ETHANOL PRODUCTION FROM XYLOSE AND WOOD HYDROLYZATE BY *MUCOR INDICUS* AT DIFFERENT AERATION RATES

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The fungus *Mucor indicus* is able to produce ethanol from xylose as well as dilute-acid lignocellulosic hydrolyzates. The fungus completely assimilated 10 g/L xylose as the sole carbon and energy source within 32 to 65 h at an aeration rate of 0.1 to 1.0 vvm. The highest ethanol yield was 0.16 g/g at 0.1 vvm. Xylitol was formed intermediately with a maximum yield of 0.22 g/g at 0.5 vvm, but disappeared towards the end of experiments. During cultivation in a mixture of xylose and glucose, the fungus did not assimilate xylose as long as glucose was present in the medium. The anaerobic cultivation of the fungus in the hydrolyzate containing 20% xylose and 80% hexoses resulted in no assimilation of xylose but complete consumption of the hexoses in less than 15 h. The ethanol yield was 0.44 g/g. However, the xylose in the hydrolyzate was consumed when the media was aerated at 0.067 to 0.333 vvm. The best ethanol yield was 0.44 g/g at 0.067 vvm. The results of this study suggest that *M. indicus* hydrolyzate can be first fermented anaerobically for hexose assimilation and subsequently continued under oxygen-limited conditions for xylose fermentation.

Keywords: Ethanol; lignocellulosic hydrolyzate; Xylose; Mucor indicus; Xylitol; Aeration rate

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INTRODUCTION

The turbulences in the oil market during the last four decades and the global desire to reduce net CO_2 emission has made ethanol a desirable fuel (Taherzadeh and Karimi 2007). Sugars and grains are the dominant feedstocks for ethanol production, but lignocellulosic biomass is considered as the key feedstock in the future. Lignocellulosic biomass contains cellulose, hemicellulose, and lignin. Glucose, which is the sugar monomer of cellulose, is preferentially fermented by most microorganisms, including baker's yeast *Saccharomyces cerevisiae*. However, this yeast cannot assimilate xylose, which comprises 15-40% of sugar monomers in hardwoods and typical agricultural crop residues (Potera 2004; Sun and Cheng 2002).

The fungus *Mucor indicus* (formerly *Mucor rouxii)* has recently been revealed as an alternative microorganism to *S. cerevisiae* with capability of utilizing both hexoses

and xylose for ethanol production from wood hydrolyzate (Millati et al. 2005; Sues et al. 2005) and rice straw hydrolyzate (Karimi et al. 2006). *M. indicus* is one of the *Mucor* species displaying dimorphism, i.e., it is able to grow as filamentous hyphae or rounded, budding yeast cells (Orlowski 1991). Growth in the filamentous or yeast-like form is influenced by the environmental conditions (Bartnicki-Garcia and Nickerson 1962; Orlowski 1991). This fungus is a saprophyte; it lives easily in wastes and is capable of consuming different kinds of sugars, including both hexoses and pentoses. Having a valuable biomass of its chitosan content, *M. indicus* has been explored for pharmaceutical and food applications (Chatterjee et al. 2005).

Under aerobic conditions, *M. indicus* is able to consume both hexoses and pentoses from dilute-acid wood hydrolyzate and produce ethanol with yields of 0.42-0.44 g/g (Millati 2005; Sues et al. 2005). When the fungus was cultivated on xylose under aerobic conditions, it yielded 0.18-0.22 g/g ethanol (Millati 2005; Sues et al. 2005; Karimi et al. 2006). However, *M. indicus* failed to grow and produce ethanol from xylose under anaerobic conditions (Bartnicki-Garcia and Nickerson 1962; Sues et al. 2005). It is generally believed that xylose-fermenting microorganisms do not grow on xylose under anaerobic conditions, but some of strains ferment xylose to ethanol anaerobically at low yields (McMillan 1996). On the other hand, the best performance for maximum ethanol productivity by many xylose-utilizing microorganisms such as *Pichia stipitis* was obtained under well-controlled levels of oxygenation (McMillan 1996). This is probably related to the fact that oxygen may have an indirect effect on fermentation capability of the microorganism, e.g., by supporting growth, stabilization of mitochondria, and energizing transport processes, or basic functions of the cell (Franzén 2003).

