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ORIGINAL RESEARCH PAPER

Fungal autolysate as a nutrient supplement for ethanol and chitosan production by *Mucor indicus*

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Abstract *Mucor indicus* can be used to produce ethanol from a variety of sugars, including pentose's. An extract of it, produced by autolysis, could replace yeast extract in culture medium with improved production of ethanol. At 10 g l^{-1}, the extract gave a higher ethanol yield (0.47 g g^{-1}) and productivity (0.71 g l^{-1} h^{-1}) compared to medium containing yeast extract (yield 0.45 g g^{-1}; productivity 0.67 g l^{-1} h^{-1}).

Keywords Autolysis · Bioethanol · Chitosan · Fungal extract · *Mucor indicus*

Introduction

Mucor indicus (formerly *M. rouxii*) can grow aerobically or anaerobically on various carbon sources, including hexoses and pentose's, and produce ethanol with yield and productivity in the same order as *Saccharomyces cerevisiae* (Karimi et al. 2005; Lennartsson et al. 2009). Its high tolerance to sugar, ethanol and various possible inhibitors suggest it may have industrial applicability (Abtahi et al. 2010; Karimi et al. 2005). Furthermore, the biomass of this fungus can be used as a source of chitosan and also as a fish feed (Zamani 2010; Zamani et al. 2010).

The majority of studies on ethanol production by zygomycetes fungi have used media containing complex growth supplement, such as yeast extract, to a achieve high ethanol yield (Karimi et al. 2008; Sues et al. 2005; Millati et al. 2005). However, the cost of this supplement is not realistic for industrial ethanol production.

Fungal biomass can be autolysed by endogenous enzymes resulting in disintegration of the cells (Perez-Leblic et al. 1982). The resulting autolysate is a nutrient-rich solution of amino acids, peptides, phosphorus, and carbohydrates (Koutinas et al. 2005).

In the current work, *M. indicus* biomass was subjected to autolysis, the cell wall was separated for chitosan production, and the fungal autolysate was used as a nutrient for ethanol production by the same fungal strain in order to reduce the nutrient consumption in the fermentation.

Materials and methods

Microorganism and growth

Mucor indicus CCUG 22424 (Culture Collection University of Gothenburg, Sweden) was grown in Erlenmeyer flasks with 300 ml medium containing (per liter):

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40 g glucose monohydrate, 5 g yeast extract, 7.5 g $(NH_4)_2SO_4$, 3.5 g K_2HPO_4, 1.0 g $CaCl_2{\cdot}2H2O$, and 0.75 g $MgSO_4{\cdot}7H_2O$ at pH 5.5 ± 0.1 (Sues et al. 2005). The flasks were incubated at 32 ± 0.5°C and 180 rpm for 30 h.

Fungal autolysis

Fungal biomass was recovered by filtration, washed, and re-suspended in sterile distilled water to give 50 g biomass l^{-1}. The pH was then adjusted to 5.2 using 1 M H_2SO_4. The autolyses were performed at 55 ± 1°C for 72 h by shaking at 120 rpm, with or without ultrasonication. The ultrasonication was carried out before autolysis for 20 min (38 kHz; Pulse 700, Italy).

After autolysis, the suspensions were centrifuged for 15 min (3,400×*g*). The solubilized cell constituents, present in the supernatant (autolysate), resulting from the autolysis process with or without ultrasonication were referred to as "ultrasonicated fungal extract" or "fungal extract", respectively. The fungal biomass and the residual solids remained after the autolysis were dried at 55 ± 1°C and were referred to as "untreated fungal biomass" and "residual cellular materials", respectively.

Ethanol production

Fermentations were carried out in 120 ml sealed anaerobic bottles with 50 ml medium containing 40 g glucose monohydrate l^{-1} and different supplements (Table 1). The media were autoclaved at 121°C for 20 min, and inoculated with 1 ml suspension containing 4.5 (±0.5) × 10^5 spores of *M. indicus*.

Table 1 The results of ethanol and glycerol production by *M. indicus* in different media

