

## Ethanol production by *Mucor indicus* using the fungal autolysate as a nutrient supplement

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**Abstract:** To develop a cost-effective fermentation medium, fungal extract (FE) of *Mucor indicus* biomass, which is a by-product of fermentation processes, was evaluated as a nutrient source for ethanol production by the fungus. Autolysis as a natural process of self-digestion of fungal cells was used to release the nutrients in surrounding medium leading to the production of the FE. Glucose consumption and ethanol production were followed using several media made with different concentrations of FE as nutrient supplementation replacing either yeast extract (YE) or whole nutrient. According to the results, 5 g/L YE could be successfully replaced with 5 g/L FE, resulting in higher ethanol yield (0.46 g/g) and productivity (0.69 g/L.h). Yield of glycerol production, the major byproduct of fermentation, was also increased by supplementation of the FE.

**Keywords:** Bioethanol; Fungal extract; Autolysis; *Mucor indicus*.

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### 1. Introduction

Amongst all liquid biofuels, bioethanol is widely recognized these days as a promising renewable and environmentally friendly source of energy. It is an alternative fuel with the recognition that the global crude oil reserve is finite, and its depletion is occurring much faster than previously predicted [1]. Recently, saprophytic zygomycetes strain *Mucor indicus* (formerly *M. rouxii*) has been identified as an ethanol-producing organism, capable to grow aerobically or anaerobically on a number of different carbon sources including hexoses and pentoses with yield and productivity in the same order as *Saccharomyces cerevisiae* [2]. Furthermore, the interest in the potential utilization of fungal biomass zygomycetes as a valuable product is increasing due to the structural composition of cell walls [3]. Ethanol production by fermentation of natural feedstocks usually requires the use of complex growth supplements, such as yeast extract (YE) [2, 4]. The high cost of YE and other commercial nutrients is a limitation to its application in industrial processes, including the fermentation of biomass to ethanol. Thus, it is desirable to develop media that are likely to perform well in conditions that are representative in microbial fermentation. Several studies have concentrated on the use of yeast autolysate as effective nutrients in wheat fermentations and ethanol production [5, 6]. Fungal biomass as a by-product of fungal fermentations can be used as a source of nutrients for microbial fermentations. This can be achieved by disintegration and releasing materials hydrolyzed in-to assimilable monomers to produce a fungal autolysate as a nutrient-rich solution containing such as amino-acids, peptides, phosphorus and carbohydrates. Cell autolysis as an economical method is the natural degradation process, which starts after the exhaustion of major nutrients and reserves [7]. The objective of the present study was to develop a low-cost and suitable fermentation medium based on the utilization of filamentous fungus biomass, *M. indicus*, as a nutrient source for production of ethanol with the same fungal strain.

### 2. Materials and Methods

#### 2.1. Microorganism strain and media

The fungus *M. indicus* 22424 CCUG (Culture Collection University of Göteborg, Sweden) was used in all experiments. The fungus was cultivated on agar slants containing (g/L):

glucose monohydrate, 40; peptone, 10; and agar 20 at pH  $5.5\pm 0.1$  and  $32\pm 0.5^\circ\text{C}$  for 5 days, where the fungus grew to form a cotton-like mycelium and spores. The agar slants were stored at  $4^\circ\text{C}$  until use.

## 2.2. Fungal spore germination

The batch cultivations were carried out in 500-ml cotton-plugged conical flasks with 300 ml working volume containing glucose monohydrate (40 g/L), supplemented with (per liter): 5 g YE, 7.5 g  $(\text{NH}_4)_2\text{SO}_4$ , 3.5 g  $\text{K}_2\text{HPO}_4$ , 1 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.75 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  at pH  $5.5\pm 0.1$ . The flasks were incubated at  $32\pm 0.5^\circ\text{C}$  and 180 rpm for 30 h, which provided initial biomass for further fungal autolysis.

## 2.3. Fungal autolysis

The produced biomass (fungal cells) from the fungal germination were recovered and separated from the liquid broth by filtration under aseptic conditions and washed at least three times with sterile distilled water to remove any residual nutrients. The clean solids were then re-suspended in sterile distilled water to achieve a concentration of 50 g/L fungal biomass. The fungal suspensions were then placed in 250 ml glass vessels immersed in a temperature-controlled shaking water bath. The initial pH was adjusted to  $5.2\pm 0.1$  using either 10% sulfuric acid or 1 N sodium hydroxide. The autolysis was carried out at  $55\pm 1^\circ\text{C}$  and 120 rpm for 72 h. After autolysis, the suspension was centrifuged for 15 min at  $4^\circ\text{C}$  and 4500 rpm, and the supernatant was designated as autolysate of fungal cells. The solubilized cell constituents in autolysate of fungal cells resulting from the autolysis were referred to as "FE".