The objective of the current work was to investigate the effect of aeration on sugar consumption and ethanol production from xylose and from sugars in dilute-acid wood hydrolyzate. The experiments were carried out under well-controlled conditions in bioreactors and different levels of aeration were investigated.

EXPERIMENTAL

Raw Material and Hydrolysis

The dilute-acid wood hydrolyzate used in this work was produced from forest residues originating mainly from spruce. Hydrolysis of the raw material was carried out in a 350-L rebuilt masonite gun batch reactor located in Rundvik, Sweden. In the hydrolysis process, an amount of material corresponding to 10 kg of dry wood splinter was impregnated with H_2SO_4 and water to obtain an initial acid concentration of 5 g/L, and a solid concentration of 33%. The impregnated wood was charged into the reactor, and the reaction was started by direct steam injection, with a heat-up time of approximately 20 s. After the heat-up period, the reactor was held at 15 bar pressure for 10 min, followed by rapid decompression and discharge of the material into a collecting vessel. The solid residue was separated from the liquid hydrolyzate by filtration. The hydrolyzate contained altogether 48.5 g/L sugar, *viz*., in g/L: galactose 3.3, glucose 10.7, mannose 24.4, xylose 10.1; and acetic acid 6.8, furfural 0.5, and hydroxymethyl furfural (HMF) 1.05. The hydrolyzate was kept at 4°C until use. It should be noticed that

sterilization of the hydrolyzate at 121°C resulted sometimes in a sugar loss, which was less than 15% in the worst conditions.

Microorganism

Mucor indicus CCUG 22424 obtained from Culture Collection University of Göteborg (Göteborg, Sweden) was used in all the experiments. The strain was maintained at pH 5.6 on potato dextrose agar slants prepared with 10 g/L neopeptone (Difco, Sparks, MD, USA), 15 g/L agar, and 40 g/L D-glucose as the additional carbon source by incubation for four days at 30°C. The slants can be stored for one year at 4°C. A spore suspension was prepared by adding 10 mL of sterile distilled water to the slant and shaking it vigorously, resulting in $5\text{-}6\times10^6$ spores/mL. Ten milliliters of the suspension were used for inoculation of the medium to initiate fungal growth.

Cultivation Conditions

A previously reported optimum medium composition supplemented with 1 g/L $CaCl_2{\cdot}2H_2O$ (further will be called synthetic medium) (Sues et al. 2005) was used in all the experiments into which different carbon and energy sources were added. Batch cultivations were carried out in a Biostat-A bioreactor (B. Braun Biotech International, Germany). The stirring rates were 200 rpm and 250 rpm for the experiments with added xylose and hydrolyzate, respectively. The lower stirring rate while cultivating on xylose was in order to prevent damaging of the cotton-like fungus, which was not the case for the growth on the hydrolyzates. The stirrer was a six-blade Rushton turbine. The temperature was set at 30±0.1°C, and the pH was controlled at 5.50±0.07 by addition of 2M NaOH throughout the experiments. Experiments with xylose medium were carried out in a volume of 1.0 L containing 10 g/L of xylose as the carbon source. Aeration was maintained by continuous air sparging at flow rates of 0.1-1 L/min, controlled by a Hi-Tech mass flow controller (Ruurlo, The Netherlands). Growth on wood hydrolyzate medium was initiated by cultivation on 500 mL of synthetic medium with a glucose concentration of 50 g/L to prepare enough biomass. The medium was inoculated with 10 mL of a spore suspension as it was also used for the experiments on xylose medium. However, the concentrations of mineral salts and vitamins were tripled in order to compensate for the dilution after addition of hydrolyzate. The stirrer at this stage was set at a low rate of 110 rpm to prevent damaging of the fungal growth. The air flow was kept constant at 0.5 L/min. One liter of hydrolyzate solution was then added to the bioreactor after complete consumption of the initial glucose. The stirring rate for hydrolyzate cultivation was raised to 250 rpm. Aeration was maintained by continuous air sparging at flow rates of 0.1-0.5 L/min unless otherwise stated. The liquid samples for metabolite analyses were stored at -20°C.

