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ORIGINAL PAPER

Production of ethanol by filamentous and yeast-like forms of *Mucor indicus* from fructose, glucose, sucrose, and molasses

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Abstract The fungus *Mucor indicus* is found in this study able to consume glucose and fructose, but not sucrose in fermentation of sugarcane and sugar beet molasses. This might be an advantage in industries which want to selectively remove glucose and fructose for crystallisation of sucrose present in the molasses. On the other hand, the fungus assimilated sucrose after hydrolysis by the enzyme invertase. The fungus efficiently grew on glucose and fructose and produced ethanol in synthetic media or from molasses. The cultivations were carried out aerobically and anaerobically, and manipulated toward filamentous or yeast-like morphology. Ethanol was the major metabolite in all the experiments. The ethanol yield in anaerobic cultivations was between 0.35 and 0.48 g/g sugars consumed, depending on the carbon source and the growth morphology, while a yield of as low as 0.16 g/g was obtained during aerobic cultivation. The yeast-like form of the fungus showed faster ethanol production with an average productivity of 0.90 g/l h from glucose, fructose and inverted sucrose, than the filamentous form with an average productivity of 0.33 g/l h. The biomass of the fungus was also analyzed with respect to alkali-insoluble material (AIM), chitin, and chitosan. The biomass of the fungus contained per g maximum 0.217 g AIM and 0.042 g chitosan in yeast-like cultivation under aerobic conditions.

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Introduction

The future risks of global warming and shortage of petroleum, as well as the superior environmental characteristics of ethanol as an oxygenated additive to gasoline that improves the knocking resistance of gasoline, promote the production and usage of bioethanol in the fuel market [1, 2]. Sugar substances, starchy materials and lignocellulosic materials are being considered as the raw materials for ethanol production. From the industrial point of view for ethanol production, the sucrose-based substrates such as cane and sugar beet juices or molasses present many advantages including their relative abundance and renewable nature [3, 4]. Molasses, which is the residue of the sugar juices after sucrose crystallisation, has additional advantages of being a relatively inexpensive raw material, readily available, and already used for industrial ethanol production. Molasses are complicated substrates for fermentation, since they contain mixtures of several fermentable and non-fermentable sugars together with different types of inhibitors [5, 6].

Mucor indicus (formerly *M. rouxii*) is a filamentous zygomycetes fungus which has recently been presented as an ethanol-producing organism, particularly from lignocellulosic hydrolyzates [7, 8]. The fungus is safe for humans, and can produce ethanol from hexoses such as glucose, mannose, and galactose with the yield and productivity of the same order as *Saccharomyces cerevisiae* [7]. The fungus is also capable of assimilating xylose and producing ethanol under aerobic conditions, with a yield as high as 0.22 g/g [8]. Sucrose and its monomers, fructose and glucose, are industrial sugars present in, e.g., cane juice and

molasses. These are important carbon sources for production of several products such as ethanol. However, there is no previous report on whether *M. indicus* could be suitable for assimilating these sugars, for e.g., production of ethanol.

The biomass of *M. indicus* is a valuable product, since it contains appreciable amounts of chitosan [9]. Chitosan is a polycationic, nontoxic, biodegradable and antimicrobial polymer with numerous industrial applications particularly in agriculture, food and pharmaceutical industries [10–12]. Furthermore, the cell wall skeleton of zygomycetes can be processed to a superabsorbent material [13].

Mucor indicus is a dimorphic fungus which can grow as yeast-like or filamentous forms to various extents, being "purely yeast-like", "mostly yeast-like", "mostly filamentous", and "purely filamentous" [14, 15]. The dimorphic nature of the fungus could be an important obstacle for its use as an industrial ethanol-producing microorganism, since in the filamentous form it may become difficult to handle in a large-scale process [16, 17]. However, there is little difference between handling of the yeast-like form of the fungus and baker's yeasts.

In this study, assimilation by *M. indicus* was investigated under aerobic and anaerobic conditions of sugarcane molasses, sugar beet molasses, glucose, fructose, sucrose, inverted sucrose sugarcane molasses, inverted sucrose sugar beet molasses, and inverted sucrose. Metabolites and chitosan content of biomass have also been analyzed and related to the different morphological forms of the fungus which appeared.

