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Original Research Paper

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

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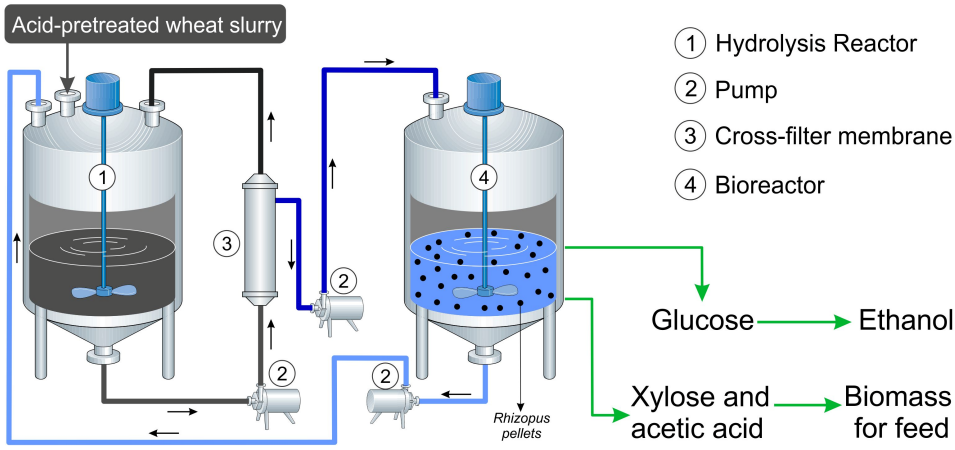
HIGHLIGHTS

- Economically viable production of 2nd generation bioethanol cannot rely on a single product.
- SSFF can be used for production of ethanol and biomass from wheat straw.
- Glucose present in the feed controlled the assimilation of xylose and acetic acid.
- The fungal growth rate was found not to be influenced by the feed composition.
- *Rhizopus* biomass yields of up 0.34 g/g and ethanol yields of 0.40 g/g were obtained.

GRAPHICAL ABSTRACT



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ABSTRACT

The economic viability of the 2nd generation bioethanol production process cannot rely on a single product but on a biorefinery built around it. In this work, ethanol and fungal biomass (animal feed) were produced from acid-pretreated wheat straw slurry under an innovative simultaneous saccharification, fermentation, and filtration (SSFF) strategy. A membrane unit separated the solids from the liquid and the latter was converted to biomass or to both biomass and ethanol in the fermentation reactor containing *Rhizopus* sp. pellets. Biomass yields of up to 0.34 g/g based on the consumed monomeric sugars and acetic acid were achieved. A surplus of glucose in the feed resulted in ethanol production and reduced the biomass yield, whereas limiting glucose concentrations resulted in higher consumption of xylose and acetic acid. The specific growth rate, in the range of 0.013-0.015/h, did not appear to be influenced by the composition of the carbon source. Under anaerobic conditions, an ethanol yield of 0.40 g/g was obtained. The present strategy benefits from the easier separation of the biomass from the medium and the fungus ability to assimilate carbon residuals in comparison with when yeast is used. More specifically, it allows *in-situ* separation of insoluble solids leading to the production of pure fungal biomass as a value-added product.

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1. Introduction

Nowadays, the production of 1st generation bioethanol from agricultural sugar- or starch-rich crops, as a replacement to gasoline, is well established at commercial scale. The leading ethanol-producing countries, USA and Brazil, use corn and sugarcane as main feedstocks, respectively (RFA, 2014). However, ethical issues related to the use of sugar- or starch-rich feedstocks for fuel production instead of being directed to human consumption have put pressure on finding alternative feedstocks (Cherubini, 2010).

The production of ethanol from lignocellulosic materials has been considered for several decades (Leonard and Hajny, 1945). Nevertheless, due to their recalcitrant structure, a feasible commercial facility producing the so-called 2nd generation bioethanol is presently inexistent and is only limited to some pilot plants (Pandey et al., 2015). Constraints include the cost-intensive pretreatment needed to open up the lignocellulosic structure, the cost of enzymes needed in the post-pretreatment stage, the lack of robust microorganisms that can cope with inhibitors, and robust cultivation strategies that can meet all requirements for feasible 2nd generation bioethanol prodution (Ishola, 2014). Another conclusion drawn by the intensive studies conducted over years is that a facility using lignocelluloses as feedstock cannot rely on a single product (i.e., ethanol) for achieving an economically-viable operation (Pandey et al., 2015).