Analyses of Metabolites

The metabolites were analyzed by HPLC. An ion exchange column (Aminex HPX-87P, Bio-Rad, USA) was used at 85°C for the analyses of glucose, xylose, galactose, and mannose. Ultra-pure water was used as eluent at 0.6 mL/ min. Ethanol, acetic acid, xylitol, furfural, and HMF concentrations were analyzed by another ion-exchange column (HPX-87H, Bio-Rad) at 60°C using 5 mM H_2SO_4 as eluent at 0.6

mL/min. A refractive index (RI) detector (Waters 410, Millipore, Milford, USA) and UV absorbance detector at 210 nm (Waters 486) were used in series. Concentrations of all metabolites except furfural and HMF were determined from the RI chromatograms. The yields were calculated from concentrations of metabolites per sugar consumed, whereas ethanol productivity was calculated as gram of produced ethanol per liter medium per hour. It should be mentioned that specific ethanol productivity (g/g·h) was not possible to measure since the profile of biomass concentration could not be determined throughout the experiment.

Biomass Determination

Since the biomass consisted of cotton-like mycelium, it was only measured at the end of the experiment, when the entire culture medium was harvested. The entire biomass was collected on a tea-strainer, rinsed with distilled water, and dried at 105^{o}C for 24 h. The biomass yield was calculated based on the total formation of biomass and the consumption of sugars.

RESULTS AND DISCUSSION

Cultivation on Xylose Medium

Batch cultivation of *M. indicus* on synthetic media with 10 g/L xylose as the carbon and energy source was performed in a bioreactor at different aeration rates (Table 1 and Fig. 1). The bioreactor was sparged with air at 100, 200, 500, and 1000 mL/min in 1.0 L medium, giving aeration rates of 0.1, 0.2, 0.5, and 1.0 vvm (volume of air per volume of liquid per minute). Ethanol was produced in all the conditions investigated. Decreasing aeration rates from 1.0 to 0.1 vvm resulted in increasing ethanol yields from 0.08 to 0.13 g/g. The difference in ethanol yields at aeration of 0.1 and 0.2 vvm was 0.01 g/g, while this difference was larger between the yields obtained at 0.2 and 0.5 vvm (0.02 g/g). Furthermore, supplementation of Tween 80 1 mL/L to the medium gave rise to increased ethanol yields for cultivation at aeration of 0.1 and 0.2 vvm. The yields of ethanol increased to 0.16 and 0.14 g/g, respectively (Table 1).

Table 1. Yields of Ethanol, Glycerol, Xylitol, and Biomass in Batch Cultivation of *M. indicus* on 10 g/L Xylose as the Sole Carbon and Energy Source at Different Aeration Rates.

Aeration rate (vvm)	Ethanol Productivity (g/L·h)	$Y_{Ethanol/S}$ (g/g)	$Y_{Glycerol/S}$ (g/g)	$Y_{Xylitol/S}$ (g/g)	$Y_{Biomass/S}$ (g/g)	t[a] (h)
0.1	0.039	0.13	0.008	0.118	0.43	65.5
0.1[b]	0.042	0.16	0.009	0.173	0.37	64.5
0.2	0.054	0.12	0.000	0.171	0.35	48.0
0.2[b]	0.071	0.14	0.000	0.135	0.48	50.0
0.5	0.052	0.10	0.011	0.224	0.49	45.7
1.0	0.062	0.08	0.014	0.055	0.52	32.0

[a] Time needed for complete xylose utilization; [b] Supplemented with 1 mL/L of Tween 80
The yields of ethanol and glycerol were taken at the time when xylose was consumed by 90-95%
The yields of xylitol were taken at the middle of the exponential phase