Materials and methods

Microorganism used and selection of the morphology

The fungus *M. indicus* CCUG 22424, obtained from the Culture Collection at the University of Göteborg (Sweden), was used in all the experiments. It was incubated on agar slants containing (g/l): glucose, 40; peptone, 10; and agar 20 at pH 5.5 and 30°C for 5 days, where the fungus grew to form a cotton-like mycelium and spores. For inoculation, spores were suspended by adding sterile distilled water to a slant and shaking it vigorously with a tube shaker. The desired morphology of *M. indicus* was obtained by using different spore concentrations.

Cultivation

Aerobic or anaerobic cultivation of *M. indicus* was carried out in 500 ml Erlenmeyer flasks containing 250 ml medium containing glucose monohydrate (22 g/l), fructose (20 g/l), sucrose (20 g/l), inverted sucrose (20 g/l), sugar beet molasses (40 g/l), inverted sugar beet molasses (40 g/l), sugarcane molasses (40 g/l), or inverted sugarcane molasses (40 g/l) as carbon sources and supplemented with (g/l): yeast extract, 5; $(NH_4)SO_4$, 7.5; $MgSO_4 \cdot 7H_2O$, 0.75; K_2HPO_4, 3.5; $CaCl_2$, 1, at pH 5.5 ± 0.1 [18]. Cultivations were performed in a shaker incubator at 32°C for 3 days. Aerobic cultivations were carried out in 500 ml cotton-plugged Erlenmeyer flasks, while a loop trap was used for anaerobic cultivation similar to the system used by Taherzadeh et al. [19]. In the anaerobic system, sterile water was used in the loop traps to prevent oxygen back-diffusion to the media, while permitting nitrogen and evolved CO_2 to leave the flask. Pure nitrogen gas was sparged into the media at the beginning of the anaerobic cultivations and during the sampling. Sugar beet molasses was obtained from Marvdasht Sugar Industries (Shiraz, Iran) and sugarcane molasses was obtained from Karoun Sugar Industries (Khouzestan, Iran).

Purification of chitin and chitosan from the fungal cell wall

The amounts of chitin and chitosan were measured according to Synowiecki and Al-Khateeb [9]. This method involves deproteinization with 2% w/v sodium hydroxide solution (30:1 v/w, 90°C, 2 h); separation of alkali-insoluble material (AIM) by centrifugation (3,500 rpm, 15 min); extraction of chitosan from AIM under reflux (10% v/v acetic acid, 40:1 v/w, 60°C, 6 h); separation of crude chitin by centrifugation (3,500 rpm, 15 min) and precipitation of chitosan from the extract at pH 10.0 using sodium hydroxide. Crude chitin and chitosan were washed with water and air-dried at 60–70°C in an oven.

Inversion of sucrose

Invertase enzyme breaks sucrose dimers into fructose and glucose. In this study, invertase was kindly donated by Novozymes A/S (Bagsvaerd, Denmark). The optimum temperature and pH of invertase were approximately 65°C and 4.5, respectively. For the inversion, 40 g of sugar beet molasses, 40 g of sugarcane molasses or 20 g pure sucrose were dissolved in one liter distilled water, and hydrolyzed for 20 h at 65°C in a shaking bath by adding 4.8 ± 0.1 mg invertase per liter of the syrup.

Analytical methods

The liquid samples of the cultivations were analyzed by high-performance liquid chromatography (HPLC), equipped with UV–VIS and RI detectors (Jasco International Co., Tokyo, Japan). Ethanol and glycerol were analyzed on an Aminex HPX-87H column (Bio-Rad, Richmond, CA, USA) at 60°C with 0.6 ml/min of 5 mM sulfuric acid as eluent. The sugars (glucose, fructose, and

sucrose) were analyzed by a Supelcogel pb column (Sigma-Aldrich, Sweden) at 80°C with 0.5 ml/min of de-ionized water as eluent. All components were detected on RI chromatograms. The biomass yields of *M. indicus*, which consisted of mycelium and yeast-like cells, were measured at the end of the fermentation. The filamentous biomass was collected on a strainer, washed with distilled water and dried at 65–70°C, while the liquid phase containing the yeast cells was centrifuged at 3,500 rpm for 15 min, washed with distilled water, centrifuged once again, and dried at 65–70°C. Morphological development of the fungus was followed periodically using light microscopy.