The most commonly used strategies for production of ethanol include simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF). Running a SSF instead of a SHF circumvents the product inhibition of cellulase enzymes due to glucose accumulation (Olofsson et al., 2008). However, SSF disadvantageously requires the use of new microorganisms at each batch since it is difficult to separate them from the medium (Wingren et al., 2003). Therefore, a new cultivation strategy, i.e., simultaneous saccharification, filtration, and fermentation (SSFF) was developed by Ishola et al. (2013). This new concept consists of a membrane unit (cross-flow membrane) connecting a hydrolysis reactor to a fermentation reactor. The enzyme-slurry mixture from the hydrolysis reactor is filtered and the sugar-rich permeate is continuously supplied to the fermentation reactor while the residues are returned to the hydrolysis reactor. The fermented medium in the fermentation reactor is also pumped back to the hydrolysis reactor for volume balance (**Fig. 1**).

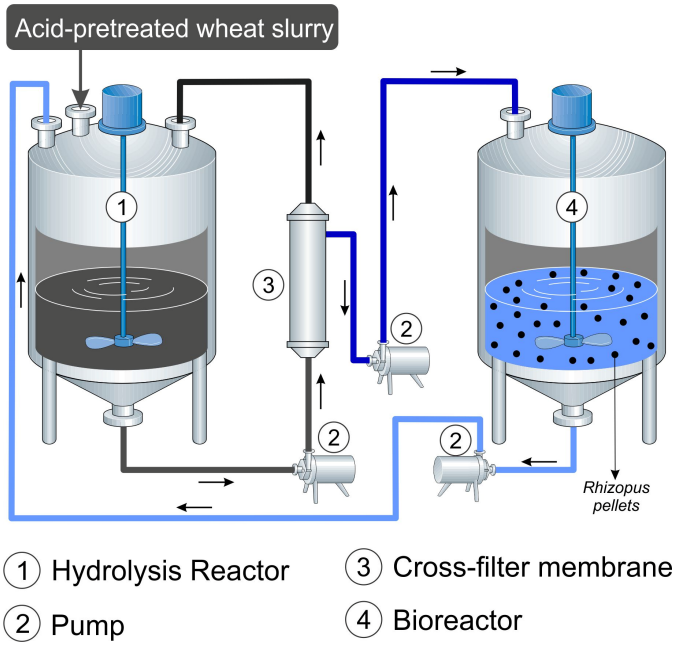

Fig.1. Schematic representation of SSFF for aerobic biomass (*Rhizopus* sp.) production.

Therefore, SSFF combines the advantages of both SSF and SHF by simultaneously solving their limitations, i.e., both hydrolysis and fermentation can be carried out at optimal conditions, the end-product inhibition is avoided, and there is also the possibility to reuse the fermenting cells. The fact that the fermenting cells is free from solid substrates (i.e., lignocellulosic materials) in the fermentation reactor under the SSFF, opens up the opportunity for the production of a second value-added product (i.e., biomass as animal feed) from the residual glucose, pentose sugars (such as xylose), and other components (such as acetic acid) contained in the pretreated wheat straw slurry. This would be a similar situation as exists for the 1st generation bioethanol plants from starch grains where both ethanol and animal feed products are generated (Kim et al., 2008).

Edible filamentous fungi have been previously used for production of protein-rich biomass (animal feed) from various types of substrates (Ferreira et al., 2013). For instance, tempe-isolated zygomycete *Rhizopus* sp. has been found to cope with inhibitors contained in lignocellulosic hydrolysates (FazeliNejad et al., 2013). Moreover, these microorganisms form pellets, and therefore, could be easily separated from the medium (Nyman et al., 2013). They also consume pentose sugars contrary to baker's yeast *Saccharomyces cerevisiae* (Wikandari et al., 2012). These attributes mark these fungi as appropriate candidates for application in the SSFF. Among the different lignocellulosic materials, wheat straw is an economic agricultural by-product available at huge amounts with potentials for production of ethanol in view of its cellulose (33%) and hemicellulose (33%) dry weight contents (Canilha et al., 2006). It is worth quoting that various studies have been carried out on the pretreatment and hydrolysis of wheat straw (Talebnia et al., 2010; Baboukani et al., 2012; Peng et al., 2012).

In the present work, SSFF was used for ethanol production from glucose using *S. cerevisiae* as well as biomass (animal feed) production from residual carbon sources using *Rhizopus* sp. in pellet form. The effects of the enzyme addition to the hydrolysis reactor, temperature in the hydrolysis and fermentation reactors, and aeration for assimilation of carbon sources in the filtered permeate for production of biomass (animal feed) were investigated. This is the first work reporting the use of SSFF with filamentous fungi *Rhizopus* sp. for the production of two value-added products, i.e., bioethanol and animal feed from lignocellulosic materials.