## 2.4. Ethanol production

The fermentation experiments were carried out anaerobically in 120 ml glass bottles with 50 ml working volume, containing 40 g/L glucose monohydrate and different media supplementation (Table 1) in 50 mM sodium citrate buffer with pH  $5.5\pm 0.1$ . The media were sterilized by autoclaving at  $121^\circ\text{C}$  for 20 min, and then inoculated with 1.0 ml of a suspension containing  $4.5(\pm 0.5) \times 10^5$  spores of *M. indicus*. The fully nutrient medium containing YE (5 g/L) supplemented with mineral salts (g/L):  $(\text{NH}_4)_2\text{SO}_4$ , 7.5,  $\text{K}_2\text{HPO}_4$ , 3.5,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 1,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.75. Table 1 shows the type of supplementation corresponding to the various media assayed. All fermentations were performed in a shaking incubator at  $32\pm 0.5^\circ\text{C}$  with the agitation speed of 180 rpm for 72 h. The fermentation samples were stored at  $-20^\circ\text{C}$  before metabolite analysis.

## 2.5. Analytical methods

For determination of the amount of materials released from the cells into the surrounding liquid phase during autolysis (FE), 10 ml of autolysate of fungal cells after autolysis process was separated and dried in an oven at  $55\pm 1^\circ\text{C}$  until constant weight was achieved. The liquid samples from fermentations were analyzed by high performance liquid chromatography (HPLC), which was equipped with UV/vis and RI detectors (Jasco International Co., Tokyo, Japan). Glucose, ethanol, glycerol were analyzed on an Aminex HPX-87H column (Bio-Rad, Richmond, CA, USA) at  $60^\circ\text{C}$  with 0.6 ml/min eluent of 5mM sulfuric acid. All components were detected on RI chromatograms. All experiments in this work were duplicated and the averages of two replications are presented.

### 3. Results

#### 3.1. Effect of the supplementation of fungal extract on ethanol production

*M. indicus* was produced in the fully supplemented medium containing 40 g/L glucose monohydrate and other nutrient components, and the produced fungal cells were recovered by filtration and were subjected to fungal autolysis process. To verify the potential of FE as an alternative nutrient supplementation replacing YE, a series of experimental fermentations were performed at two concentrations (2.5 and 5 g/L) of FE (Table 1).

Table 1. Results of ethanol production by *M. indicus* in different media.

Nutrient Supplementation	Maximum ethanol volumetric productivity (g/L h)	$Y_{E/S}^b$ (g/g)	$Y_{Gly/S}^c$ (mg/g)	Terminal time (h) <sup>d</sup>
YE (5 g/L), mineral salts <sup>a</sup>	0.67	0.45	47.3	24
YE (5 g/L)	0.37	0.43	46.4	48
FE (2.5 g/L)	0.34	0.39	43.3	72
FE (2.5 g/L), mineral salts	0.53	0.40	42.0	36
FE (5 g/L)	0.54	0.43	45.1	36
FE (5 g/L), mineral salts	0.69	0.46	49.0	24

<sup>a</sup> Mineral salts supplementation (g/L):  $(NH_4)_2SO_4$  (7.5),  $K_2HPO_4$  (3.5),  $MgSO_4 \cdot 7H_2O$  (0.75),  $CaCl_2 \cdot 2H_2O$  (1)

<sup>b</sup> Maximum ethanol yield on consumed glucose.

<sup>c</sup> Maximum glycerol yield on consumed glucose.

<sup>d</sup> Time needed for total consumption was defined as the period between addition of glucose and its exhaustion to concentration below 0.5 g/L.

