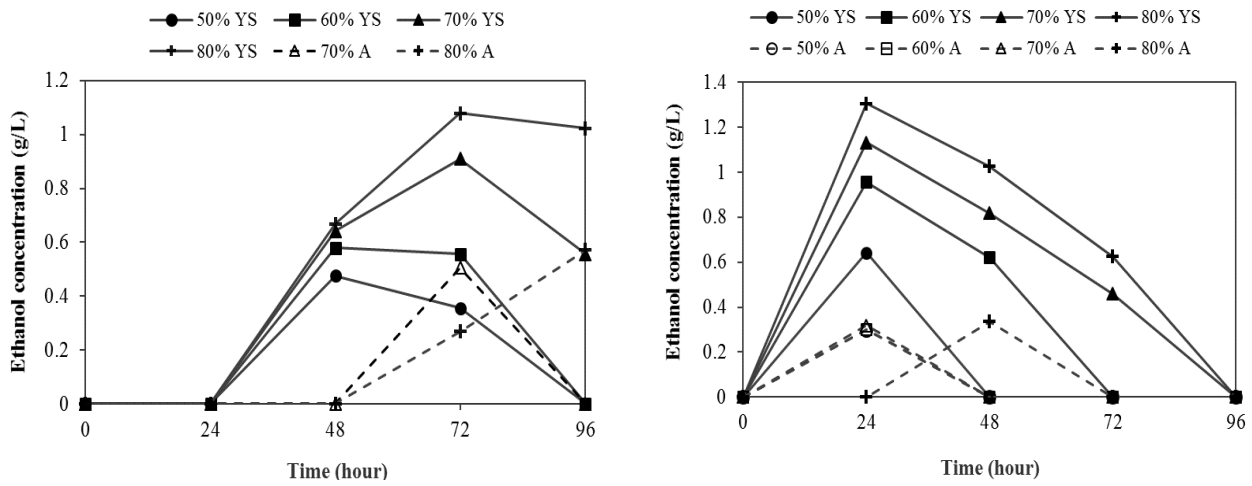
Fig. 1 Concentration of total monomeric sugars in cultivation of (a) *A. oryzae*, (b) *M. indicus*, and (c) *R. oryzae* on SSL50% (circles), SSL60% (squares), SSL70% (triangles), and SSL80% (plus sign) with yeast extract, $(\text{NH}_4)_2\text{SO}_4$, KH_2PO_4 , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (straight lines) and $\text{NH}_4\text{H}_2\text{PO}_4$ and ammonia (dashed lines)





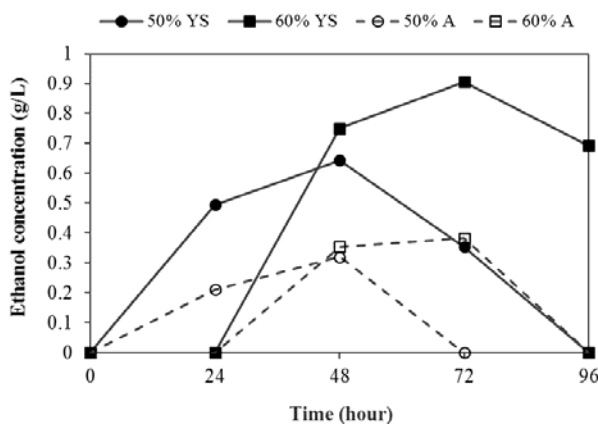
(c)

Fig. 2 Concentration of acetic acid in cultivation of (a) *A. oryzae*, (b) *M. indicus*, and (c) *R. oryzae* on SSL50% (circles), SSL60% (squares), SSL70% (triangles), and SSL80% (plus sign) with yeast extract, $(\text{NH}_4)_2\text{SO}_4$, KH_2PO_4 , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (straight lines) and $\text{NH}_4\text{H}_2\text{PO}_4$ and ammonia (dashed lines)



(a)

(b)



(c)

Fig. 3 Ethanol profiles during cultivation of (a) *A. oryzae*, (b) *M. indicus*, and (c) *R. oryzae* on SSL50% (circles), SSL60% (squares), SSL70% (triangles), and SSL80% (plus sign) with yeast extract, $(\text{NH}_4)_2\text{SO}_4$, KH_2PO_4 , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (straight lines) and $\text{NH}_4\text{H}_2\text{PO}_4$ and ammonia (dashed lines)

The performance of the three filamentous fungi *A. oryzae*, *M. indicus*, and *R. oryzae* in the medium containing SSL with

different dilutions and two nutrients supplementations on ethanol formation was investigated (Fig. 3). At the first 48h cultivation, *A. oryzae* started ethanol production in the medium supplemented with yeast extract, $(\text{NH}_4)_2\text{SO}_4$, KH_2PO_4 , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and maximum ethanol concentrations in SSL50%, SSL60%, SSL70%, and SSL80% were 0.48, 0.58, 0.91, and 1.08 g/L, respectively. In cultivation of *A. oryzae* on SSL50% and SSL60% with $\text{NH}_4\text{H}_2\text{PO}_4$ and ammonia, no ethanol was formed and biomass was the only final product. In contrast, when SSL was more diluted (70% and 80%), formation of ethanol with a maximum concentration of 0.51 and 0.57 g/L respectively were detected. In the medium containing $\text{NH}_4\text{H}_2\text{PO}_4$ and ammonia, ethanol production was started during the first 72h (Fig. 3 (a)). When yeast extract and salts were used as nutrients supplementations, *M. indicus* was the best and fastest ethanol producer from SSL among the tested fungi and the fungus produced ethanol with maximum concentrations of 0.64, 0.96, 1.13, and 1.31 g/L in SSL50%, SSL60%, SSL70%, and SSL80%, respectively. Ethanol production rate was higher for *M. indicus*, since the consumption of sugars was faster during cultivation (Fig. 3 (b)). *R. oryzae* produced ethanol with maximum concentrations of 0.64 and 0.91 g/L in SSL50% and SSL60%, respectively when yeast extract and salts were applied in the medium (Fig. 3 (c)). The results indicated that the medium supplemented with $\text{NH}_4\text{H}_2\text{PO}_4$ and ammonia in all cultivations resulted in a less ethanol production and longer lag phase compared to the medium containing yeast extract and salts which is probably related to the presence of yeast extract in the medium. All fungi consumed the ethanol during cultivation. Therefore, the concentrations of ethanol decreased slowly at the end of cultivation. This was confirmed by growing *M. indicus* on ethanol as a sole carbon resource (data not shown) [15].

IV. CONCLUSION

It can be concluded that the SSL as an undesirable by-product of pulp and paper industries can be fermented to ethanol and mycelial biomass by filamentous fungi. In addition to good performance of filamentous fungi in hexoses and pentoses assimilating and also aerobic and anaerobic cultivation, they are able to produce fungal biomass which can be further used for the production of valuable products such as protein and chitosan. On the other hand, the type of nutrients supplementations and dilution rates has considerable effect on sugar and acetic acid consumption as well as fungal biomass and ethanol production from SSL.

ACKNOWLEDGMENT

The work was financed through the Swedish Centre for Resource Recovery, University of Borås. The authors would like to thank Stora Enso Nymölla Mill for providing the spent sulfite liquor and all the lab personnel that made this work possible.

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