Nutrient supplementation	Maximum ethanol productivity (g l^{-1} h^{-1})	$Y^{d}_{E/S}$ (g g^{-1})	$Y^{e}_{Gly/S}$ (mg g^{-1})	Terminal time (h)[f]
Yeast extract (5 g l^{-1}) + mineral salts[a]	0.67	0.45	47.3	24
Yeast extract (5 g l^{-1})	0.37	0.43	46.4	48
Untreated fungal biomass (5 g l^{-1})	0.19	0.36	36.9	>72
Untreated fungal biomass (5 g l^{-1}) + mineral salts	0.51	0.39	49.3	48
Fungal extract (2.5 g l^{-1})	0.34	0.39	43.3	72
Fungal extract (2.5 g l^{-1}) + mineral salts	0.53	0.40	42.0	36
Fungal extract (5 g l^{-1})	0.54	0.43	45.1	36
Fungal extract (5 g l^{-1}) + mineral salts	0.69	0.46	49.0	24
Fungal extract (5 g l^{-1}) + (Mg and Ca)[b]	0.63	0.46	47.2	36
Fungal extract (10 g l^{-1})	0.71	0.47	38.4	24
Ultrasonicated fungal extract[c]	0.64	0.44	41.5	36
Ultrasonicated fungal extract + mineral salts	0.67	0.45	44.6	24
Ultrasonicated fungal extract + (Mg and Ca)	0.69	0.46	37.1	24
Residual cellular materials (5 g l^{-1})	0.12	0.29	17.1	>72
Residual cellular materials (5 g l^{-1}) + mineral salts	0.41	0.38	46.3	48

All fermentations were performed under anaerobic conditions in a shaker incubator at 32 ± 0.5°C with 180 rpm for 72 h. The pH was adjusted to 5.5 ± 0.1 (50 mM sodium citrate buffer)

Concentrations of fungal extract, untreated fungal biomass, and residual cellular materials are based on the dry weight

[a] Mineral salts supplementation (g l^{-1}): $(NH_4)_2SO_4$ (7.5); K_2HPO_4 (3.5); $MgSO_4{\cdot}7H_2O$ (0.75); $CaCl_2{\cdot}2H_2O$ (1.0)

[b] (Mg and Ca) supplementation (g l^{-1}): $MgSO_4{\cdot}7H_2O$ (0.75) and $CaCl_2{\cdot}2H_2O$ (1.0)

[c] Similar volume of the autolysate as the previously mentioned for fungal extract (5 g l^{-1}) was used

[d] Maximum ethanol yield on consumed glucose: [Produced ethanol (g l^{-1})/Consumed glucose (g l^{-1})]

[e] Maximum glycerol yield on consumed glucose: [Produced glycerol (mg l^{-1})/Consumed glucose (g l^{-1})]

[f] Time needed to reach the glucose concentration of less than 0.1 g l^{-1}

Analytical methods

The amount of the fungal extract in autolysates was measure by drying of 10 ml autolysate at 55 ± 1°C. Glucose, ethanol, glycerol, and other metabolites were determined by HPLC according to Karimi et al. (2008).

Results and discussion

In all previous studies on *M. indicus*, high yields of ethanol have been obtained only when a fully supplemented media, containing at least 5 g yeast extract l^{-1}, was used (Sues et al. 2005; Karimi et al. 2008; Lennartsson et al. 2009). In the current work, the fungal biomass, an unavoidable byproduct of the ethanol production, was subjected to autolysis, and the autolysate was used as a supplementary nutrient which replaced yeast extract and other nutrients (mineral salts) in fermentation media. The nutritional effects of untreated fungal biomass and residual cellular materials were also investigated. Cultivation of the fungus with 5 g yeast extract l^{-1} and mineral salts supplementation as a fully supplemented medium (Sues et al. 2005) was used to establish a basis for the comparison in ethanol production (Fig. 1a, b).

In all cultivations, glycerol was the most abundant byproduct of the fermentation (Table 1). The other metabolites such as pyruvic, acetic, and succinic acids were also detected but at less than 10 mg g^{-1} in all experiments (data not shown).

Evaluation of untreated fungal biomass for media supplementation

Using a non-supplemented medium (40 g glucose l^{-1} with no nutrient supplementation), no ethanol was produced even after 72 h (Fig. 1a, b). Untreated fungal biomass as a supplement gave low ethanol yields with incomplete sugar consumption even after 72 h (Table 1). The low performance of the untreated fungal biomass may be attributed to *M. indicus* being unable to use unhydrolysed proteins or other polymeric materials of the fungal cell. Supplementation of the mineral salts together with the untreated fungal biomass improved ethanol yield and productivity; however, the results were low (Table 1; Fig. 1).