2. Materials and methods

2.1. Microorganisms

Rhizopus sp. CCUG 61147 (Culture Collection University of Gothenburg, Sweden) isolated from Indonesian leaves traditionally used for preparation of tempe, was used in this work. The strain was identified as RM4 in a previous publication (Wikandari et al., 2012). The fungus was kept in Potato Dextrose Agar (PDA) plates and its incubation and preparation for inoculation were carried out according to FazeliNejad et al. (2013). A strain of *S. cerevisiae*, Ethanol Red, kindly provided by Fermentis (France) in dry form was also used.

2.2. Pretreated wheat straw slurry

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

2.3. Enzyme cocktail

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

2.4. Inoculant preparation and cultivations in shake-flasks

A complex medium containing (in g/L): xylose, 20; potato extract, 4; soybean peptone, 6; and $CaCO_3$, 6 was used for preparation of *Rhizopus* sp. pellets as inoculant. The medium (50 mL) was transferred to 250 mL

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cotton-plugged Erlenmeyer flasks followed by sterilization in an autoclave at 121 °C for 20 min. It should be noted that xylose was autoclaved separately. After mixing and inoculation with 1.0×10^5 spores/mL of *Rhizopus* sp., the flasks were kept in a water-bath at 30 °C and 150 rpm for 72 h. The produced *Rhizopus* sp. pellets were transferred to new cultivations to a cell concentration of 1.65 ± 0.10 g/L (dry weight ± 1 SD). The new cultivations were carried out in 250-mL Erlenmeyer flasks containing 100 mL of the same medium which also consisted of (in g/L): $(NH_4)_2SO_4$, 7.5; KH_2PO_4, 3.5; $CaCl_2{\cdot}2H_2O$, 1; $MgSO_4{\cdot}7H_2O$, 0.75; and one of the following carbon sources namely acetic acid, 5.0; ethanol, 10; glucose, 10; lactic acid, 10; and xylose, 10. The cultivations were kept in a water-bath at 30 °C while being shaken at 150 rpm.

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

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

2.5. Cultivations under SSFF

The SSFF as previously described by Ishola et al. (2013) was employed. The lignocellulosic feedstock was hydrolysed enzymatically in a separate vessel (hydrolysis reactor) and the resulting sugar-rich liquid was circulated through the fermentation reactor, where the fungal biomass production took place. The solid fraction was separated from the sugar-rich stream by a cross-flow membrane. However, a cell retention system was not needed in this work since 5 mm spherical pellets of *Rhizopus* sp. were used and they maintained this morphology throughout the cultivations.

For the SSFF trials, pellets were prepared as described above and transferred to a 750 mL fermentor (Ant, Belach Bioteknik AB, Sweden) containing sterilized salt solution as described above and 0.1 g/L antifoam. The transferred *Rhizopus* sp. pellets had an initial dry weight within the range 1.5-2.1 g and the volume was adjusted to a total volume of 500 mL. Wheat straw slurry was transferred to a parallel hydrolysis reactor (Memma, Belach Bioteknik AB, Sweden) and diluted with deionized water to 5.0% SS to a total volume of 3.5 L. The salt and antifoam content was the same as in the fermentation reactor. The cross-flow membrane unit was set up according to Ishola et al. (2013). After integration (**Fig. 1**), the flow of the filtrate through the fermentation bioreactor was 40 mL/h.

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

In a similarly initiated experiment, the effect of aeration in the fermentation reactor was investigated by switching off the air supply after 72 h. Moreover, 10 FPU/g SS enzyme was simultaneously added into the hydrolysis reactor. This anaerobic fermentation phase lasted for 94 h.

Another SSFF trial was also carried out where the temperature was adjusted to 35 °C in both reactors. Enzyme (10 FPU/g SS) and 15 g of dry baker's yeast were added to the hydrolysis reactor. The experiment was carried out in duplicate where the integration phase lasted for 96 and 120 h.

In a different set-up, the similar cultivation as of above (i.e., 35 °C, 10 FPU/g SS, and 15 g dry yeast) was initially performed without any integration for 54 h. The resulting fermented slurry was then distilled using a rotary evaporator (Labinett, Sweden) at 140 °C (oil bath), and 30 rpm rotation speed at atmospheric pressure. The water content lost during distillation was re-adjusted by the addition of sterile ultrapure water. The resulting slurry, now without ethanol, was used for integration with the SSFF and aerobic production of *Rhizopus* sp. biomass as described above during 96 h.