All of the cultivation conditions displayed a lag phase of 10-25 h to produce ethanol, where the longest lag phase appeared at aeration of 0.1 vvm (Fig. 1b). The volumetric ethanol productivity was directly affected by aeration. The highest ethanol productivity obtained was 0.062 g/L·h, at an aeration rate of 1.0 vvm with no addition of Tween 80. However, addition of Tween 80 improved ethanol productivity by 31% at 0.2 vvm aeration (Table 1). The ethanol concentration was at the maximum level when xylose and also xylitol earlier released into the medium were nearly depleted. Ethanol was then consumed by the fungus (Fig. 1b). The cells were not able to consume ethanol while aerated at 0.1 vvm as fast as in the experiments with higher aeration rates. The concentration of ethanol decreased only 8.8% in 5.5 h from the peak at aeration of 0.1 vvm, while it decreased 47% in 4 h from the peak at aeration of 0.2 vvm. There was a direct correlation between ethanol production and xylose consumption, where xylose was consumed more quickly at higher aeration rates (Fig. 1). The total cultivation time for complete assimilation of xylose at the slowest aeration rate of 0.1 vvm was 65.5 h, which is twice as long as that at a rate of 1 vvm (Table 1).

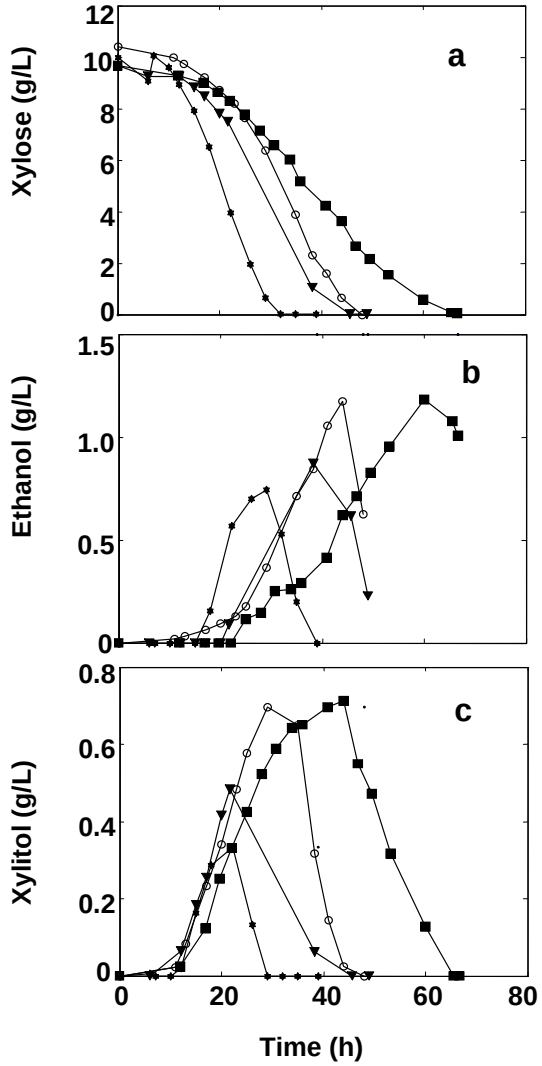

Fig. 1. The profiles of (a) xylose assimilation (b) ethanol production and (c) xylitol production in batch cultivation of *M. indicus* on synthetic media containing 10 g/L xylose as the carbon and energy source at different aeration rates of (■) 0.1 vvm, (○) 0.2 vvm, (▼) 0.5 vvm, (✶) 1 vvm.

Xylitol and glycerol were the two metabolites considered as major by-products in the consumption of xylose by *M. indicus* (Table 1). Concentrations of xylitol higher than 0.8 g/L were recorded. The formation of glycerol was not as high as that of xylitol, and its concentration did not exceed 0.2 g/L in any experiment. Both xylitol and glycerol were consumed by the fungus. Other by-products such as acetic and pyruvic acids were detected in trace amounts. The maximum concentration of acetic acid was 0.3 g/L, whereas pyruvic acid did not exceed 0.03 g/L.