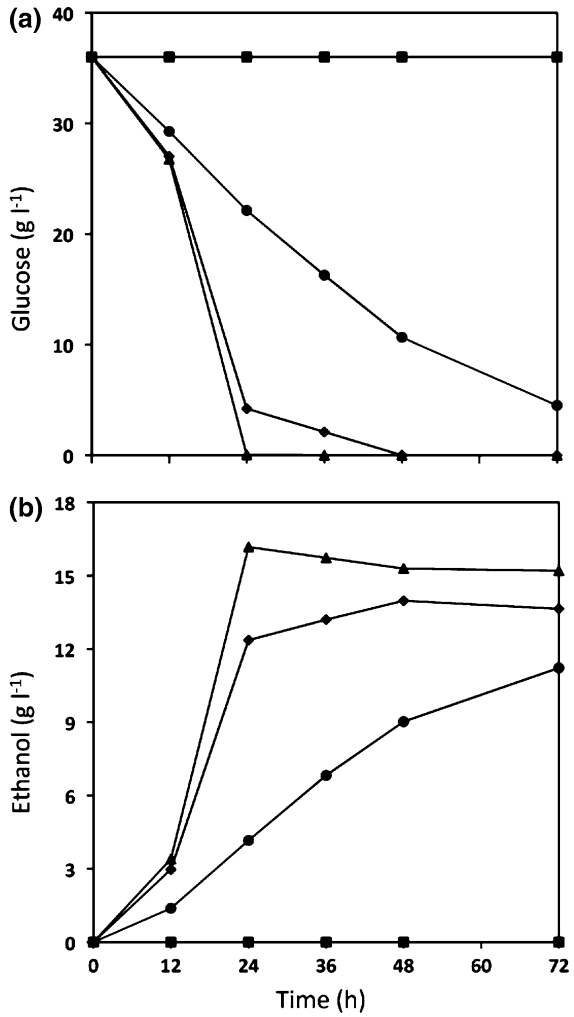

Fig. 1 Effect of the supplementation of untreated fungal biomass on **a** glucose assimilation and **b** ethanol production. The symbols = non-supplemented medium (40 g glucose l^{-1} with no nutrient supplementation) (*filled square*); yeast extract (5 g l^{-1}) and mineral salts (*filled triangle*); untreated fungal biomass (5 g l^{-1}) (*filled circle*); untreated fungal biomass (5 g l^{-1}) and mineral salts (*filled diamond*). All fermentations were performed at 32 ± 0.5°C with 180 rpm for 72 h

Evaluation of fungal autolysate for media supplementation

Supplementation of fungal autolysate, to give 2.5, 5, and 10 g fungal extract l^{-1} in fermentation media, was investigated (Table 1). Fungal extract at 5 g l^{-1} successfully replaced 5 g yeast extract l^{-1} with a better production of ethanol (Table 1; Fig. 2). The extract at 10 g l^{-1} gave complete sugar consumption in less than 24 h and an even higher ethanol yield and

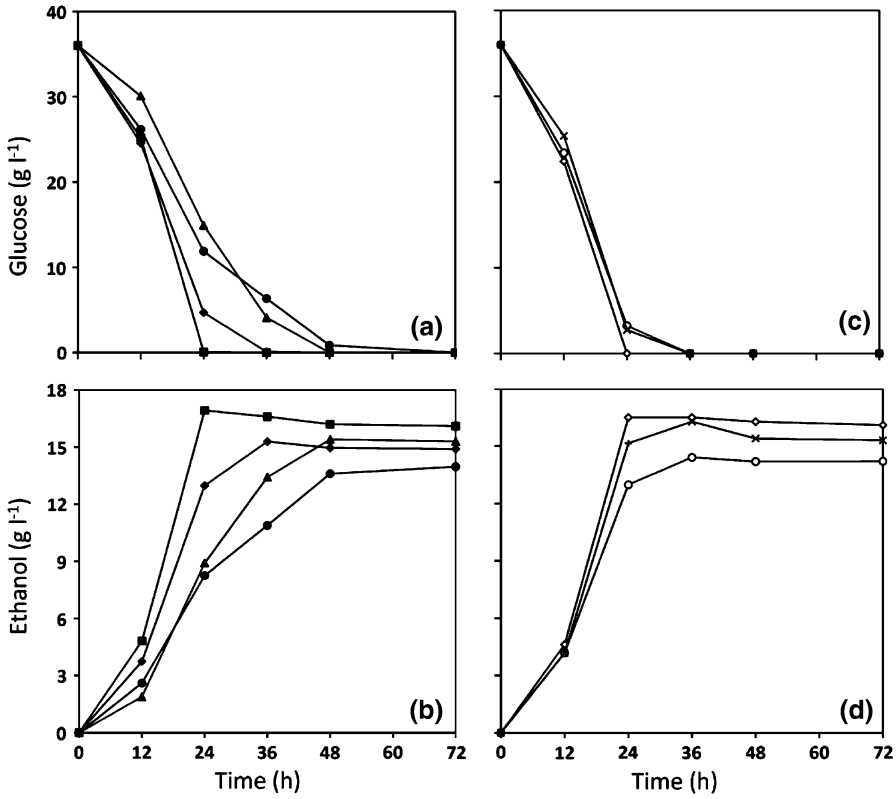

Fig. 2 Effect of supplementation of different fungal extract concentrations on (**a** and **c**) glucose assimilation and (**b** and **d**) ethanol production. The symbols = yeast extract (5 g l^{-1}) (*filled triangle*); fungal extract (2.5 g l^{-1}) (*filled circle*); fungal extract (5 g l^{-1}) (*filled diamond*); fungal extract (10 g l^{-1}) (*filled square*); fungal extract (2.5 g l^{-1}) and mineral salts (*white circle*); fungal extract (5 g l^{-1}) and mineral salts (*lozenge*); fungal extract (5 g l^{-1}) and (Mg and Ca salts) (*times symbol*). All fermentations were performed at 32 ± 0.5°C with 180 rpm for 72 h