To establish a basis for comparison, a fermentation run was carried out in a fully supplemented medium (YE (5 g/L) and mineral salts). The results showed that, glucose was rapidly consumed and mainly converted to ethanol (Fig. 1a and b), reaching a maximum yield of 0.45 g/(g glucose) and a volumetric productivity of 0.67 g/L h in less than 24 h cultivation under anaerobic conditions. Glycerol was the most important byproduct of the fermentation with maximum yield of 47.3 mg/g glucose (Table 1). As can be seen from Table 1, *M. indicus* gave the ethanol yield of 0.43 g/g and low productivity with the supplementation of only 5 g/L YE (Table 1). A preliminary experiment was carried out using 2.5 g/L of FE as unique supplementation (Table 1). As a result, the low glucose consumption indicated the existence of nutrient limitation in the medium and the maximum ethanol concentration was reached after a relatively long reaction time (about 72 h). However, the experiment with supplementation of 2.5 g/L FE gave a poor performance in ethanol production because of nutrient limitation in fermentation media relative to YE or whole nutrient supplementations.

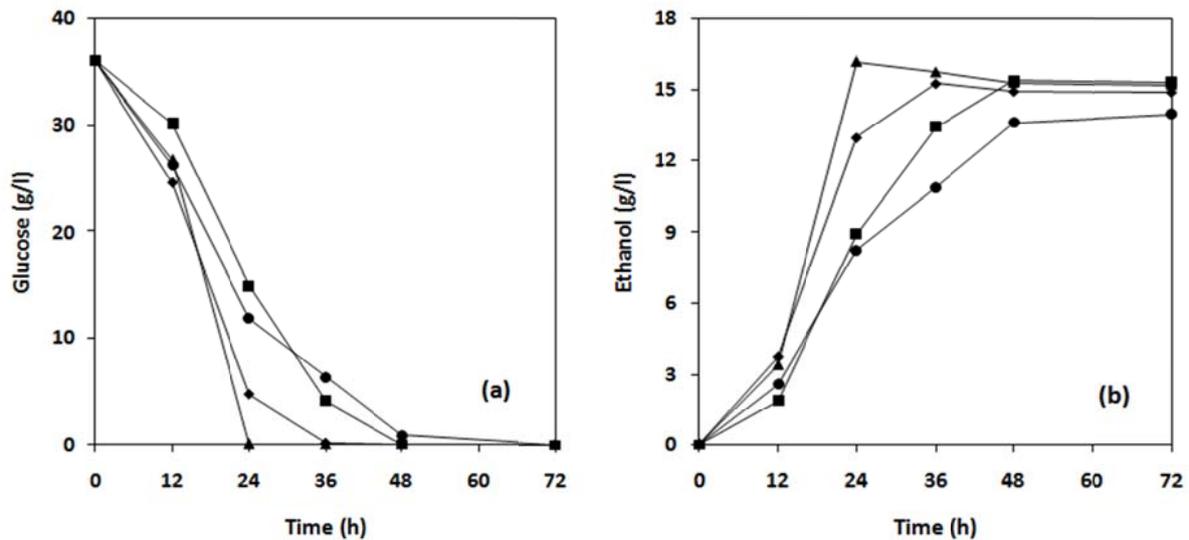


Fig.1. Effect of the supplementation of fungal extract on glucose assimilation (a) and ethanol production (b). The symbols represent of supplementation of YE (5 g/L) (■); YE (5 g/L) with mineral salts (▲); FE (2.5 g/L) (●) and FE (5 g/L) (◆).

In this direction, an additional experiment performed in order to increase the nutrient concentration with 5 g/L FE of *M. indicus* as unique supplementation (Table 1). Compared with 5 g/L YE, 5 g/L FE resulted in a higher ethanol yield with a maximum of 0.43 g/g. However, the low glucose consumption indicated the existence of nutrient limitation in the medium. As a result, the maximum yield and productivity of ethanol in this medium was still lower than the fully supplemented medium (Table 1).

### 3.2. Evaluation of fungal extract for media supplementation replacing yeast extract

The possibility of supplementing of FE (2.5 g/L) in the fermentation media with combination of mineral salts (Table 1) was assessed in an additional experiment to overcome nutrient limitation. According to Table 1, addition of the mineral salts provided a gradual increase in ethanol yield and volumetric productivity in comparison with addition of only 2.5 g/L FE. However, it was comparatively low due to result obtained in a fully supplemented medium. Considering that a deficit in mineral salts with 5 g/L FE supplementation could be partially responsible for the prolonged fermentation time, additional experiment was prepared by adding the mineral salts presented in the full nutrient medium. As a result of this modification, the bioconversion to ethanol was further improved and showed results closely related to the ones observed for the fully supplemented medium (Fig. 2a and b), with a maximum ethanol yield and productivity of 0.46 g/g and 0.69 g/L h in less than 24 h, respectively. The maximum glycerol yield of 0.49 mg/g was achieved at this condition. It came, therefore, to the conclusion that autolysis of *M. indicus* biomass as a valuable by product from ethanol fermentation could be used as a microbial nutrient source for further fermentation with supplementation of 5 g/L FE replacing 5 g/L YE.