All the experiments were performed in duplicate and the average standard deviation in the results of the duplicated cultivations was less than 4.2%. All data reported in this paper are the average of the two replications. The maximum ethanol yield was calculated based on the maximum ethanol concentration divided by the total sugar consumed. The maximum volumetric ethanol productivity was calculated based on the difference in ethanol concentration (g/l) in a specific cultivation time (h).

Results

Cultivation on glucose and fructose

Aerobic and anaerobic cultivations of *M. indicus* were performed in glucose- and fructose-containing media. When the growth was examined microscopically, the fungus grew with filamentous and yeast-like morphology, depending on the size of the inoculation (Fig. 1). With a large inoculation of spores, $6(\pm 3) \times 10^6$ spores/ml, under anaerobic conditions, the morphology consisted of purely yeast-like cells (Fig. 1a). Mostly yeast-like cells with a small number of short filaments were observed when a high initial spore concentration, $6(\pm 3) \times 10^6$ spores/ml, was used under aerobic conditions (Fig. 1b). At lower inoculum levels, $3(\pm 1) \times 10^4$ spores/ml, growth was filamentous under both aerobic and anaerobic conditions (Fig. 1c).

The most important results are summarized in Fig. 2 and Table 1. Among the metabolites produced by *M. indicus*, ethanol and glycerol were by far the most important in terms of concentrations under both aerobic and anaerobic conditions with both kinds of morphological growth. Anaerobic fermentation led to higher ethanol yields, although accompanied by lower biomass production. The maximum yields of ethanol from glucose were 0.47 and 0.43 g/g consumed glucose under anaerobic conditions with mostly filamentous and purely yeast-like morphology, respectively (Table 1). The ethanol yield from fructose by the fungus was similar to that from glucose, while the ethanol productivity was somewhat lower than the productivity from glucose, by either yeast-like or filamentous forms (Table 1).