2.6. Analytical methods

The measurements of glucose, metabolites, and inhibitors concentrations as well as spore counting were performed according to FazeliNejad et al. (2013). The SS was determined by filtration with Munktell filters, Grade 3 (5-8 µm) while the TS were determined by drying the samples to a constant weight at 105 °C overnight. The solid fraction of the wheat straw slurry and the enzyme activity were analyzed according to the NREL protocols (Adney and Baker, 2008; Sluiter et al., 2011).

3. Results and discussion

3.1. SSFF of wheat straw slurry with Rhizopus sp. pellets

Production of additional products in a biorefinery concept has been proposed to improve the process economy of ethanol production from cellulosic raw materials (Wheals et al., 1999; Gnansounou and Dauriat, 2010). Animal feed in the form of *Rhizopus* sp. biomass has been suggested as a valuable co-product for ligno-ethanol in the present study. Implementing SSFF for aerobic production of *Rhizopus* sp. biomass entails the application of a cross-flow membrane to separate available sugars and other organic compounds from a pretreated lignocellulosic slurry (**Fig. 1**). The filtrate is supplied to an aerated fermentor, where carbon sources are consumed by *Rhizopus* sp. pellets in order to produce biomass (animal feed).

The pellet morphology is useful in order to prevent leakage of biomass when liquid is pumped back to the hydrolysis reactor. This reflux is necessary in order to maintain the liquid balance between the vessels and to prevent increasing the dry matter content of the slurry. Besides, the glucose concentration, which would increase as a result of enzymatic decomposition of cellulose and could inhibit the enzymes, can be controlled in this way.

On the other hand, the filtration of the slurry is in itself a very important operation since the solid fraction must not be mixed with the biomass, which would result in a downstream separation problem. In addition to biomass, the *Rhizopus* sp. used in this work is also a potential producer of ethanol (Wikandari et al., 2012).

The implementation of SSFF for production of ethanol and biomass includes the use of continuous cross-flow membrane as described earlier. The results obtained revealed that the filtration unit was used for up to 168 h without regeneration of the membrane and without any fouling. In a similar experiment, involving the slurry of pretreated spruce, the same operation was performed during 28 d without interruption, regeneration, or fouling (Ishola et al., 2013).

3.2. Specific growth rate and biomass yield

Various SSFF experiments with *Rhizopus* sp. production from wheat straw slurry were carried out in order to validate this concept. The main difference between the different trials was the composition of the substrate, notably its glucose content. Enzymatic decomposition of cellulose in the solid fraction prior to integration with SSFF (**Fig. 2**) produced a relatively high initial glucose concentration in contrast to a similar experiment without enzyme addition (**Table 1**).

The addition of baker's yeast to the hydrolysis vessel in a different experiment nearly eliminated the glucose in the inflow to the fermentation reactor. Furthermore, an experiment was carried out where the amount of glucose was reduced by the addition of baker's yeast and the produced ethanol was also removed by distillation. The resulting mix was used for *Rhizopus* sp. production by SSFF (**Table 1**).