productivity, compared to the fully supplemented medium (Fig. 2a, b). This demonstrates that the fungal autolysate contains sufficient essential nutrients for ethanol fermentation. However, the rates of glucose consumption and ethanol production in media with 2.5 g fungal extract l^{-1} supplementation were low which indicated the existence of nutrient limitation.

Effect of ultrasonic pretreatment on fungal autolysate for media supplementation

Ultrasonic treatment of fungal biomass before autolysis released more intracellular components and produced an autolysate with higher concentrations of fungal extract. Supplementation of the fungal autolysate containing ultrasonicated fungal extract gave higher ethanol yields with a higher productivity than those obtained from 5 g fungal extract l^{-1} supplementation (Table 1). Addition of either mineral salts or $CaCl_2$ and $MgSO_4$ to media containing untrasonicated fungal extract improved the results to the level which was achieved fermentation of the fully supplemented medium.

Evaluation of the residual cellular materials for media supplementation

Supplementation of the residual cellular materials in fermentation media resulted in incomplete sugar assimilation and low ethanol yield and productivity even after addition of mineral salts (Table 1). Therefore, the residual cellular materials, which mainly contain cell walls, have low nutritional values, and can probably be used for production of chitosan.

Chitosan extraction

The chitosan contents of residual cellular materials and untreated fungal biomass were extracted according to the method described by Zamani et al. (2010). The results showed that the residual cellular materials contain higher amounts of chitosan per dry alkali-insoluble material (AIM) compared with untreated

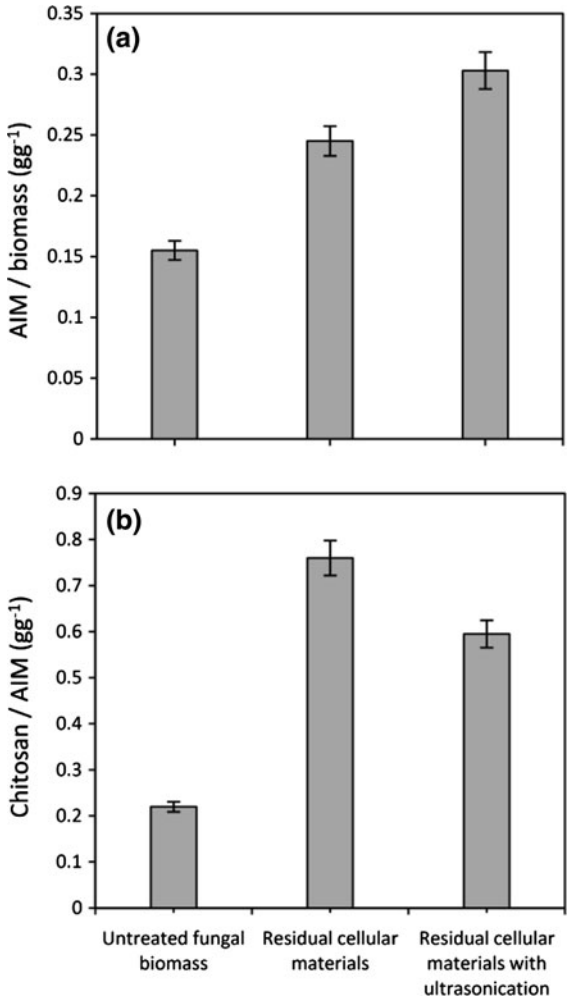

Fig. 3 **a** Concentration of alkali-insoluble cell wall material (*AIM*) of *M. indicus* before and after autolysis process, measured as dry AIM per dry biomass (g g^{-1}) and **b** concentration of chitosan in the AIM of materials, measured as chitosan per dry AIM (g g^{-1}). The dried biomass was treated with 0.5 M NaOH at 120°C for 20 min to obtain AIM. The AIM was then subjected to two-steps treatment with dilute sulfuric acid, and the chitosan was subsequently recovered from the acid solution by precipitation at lowered temperature (Zamani et al. 2010)

fungal biomass (Fig. 3). Therefore, the residual cellular materials have a high potential for biological production of superabsorbent (Zamani 2010).

Conclusions

A fermentation medium based on application of *M. indicus* biomass autolysate can be used for bioethanol production with a minimal or no additional nutrient requirement. The residual cellular materials as a byproduct of the autolysis process contain appreciable amounts of chitosan.

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