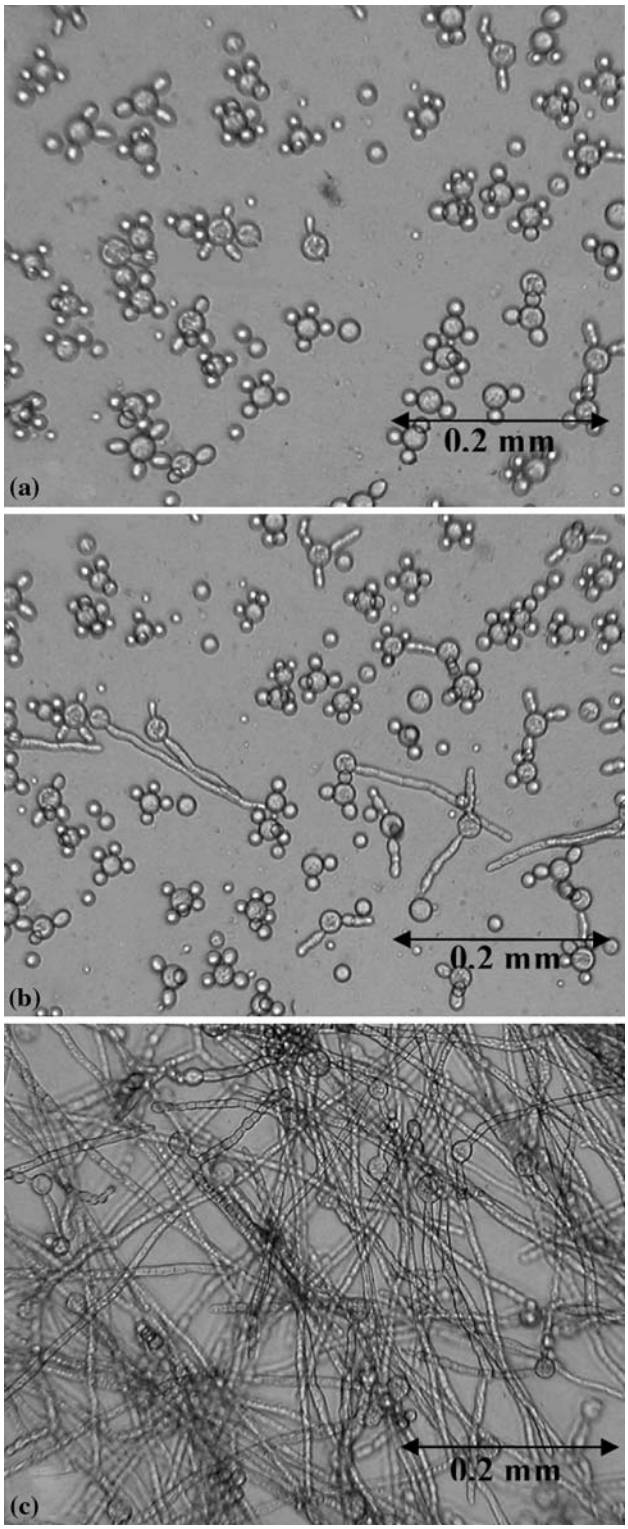

Fig. 1 Growth of *M. indicus* in purely yeast-like form induced by large inoculum under anaerobic conditions (**a**), mostly yeast like form induced by large inoculum under aerobic conditions (**b**), and filamentous form induced by small size of inoculum (**c**)

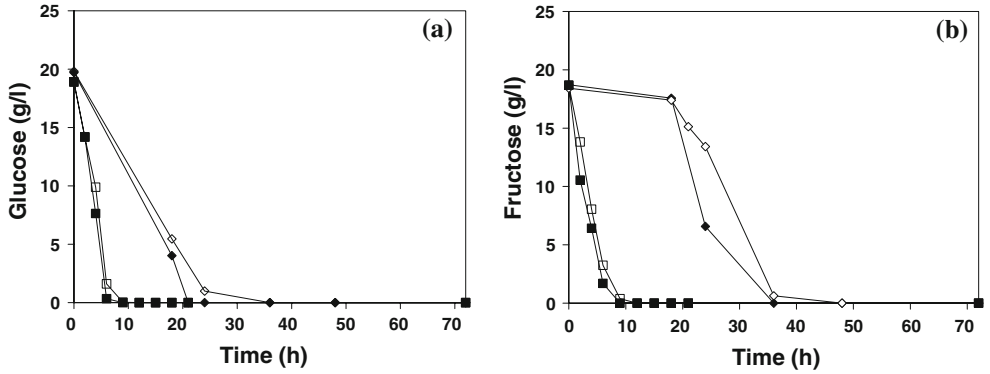

Fig. 2 Assimilation of glucose (**a**) or fructose (**b**) by *M. indicus*. The symbols represent: aerobic cultivation by filamentous form (*filled diamonds*); anaerobic cultivation by filamentous form (*open diamonds*); aerobic cultivation by yeast-like form (*filled squares*); anaerobic cultivation by yeast-like form (*open squares*)

Table 1 The results of aerobic and anaerobic cultivations of *M. indicus* grew with different morphologies induced by large or small inocula

Fermentation condition	Substrate	Morphology	$Y_{E/S}$[a] (g/g)	q_e[b] (g/l h)	$Y_{Gly/S}$[c] (g/g)	$Y_{X/S}$[d] (g/g)	$Y_{AIM/X}$[e] (g/g)	$Y_{Ch/X}$[f] (g/g)
Anaerobic	Glucose	PY	0.43	0.89	0.044	0.19	0.217	0.042
Anaerobic	Fructose	PY	0.45	0.69	0.058	0.18	0.166	0.014
Anaerobic	Inverted sucrose	PY	0.46	0.89	0.055	0.21	0.149	0.036
Aerobic	Glucose	MY	0.41	1.27	0.042	0.31	0.155	0.008
Aerobic	Fructose	MY	0.42	0.87	0.042	0.29	0.173	0.015
Aerobic	Inverted sucrose	MY	0.41	0.79	0.036	0.31	0.169	0.029
Anaerobic	Glucose	MF	0.47	0.34	0.064	0.10	0.203	0.023
Anaerobic	Fructose	MF	0.47	0.21	0.073	0.11	0.178	0.010
Anaerobic	Inverted sucrose	MF	0.46	0.44	0.048	0.12	0.160	0.017
Aerobic	Glucose	MF	0.42	0.39	0.037	0.20	0.210	0.029
Aerobic	Fructose	MF	0.43	0.22	0.035	0.24	0.216	0.018
Aerobic	Inverted sucrose	MF	0.38	0.42	0.036	0.20	0.207	0.005