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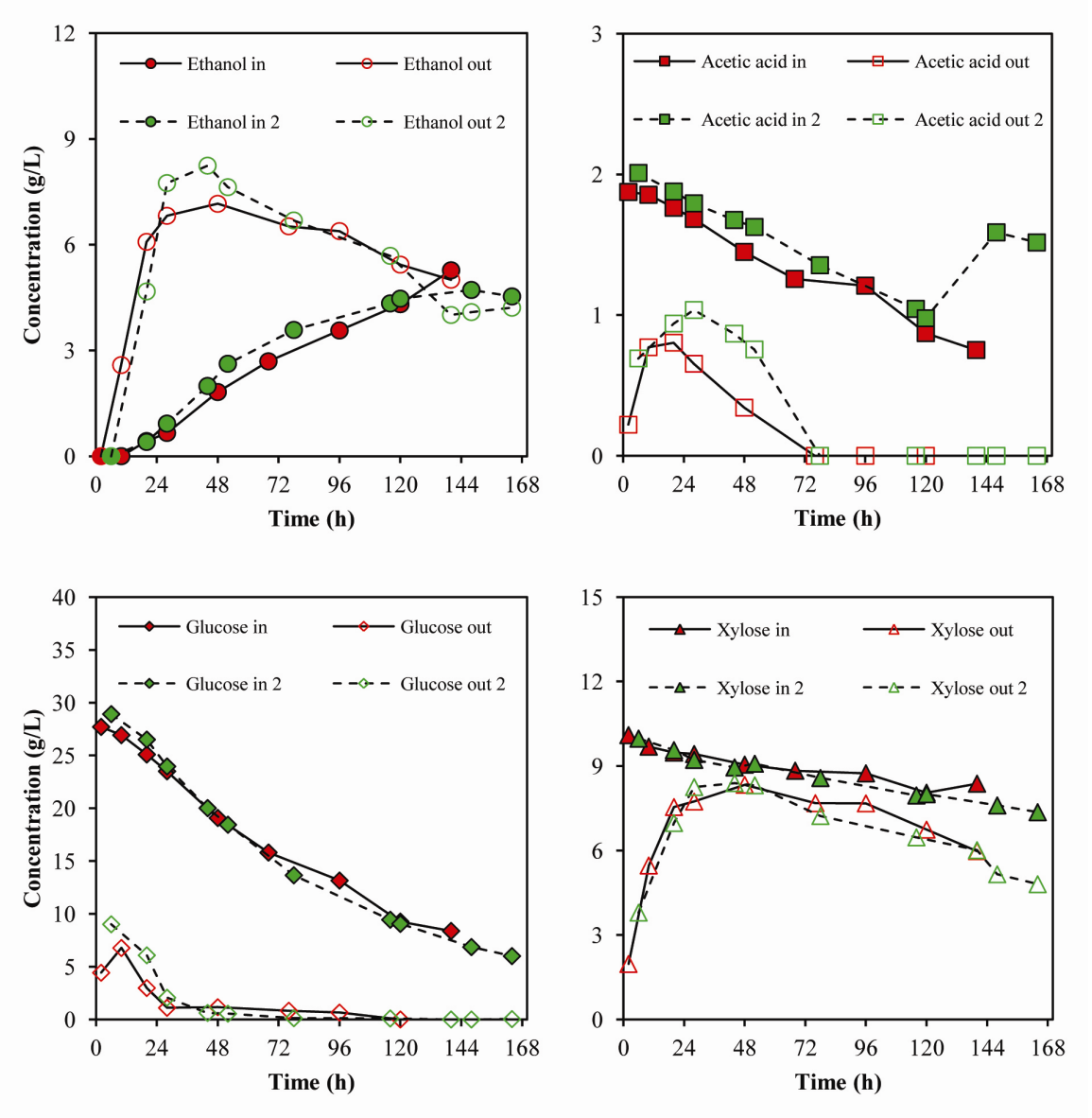

Fig.2. Concentrations of ethanol, acetic acid, glucose, and xylose in SSFF for aerobic production of Rhizopus sp. biomass and ethanol. The integration (connection between the hydrolysis reactor and bioreactor by a filtration unit) was preceded by 24 h of enzymatic hydrolysis. Closed symbols denote concentrations in the ingoing feed to the bioreactor (number 4 in Fig. 1), open symbols denote concentrations in the recirculation feed from the bioreactor to the hydrolysis reactor. The expressions "in" and "out" represent the medium going from the filtration unit into the bioreactor and the medium that leaves the bioreactor to the hydrolysis reactor, respectively, whereas "2" stands for the replicate 2 of the experiment.

The specific growth rate, μ (/h), was calculated according to the following equation (Eq. 1):

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

Where x_0 denotes the initial biomass concentration and x is the biomass concentration after the elapsed time *t*. Assuming a constant μ is debatable because of the dynamic conditions such as substrate concentrations and the fact that the results are sensitive to the accuracy of wet weight measurements of the initial biomass. Growth in the form of pellets is also known to be different from that of free cells (Metz and Kossen, 1977), but considering the small size of the used pellets, this effect can be assumed to be relatively low. However, the results tabulated in Table 1 show that μ as measured, hardly changed corresponding to the substrate composition, i.e., 0.013/h < μ < 0.015/h under aerobic conditions. It could be concluded that the growth rate showed no tendency to be affected by glucose concentrations as long as alternative carbon sources such as acetic acid, ethanol, and xylose were present. However, more efficient aeration could have resulted in a higher growth rate.

The biomass yields reported in Table 1 ranged between 0.24 and 0.34 g/g consumed monomeric sugars (arabinose, galactose, glucose, and xylose), acetic acid, and ethanol, except for the case with enzymatic hydrolysis (i.e., high initial glucose concentration, Fig. 2) where the biomass yield dropped due to ethanol production. These biomass yields were in harmony with the yield obtained in separate batch experiments with synthetic medium containing individual carbon sources (10 g/L of each compound except for acetic acid; 5 g/L). The measured yields of biomass for acetic acid, ethanol, and xylose in these trials were 0.30 g/g, 0.30 g/g, and 0.29 g/g, respectively, after 140 h batch cultivation (96 h for acetic acid, data not shown). The corresponding consumption of glucose was faster (less than 42 h), but the resulting biomass yield was as low as

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Table 1.
Overview of SSFF trials including: (**1**) integration preceded by 24 h of enzymatic hydrolysis; (**2**) no addition of enzyme to the hydrolysis reactor; (**3**) integration preceded by 54 h of enzymatic hydrolysis and ethanol production with yeast in the hydrolysis reactor followed by evaporation of the ethanol; (**4**) Enzyme and yeast were added to the hydrolysis reactor with no ethanol evaporation before integration; (**5**) The air supply to the bioreactor was switched off at t = 72 h with concomitant addition of enzymes to the hydrolysis reactor. The glucose column refers to the concentration of glucose in the bioreactor and how it developed (the uptake of arabinose and galactose are not reported).