The results show that the rate of aeration had a crucial effect in aerobic growth on xylose and the final ethanol yield, especially since *M. indicus* can grow respiratively on ethanol. Limitation of oxygen supply results in higher ethanol yield, while the ethanol productivity is decreased. On the other hand, faster assimilation of xylose demands a higher aeration rate, but results in lower ethanol yield (Table 1). A comparison between time course of the external concentration of xylitol and ethanol under different aeration rates could help in understanding this phenomenon (Fig. 1). With 0.1 vvm aeration to the cultivation with xylose, there was still no ethanol after 22 h, whereas 0.33 g/L xylitol was detected, which corresponded to a yield of 0.24 g/g (Fig. 1). At higher aeration rates, i.e. 0.5, and 1.0 vvm, ethanol appeared much earlier, but also started to decrease much earlier, when xylose and xylitol were nearly depleted. In these cases, the cells have probably had sufficient access to oxygen to take up all xylitol and produce biomass at an early stage. This phenomena indicates that xylose fermentation by *M indicus* is likely to follow the established pattern that is exhibited by well-known xylose-utilizing microorganisms such as *P. stipitis*, i.e. xylose is converted to xylitol and sequentially to xylulose to further enter the pentose phosphate pathway and the central metabolism (the Embden-Meyerhof pathway) and produce ethanol as one of the end-products (e.g. Aristidou and Penttila 2000; Prior et al. 1989). This hypothesis is further supported by the fact that the degree of aeration and xylitol production (Fig. 1c) are in inverse relationship. In the case of aerobic fermentation, oxygen acts as a terminal electron acceptor for conversion of xylose to xylitol.

The biomass yields were measured when the cultivations were harvested. The results cannot be fairly compared to each other, since the cultivations were not carried out within the same period of time. In general, biomass yields from the higher aeration rates 0.5 and 1.0 vvm were higher than those obtained at lower rates (Table 1).

Co-metabolisms of Xylose and Glucose

In order to study the performance of *M. indicus* to co-metabolize a mixture of xylose and hexoses, batch cultivation was performed with a synthetic medium containing 40 g/L glucose and 10 g/L xylose (Fig. 2). The bioreactor was sparged with air at a rate of 0.2 vvm. With both glucose and xylose present in the medium, a sequential pattern of sugar consumption was observed. Glucose was first consumed and fermented to ethanol. After glucose was depleted, xylose was then utilized. During the consumption of xylose, the concentration of ethanol was almost constant. This means that the fungus prefers xylose to ethanol as the carbon source. When xylose was depleted, ethanol started being a carbon source for the fungus.

It is also shown in Fig. 2 that *M. indicus* was able to produce ethanol with total sugar of 50 g/L in the medium at an aeration rate of 0.2 vvm. The ability of the fungus to

produce ethanol under aerobic conditions indicates that *M. indicus* is a "Crabtree-positive" organism, i.e. ethanol production occurs under aerobic conditions in the presence of excess sugar. This result is in line with that found for another species of *Mucor*, *M. circinelloides*, which also could produce ethanol under aerobic conditions (Lubbehusen et al. 2004; McIntyre et al. 2002).

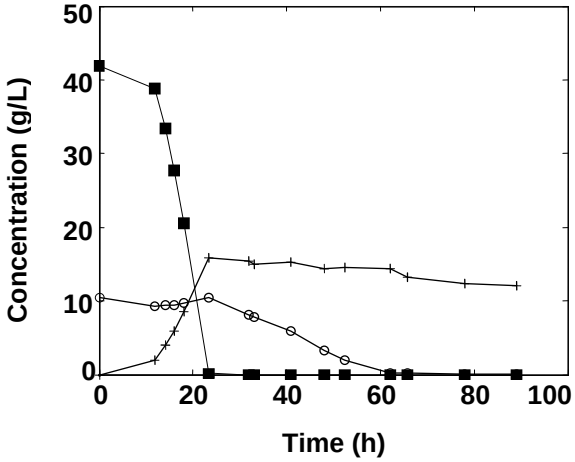

Fig. 2. The profile of batch cultivation of *M. indicus* on a mixture of 40 g/L glucose and 10 g/L xylose as the carbon and energy sources. The bioreactor was sparged with air at a rate of 0.2 vvm. The symbols represent (■) glucose, (○) xylose, and (+) ethanol.

Glycerol was produced during the growth on glucose with a yield of 0.08 g/g, but it disappeared slowly when xylose was being consumed. Xylitol was produced only during the xylose consumption phase. The amount was traced to a yield of 0.007 g/g. Biomass was measured at the end of the cultivation, and the yield was 0.14 g/g of the total sugars.