PY purely yeast like; *MY* mostly yeast like; *MF* mostly filamentous

[a] Maximum ethanol yield on sugar consumed

[b] Maximum volumetric ethanol productivity

[c] Maximum glycerol yield on sugar consumed

[d] Final biomass yield on sugar consumed

[e] AIM yield, gram of alkali-insoluble material per gram of biomass

[f] Chitosan yield, gram chitosan per gram of biomass

Aerobically, glucose was consumed by the fungus within less than 6 and 21 h by mostly yeast-like and filamentous forms of the fungus, respectively (Fig. 2a). Similarly, the required cultivation times for total fructose assimilation were 9 and 36 h by mostly yeast-like and filamentous forms, respectively (Fig. 2b). The metabolite concentrations, mainly ethanol and glycerol, increased sharply during sugar assimilation. Moreover, the fungus gradually consumed part of the ethanol and glycerol produced after complete consumption of the sugars, which took place typically 35 h after the fermentation started (data not shown).

Cultivation on sucrose and inverted sucrose

The fungus *M. indicus* was not able to assimilate sucrose in any of the forms of growth aerobically or anaerobically (Fig. 3a, c). Sucrose was therefore inverted enzymatically into a mixture of glucose and fructose, and used for cultivation of *M. indicus*. Sugar concentration profiles are shown in Fig. 3b, d. The profile of assimilation of fructose and glucose, which were produced by inversion of sucrose, was similar to the assimilation of the pure hexoses. However, when these sugars existed in a mixture, the fungus consumed most of the fructose after consumption of

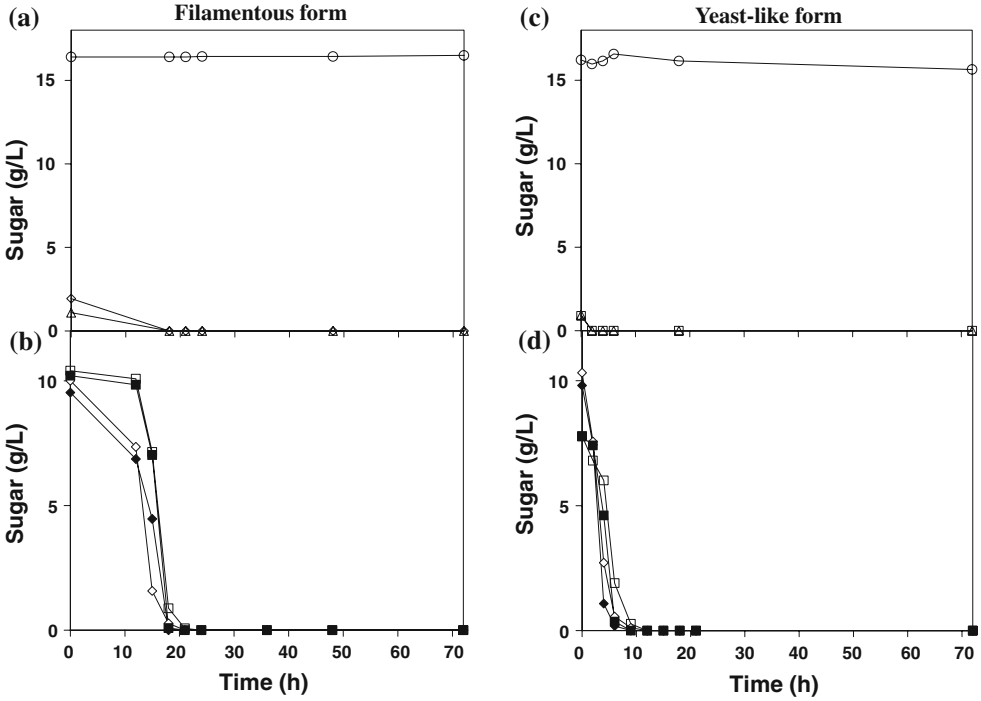

Fig. 3 The profiles of cultivation of sucrose (**a**, **c**) and inverted sucrose (which is a mixture of glucose and fructose) (**b**, **d**) by different forms of *M. indicus* including filamentous and yeast-like (purely yeast-like under anaerobic and mostly yeast-like under aerobic conditions). The symbols represent sucrose (*open circles*), glucose under aerobic (*filled diamonds*); glucose under anaerobic (*open diamonds*); fructose under aerobic (*filled squares*); fructose under anaerobic (*open squares*) conditions

glucose (Fig. 3b, d). The results showed that consumption of fructose increased the concentration of metabolites as well as the produced biomass and the required cultivation time, but it did not significantly affect the yields (Fig. 3b, d; Table 1).