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

$Y_{X/S}$ = yield (g of fungal biomass/g of consumed carbon source) $Y_{E/S}$ = yield (g of ethanol/g of consumed carbon source) "Cons." = consumed "ND" = not detected.
[a] Biomass and ethanol yields related to consumed amounts of acetic acid, arabinose, ethanol, glucose, galactose, and xylose.
[b] This treatment resulted in reduced amounts of glucose and ethanol prior to SSFF integration.
[c] This method sharply reduced the glucose content in the flux into the bioreactor.
[d] Biomass and ethanol yields related to consumed amounts of glucose and xylose.

0.11 g/g due to formation of ethanol and glycerol (data not shown), confirming overflow metabolism (Crabtree effect) for *Rhizopus* sp. (Millati et al., 2005; Lennartsson et al., 2009). The pooled standard deviation for the biomass yields was 0.042 (± 1 SD).

3.3. Steering the uptake of carbon sources

In a separate experiment with synthetic medium, the uptake pattern was studied in a cultivation, where acetic acid, ethanol, glucose, lactic acid, and xylose were added to the same cultivation of *Rhizopus* sp. in aerobic shake-flasks. The results showed a relatively rapid consumption of glucose, followed by acetic acid, whereas xylose and ethanol with similar consumption trends were not totally consumed after 72 h of cultivation (**Fig. 3**). Lactic acid frequently occurs as an undesired metabolite produced by contaminants (Skinner and Leathers, 2004) and its uptake by other zygomycetes is documented (Ferreira et al., 2013). However, no measurable consumption of lactic acid by the *Rhizopus* sp. strain was confirmed in this experiment. It is observed that the preference of carbon source, among those examined, under the examined conditions can be ranked as follows: glucose > acetic acid > xylose & ethanol (**Fig. 3**). The measured specific growth rate, μ, was 0.013/h, i.e., similar to the level in the SSFF experiments with wheat straw hydrolysate (**Table 1**), but it is difficult to differentiate the effects of inhibitors and different aeration rates.

In the SSFF experiment with cellulase addition, it is clearly visible that the glucose uptake was relatively efficient but had no visible positive effects on the specific growth rate. Instead, ethanol was produced in a respire-fermentative pattern (**Fig. 2 and Table 1**). In a biorefinery context, it is probable that glucose, if available, would be used for other

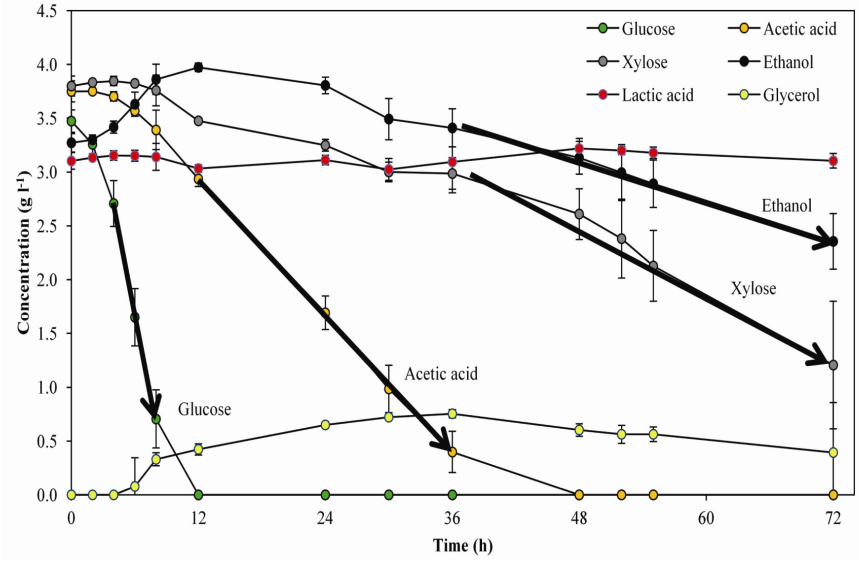

Fig.3. Concentration profiles of glucose, acetic acid, xylose, ethanol, lactic acid, and glycerol in an aerated shake-flask experiment inoculated with Rhizopus sp. pellets. At the beginning of the cultivation, the concentration of all mixed components except glycerol, which was produced during cultivation, was 3.5 g/L. Error bars denote ±1 SD.