Cultivation on Dilute-acid Wood Hydrolyzate

Dilute-acid wood hydrolyzate was used as carbon and energy sources for cultivation of *M. indicus* under different aeration rates of 100, 200, and 500 mL/min, which correspond to 0.067, 0.133, and 0.333 vvm, respectively. In order to make a comparison, anaerobic cultivation of hydrolyzate aerated with 0.5 L/min of nitrogen was conducted. The most important results are presented in Table 2 and Fig. 3. The experiments started with the seeding of spores in 500 mL synthetic medium containing 50 g/L glucose as the carbon source to prepare initial biomass. During spore germination, the culture was sparged with air at a rate of 1.0 vvm. The initial glucose was completely consumed by 24 h, when 1 L of hydrolyzate was added into the medium.

All the hexoses present in the hydrolyzate including glucose, mannose, and galactose were assimilated in less than 15 h regardless of the aeration rate (Fig. 3). However, no assimilation of xylose was obtained as long as the hexoses were present. This phenomenon was similar to the results obtained by co-metabolism of glucose and xylose on synthetic medium discussed previously (Fig. 2). The fungus consumed xylose after complete depletion of the hexoses. The rates of xylose consumption at aeration rates of 0.067, 0.133, and 0.333 vvm were 0.12, 0.19, and 0.26 g/L· h, respectively. Furthermore, xylose concentration remained constant during the cultivation under anaerobic conditions (Fig. 3d). Hence, *M. indicus* exhibits the so-called "a diauxic behaviour", i.e. a sequential use of hexose and pentose (in this case is xylose). This phenomenon has been studied for *P. stipitis*, which was described as a competitive

inhibition of one sugar on the uptake of the other sugar (Grootjen et al. 1991). The influence of xylose on the glucose conversion was negligible. On the contrary, hexoses apparently have an inhibitory effect on the xylose conversion. Similary, as for other filamentous fungi (Lubbehusen et al. 2004), xylose consumption was repressed by glucose in aerobic cultivation both on synthetic medium and on hydrolyzate (Figs. 2, 3). It should also be noticed that there was a quick adaptation from one carbon source (hexoses) to another one (xylose). Ethanol consumption is also subject to repression in the presence of sugars, and it was consumed when no sugars available in the medium.

Table 2. Yields of Ethanol, Glycerol, Xylitol, and Biomass in Batch Cultivation of *M. indicus* on Dilute-Acid Wood Hydrolyzate at Different Aeration Rates.

Aeration rate vvm	$Y_{Ethanol/S}$ g/g	$Y^{a}_{Ethanol/S}$ g/g	$Y_{Glycerol/S}$ g/g	$Y_{Xylitol/S}$ g/g	$Y_{Biomass/S}$ g/g
0.000[b]	0.44	0.35	0.113	0.000	0.024
0.067	0.44	0.37	0.053	0.036	0.050
0.133	0.41	0.35	0.050	0.050	0.069
0.333	0.41	0.35	0.062	0.028	0.124