Cultivation on molasses

Sugarcane and sugar beet molasses and their enzymatically inverted hydrolyzates were aerobically and anaerobically cultivated by *M. indicus*, and the most important results are summarized in Table 2.

The diluted solution of sugar beet molasses contained initially 16.0 ± 0.1, 1 ± 0.1 and 1.3 ± 0.3 g/l sucrose, glucose and fructose, respectively. Cultivation of this solution with *M. indicus* resulted in rapid consumption of glucose and fructose, but not sucrose. The fungus in yeast-like form consumed these two sugars completely in less than 2 h, while the filamentous form needed about 12 h for the same purpose. Ethanol was the major metabolite produced from the molasses (Table 2).

The sugarcane molasses had higher concentration of the inverted sugars compared to the sugar beet molasses. The concentrations of sucrose, glucose and fructose in the diluted sugarcane molasses used in this work were 7.8 ± 1, 4.7 ± 0.5, and 5.7 ± 0.6 g/l, respectively. The fungus consumed the glucose and fructose present in this solution, but not the sucrose. The maximum ethanol yield was obtained from anaerobic cultivation of this substrate (Table 2).

Since *M. indicus* could not ferment sucrose present in the molasses, both the sugarcane and sugar beet molasses were first hydrolyzed by invertase enzyme at 65°C for 20 h before the cultivations. It resulted in complete conversion of sucrose to glucose and fructose. The hydrolyzed beet sugar had 9.4 ± 1 and 8.7 ± 0.6 glucose and fructose, respectively. The corresponding concentrations in sugarcane molasses were 9.4 ± 1 and 9.6 ± 1 g/l. Cultivations of these hydrolyzates were successfully carried out by *M. indicus* with two different morphologies under aerobic and anaerobic conditions and the results are presented in Table 2.

Biomass and cell wall analyses

The biomass of *M. indicus* was separated after 72 h cultivations and analyzed for chitin and chitosan and the results are summarized in Tables 1 and 2. The results show that the yield of biomass and chitosan depended on the type of substrate, availability of oxygen in the culture, and morphology (Tables 1, 2). As expected, the yield of biomass was higher under aerobic conditions than the anaerobic ones (Tables 1, 2). Furthermore, the yield of biomass was higher during cultivation of the fungus with yeast-like morphology compared to filamentous form.

Chitin and chitosan analysis of the biomass showed that the cell wall composition of the fungus was affected by the morphology, conditions of cultivation, and the substrate used. The yields of AIM were in the range of 0.15–0.53 g/g, while the biomass of the fungus contained 0.008–0.230 g chitosan per g of biomass (Tables 1, 2).

Table 2 The results of cultivation of the purely yeast-like, mostly yeast like, and mostly filamentous morphologies of *M. indicus*

Fermentation condition	Substrate	Morphology	$Y_{E/S}$[a] (g/g)	q_e[b] (g/l h)	$Y_{Gly/S}$[c] (g/g)	$Y_{X/S}$[d] (g/g)	$Y_{AIM/X}$[e] (g/g)	$Y_{Ch/X}$[f] (g/g)
Aerobic	Sugarcane molasses	MY	0.39	0.83	0.049	0.37	0.247	0.075
Aerobic	Sugar beet molasses	MY	0.16	0.16	0.000	ND	0.246	0.093
Aerobic	Inverted sugarcane molasses	MY	0.42	1.13	0.043	0.35	0.242	0.090
Aerobic	Inverted sugar beet molasses	MY	0.42	1.15	0.045	0.31	0.211	0.080
Anaerobic	Sugarcane molasses	PY	0.48	0.74	0.060	0.31	0.198	0.080
Anaerobic	Sugar beet molasses	PY	0.39	0.33	0.000	ND	0.231	0.075
Anaerobic	Inverted sugarcane molasses	PY	0.46	1.11	0.056	0.24	0.254	0.109
Anaerobic	Inverted sugar beet molasses	PY	0.47	0.85	0.053	0.20	0.226	0.094
Aerobic	Sugarcane molasses	MF	0.44	0.27	0.026	0.42	0.222	0.066
Aerobic	Sugar beet molasses	MF	0.23	0.05	0.000	ND	0.344	0.117
Aerobic	Inverted sugarcane molasses	MF	0.38	0.39	0.031	0.22	0.192	0.075
Aerobic	Inverted sugar beet molasses	MF	0.42	0.46	0.039	0.13	0.170	0.060
Anaerobic	Sugarcane molasses	MF	0.47	0.12	0.057	0.19	0.266	0.112
Anaerobic	Sugar beet molasses	MF	0.35	0.07	0.000	ND	0.530	0.230
Anaerobic	Inverted sugarcane molasses	MF	0.41	0.41	0.052	0.17	0.253	0.102
Anaerobic	Inverted sugar beet molasses	MF	0.48	0.52	0.060	0.09	0.210	0.089