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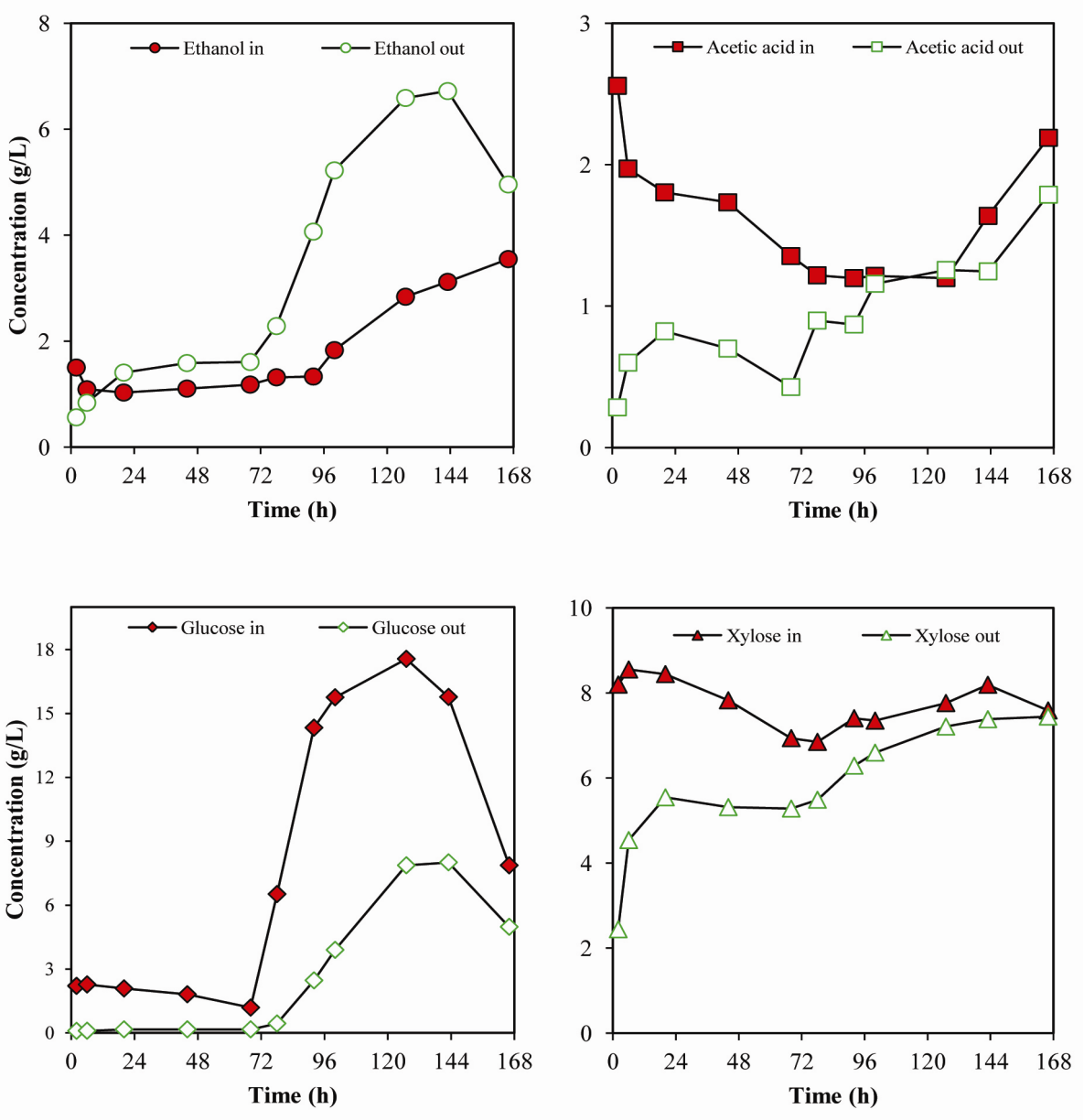

Fig.4. Concentrations of ethanol, acetic acid, glucose, and xylose in the SSFF for aerobic production of biomass followed by anaerobic fermentation by *Rhizopus* sp. The air supply to the bioreactor was switched off and enzymes were added into the hydrolysis reactor at t = 72 h. The expressions "in" and "out" represent the medium going from the filtration unit into the bioreactor and the medium that leaves the bioreactor to the hydrolysis reactor, respectively.