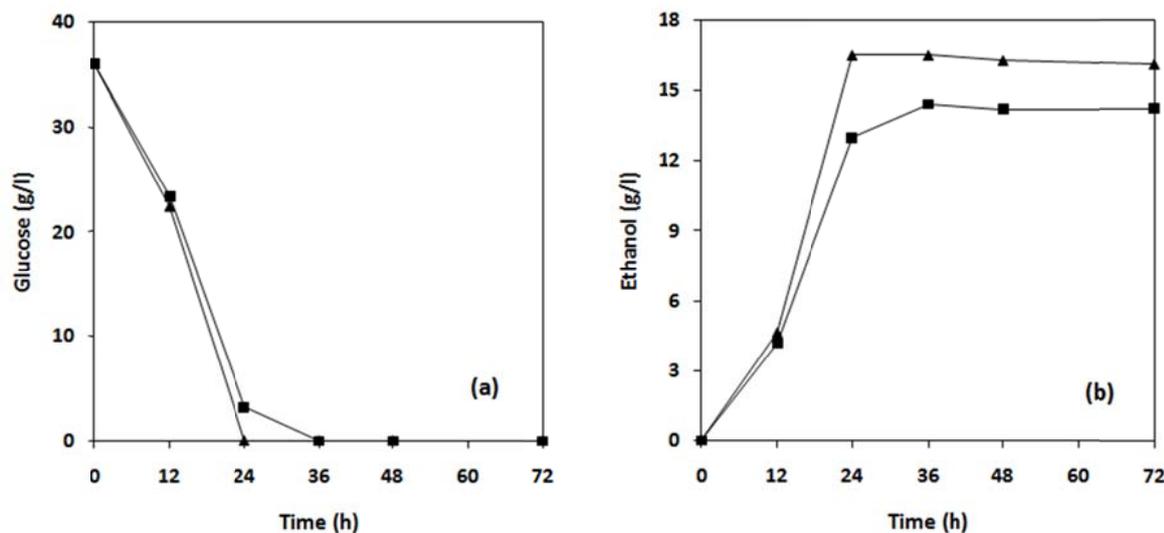


Fig.2. Effect of the supplementation of fungal extract with combination of mineral salts on glucose assimilation (a) and ethanol production (b). The symbols represent of supplementation of FE (2.5 g/L) with mineral salts (■); FE (5 g/L) with mineral salts (▲).

#### 4. Discussion

The main purpose of the current work was the fermentative production of ethanol by the filamentous fungus, *M. indicus*, using a fungal autolysate as a low-cost complex nutrient solution. *M. indicus* is a fungus that has recently been identified as a candidate for industrial production of ethanol [2, 4]. Considering the similarity of chemical components between *M. indicus* and yeasts, it might be assumed that the fungal extract might be a feasible alternative to yeast extract as a nutrient source for fermentation media. Thus, the fungal cells of *M. indicus*, as a by-product of fermentation processes, were then subjected to autolysis to produce nutrient supplements for the following fermentations by similar fungus *M. indicus*. Therefore, the autolysis of the fungal cells biomass produced during fermentation may be considered as a suitable replacement for YE. Thus, the natural enzymatic process of fungal autolysis under oxygen starvation conditions was used in order to disrupt *M. indicus* cells and release various nutrients into the surrounding liquid. On the other hand, this process could be applied as an effective approach to nutrient regeneration/ production due to its simplicity [7]. In this study, autolysate of fungal cells, referred to as FE resulted in high performance in ethanol production. Media containing FE (5 g/L) replacing YE as nutrient source led to high-yield and high-volumetric productivity of ethanol. In addition high-yield of glycerol was obtained in FE concentration (5 g/L) relative to fully supplemented medium. This demonstrates clearly that the FE of *M. indicus* contains sufficient essential nutrients for the ethanol fermentation.

#### 5. Conclusion

The biomass of *M. indicus* can be used as a nutrient source for ethanol production by this fungus. Autolysate of the fungal cells could successfully replace the major nutrients which are necessary for the fermentation.

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