PY purely yeast like; *MY* mostly yeast like; *MF* mostly filamentous; *ND* not detected

[a] Maximum ethanol yield on sugar consumed

[b] Maximum volumetric ethanol productivity

[c] Maximum glycerol yield on sugar consumed

[d] Final biomass yield on sugar consumed

[e] AIM yield, gram of alkali-insoluble material per gram of biomass

[f] Chitosan yield, gram chitosan per gram of biomass

Discussion

In previous works, *M. indicus* was presented as a suitable alternative to the baker's yeast in cultivating lignocellulosic hydrolyzates, with several advantages (e.g. cf. [7, 8]). The fungus was already shown to be able to consume glucose, galactose, mannose, xylose and arabinose and to produce ethanol with a high yield and productivity. Furthermore, its biomass is a source of different products such as chitosan [12].

The current work concerns whether *M. indicus* can assimilate sugars present in molasses. Molasses is a relatively inexpensive raw material that has been used for industrial ethanol production for many years [3]. The results show that *M. indicus* is able to consume glucose and fructose present in molasses. However, it is unable to hydrolyze sucrose, but is able to assimilate hydrolyzed sucrose.

Difficulty in handling of the biomass in a large-scale process is one of the major obstacles in industrial application of filamentous fungi. Such problems are mixing, non-uniformity, growing of the fungi on the oxygen, pH, and temperature sensor of the bioreactor [16, 17]. Therefore, it is usually preferred to use yeasts rather than filamentous fungi in industrial processes, if possible. The fungus used in this work is a dimorphic microorganism [20, 21], which is easily grown in yeast-like form.

Generally the fermenting microorganism for ethanol production should have a high ethanol yield and productivity. In the current study, it has been shown that both forms of *M. indicus*, yeast-like and filamentous, can efficiently produce ethanol with a high yield and productivity. The results show that the yield of ethanol production by the filamentous form is slightly higher than by the yeast-like. On the other hand, the ethanol productivity in yeast-like morphology is higher than that of the filamentous form. These differences might be due to variations between the structures of yeast-like and filamentous cells. The cell wall of yeast-like form of this fungus is much thicker, more diffuse, and more fibrous than the cell walls of filamentous form [9]. Difficulty in mass transfer in filamentous form may also decrease the rate of sugar consumption and consequently decrease the productivity. More studies are necessary to find the reason.

One of the major advantages of using *M. indicus* as an ethanol-producing microorganism is its biomass which can be further used for production of valuable products such as chitosan [12]. Chitosan is a nontoxic and biodegradable

polycationic material which shows antimicrobial properties. Chitosan has a variety of applications in the agricultural, food, and pharmaceutical industries. It is used for food preservation, fruit juice clarification, water purification particularly for removal of heavy metal ions, sorption for dyes, and as a flocculating agent [12]. The yield of biomass and chitosan in this study showed that the fungus is a suitable microorganism for chitosan production. Furthermore, the morphology of the fungus is an effective parameter that influences the yield of biomass and chitosan. The maximum yield of biomass is obtained in cultivation of yeast-like morphology, while chitin and chitosan are higher in the cell wall of filamentous form of *M. indicus*, which is in line with the previous work on the study of the cell wall of the fungus [14, 15, 22, 23].

Conclusion

The general conclusion of this work is that *M. indicus* can utilize the fructose which is available in many substrates including molasses but not sucrose, due to inability of sucrose hydrolysis. The fungus can therefore be used as a tool for selective removal of glucose and fructose from molasses while retaining the crystallizable sucrose in the solution. Furthermore, it is easily possible to change the morphology of *M. indicus*. The fungus in different morphologies can produce ethanol with relatively high yields and productivity.

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