[a] Ethanol yield based on total initial sugars in the hydrolyzate
[b] Anaerobic

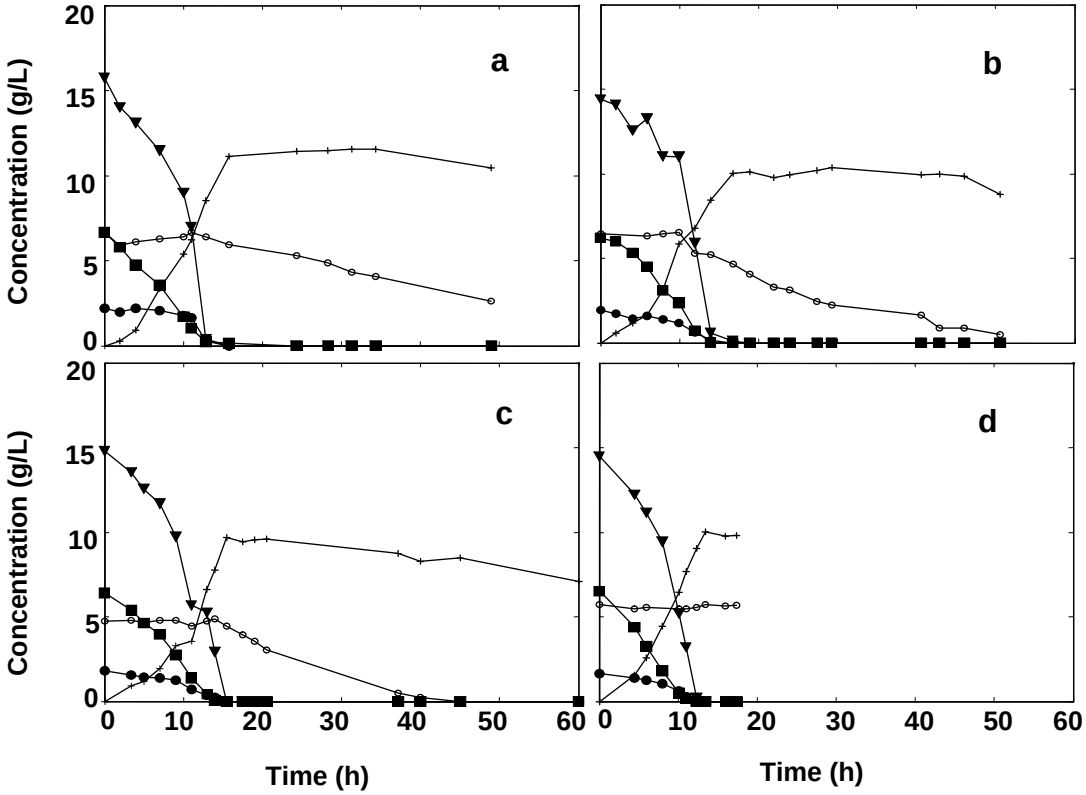

Fig. 3. Batch cultivation of *M. indicus* on dilute-acid hydrolyzate at different aeration rates of (a) 0.067 (b) 0.133 and (c) 0.333 vvm and (d) anaerobic condition. The symbols represent the sugars (■) glucose, (○) xylose, (▼) mannose, (•) galactose, and (+) ethanol.

The highest ethanol yield obtained based on the total consumed sugars was 0.44 g/g, which was recorded from the cultivations under an anaerobic condition and under aeration at 0.067 vvm. However, since xylose was only partly consumed in the latter experiment, a maximum ethanol yield of 0.37 g/g was obtained based on the total initial sugars present in the hydrolyzate (Table 2).

With the exception of anaerobic cultivation, the ethanol produced was ultimately consumed, albeit at different rates. Similar to the previous results in cultivations on xylose, increasing aeration led to increase in ethanol consumption. The concentration of ethanol decreased 9.6%, 15.3%, and 26.5% from the peaks at the end of the cultivations at rates of 0.067, 0.133 and 0.333 vvm, respectively.

Anaerobic fermentation resulted in greater formation of glycerol, up to 2.7 g/L, than those obtained in the other cultivations. The glycerol concentrations in the aerobic cultivation were less than 1.5 g/L. Xylitol was the second important secreted metabolite with respect to concentration. The highest xylitol concentration obtained was 1.5 g/L during the cultivation performed at 0.133 vvm aeration rate. No xylitol was detected in anaerobic cultivation of the fungus. Biomass, harvested at the end of the cultivation amounted to a maximum 0.124 g/g from the 0.333 vvm-cultivation.

Acetic acid, furfural, and 5-hydroxymethyl furfural (HMF), which were present in the hydrolyzate (and are known to be toxic to most microorganisms) were utilized or converted. Acetic acid was present at about 4 g/L and was partially consumed. The assimilation of acetic acid was less than 0.5 g/L in anaerobic cultivation and up to 3 g/L with the highest aeration rate. HMF and furfural were entirely converted. The conversion of furfural by the fungus did not take more than one hour.

CONCLUSIONS

1. This study shows that *M. indicus* assimilates xylose aerobically and is able to produce ethanol from xylose. However, the aeration rate is an important factor to obtain a reasonable yield of ethanol from xylose.
2. The fungus prefers hexoses (glucose, mannose and galactose) to xylose for the growth under aerobic cultivation, but it is quickly adapted to xylose once the hexoses are exhausted in the medium.
3. While both ethanol (from previously fermented hexoses) and xylose were present in the culture, the fungus assimilated xylose, but ethanol concentration remained practically constant.

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