purposes, such as ethanol production by fermentation. Therefore, it may be advantageous to utilize other compounds than glucose for biomass formation. By excluding the enzymatic decomposition, the glucose concentration was sharply reduced, resulting in a higher uptake of xylose and acetic acid. Combining the cultivation of *Rhizopus* sp. by SSFF with the addition of *S. cerevisiae* in the hydrolysis reactor further reduced the glucose concentration in the feed and increased the consumption of xylose, acetic acid, and ethanol. The use of enzymatic hydrolysis and fermentation followed by distillation prior to SSFF cultivation produced a result remarkably similar to the case with the untreated slurry, i.e., without enzyme addition (Table 1).

In conclusion, reducing the glucose concentration in the present study steered the uptake by *Rhizopus sp.* to xylose and acetic acid, which both can be present as residual compounds in a biorefinery, without reducing either the biomass yield or the specific growth rate.

3.4. Fermentation by Rhizopus sp.

Rhizopus sp. is also useful as a fermenting organism for ethanol production and the combined production of valuable biomass and ethanol is interesting in a biorefinery perspective. Performing a complete list of process possibilities is beyond the scope of this study, and only the production of ethanol and animal feed using *Rhizopus* sp. as producing organism was investigated herein.

Two experiments were carried out, where *Rhizopus* sp. was initially grown aerobically on straw hydrolysate in order to build up biomass. One of the trials was stopped after 72 h (referred to in Table 1 as cultivation without enzyme addition), and the biomass was harvested and measured (4.4 g). The second experiment was initiated in a similar way, but after 72 h, cellulase (10 FPU/g SS) was added and the air supply to the fermentation reactor was switched off (Fig. 4).

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During the subsequent 94 h, the amount of biomass increased to 6.4 g, suggesting a specific growth rate (μ) of 0.034/h during the anaerobic phase (**Table 1**).

In the time span from 72 to 166 h, at least 11.5 g of ethanol was produced (some may have evaporated), which would suggest an ethanol yield of 0.40 g/g consumed glucose and xylose, and an ethanol productivity of 0.023 g/g/h, based on the average biomass concentration. The volumetric productivity of ethanol, 0.24 g/L/h, was relatively low if compared with optimized fermentations with *S. cerevisiae* (Balat, 2011). The xylose uptake corresponded to only 5% of the consumed carbon source and the uptake should be a sign of leakage of oxygen into the fermentation vessel, considering that fungi normally do not consume xylose under anaerobic conditions. According to the measurements, the concentration of xylose in the hydrolysis vessel increased after 72 h, indicating the release of xylose from the solid fraction as a result of the enzymatic decomposition.

3.5. Impact of inhibitors

After dilution to 5.0% SS, the concentrations of acetic acid, furfural, and HMF were 1.8 g/L, 0.65 g/L, and 1.3 g/L, respectively. Considering a previous study (FazeliNejad et al., 2013), these levels should not be very inhibiting by themselves. It is worth quoting that hydrolysates of lignocellulosic material usually contain other inhibitors as well, such as phenolic compounds, whose concentrations were not measured in the present study. It was observed in all the SSFF trials that ingoing furfural and HMF were completely converted (i.e., not detected in the outflow) after 6-8 h of the integrated fermentation, confirming *in situ* conversion of these compounds by *Rhizopus* sp. (FazeliNejad et al., 2013). Furthermore, the specific growth rate, μ, was similar in the hydrolysate-based SSFF experiments and the shake-flask experiments with synthetic medium. It could thus be assumed that the impact of inhibitors was limited.

4. Conclusions

Rhizopus sp. in pellet form was successfully used for aerobic production of biomass (animal feed) by SSFF from acid-pretreated wheat straw slurry with biomass yields of up to 0.34 g biomass/g consumed monomeric sugars and acetic acid. A surplus of glucose in the feed resulted in ethanol production and reduced the biomass yield, whereas limiting glucose concentrations resulted in higher consumption of xylose and acetic acid. The specific growth rate was in the range of 0.013/h and 0.015/h and did not appear to be influenced by the composition of the carbon source. Under anaerobic conditions, an ethanol yield of 0.40 g/g and an ethanol productivity of 0.023 g/g/h were obtained using the *Rhizopus* sp. pellets. Overall, the present strategy benefits from the easier separation of the biomass from the medium and the fungus ability to assimilate carbon residuals in comparison with when yeast is used. More specifically, it allows *in situ* separation of insoluble solids and hence, a two-stage cultivation system practiced for production of biomass and ethanol from whole stillage is not needed to be applied if biomass is desired as a separate value-added product.

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