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Research article

Effects of fermentation conditions on valuable products of ethanolic fungus *Mucor indicus*

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ABSTRACT

Background: Mucor indicus is a dimorphic fungus used in the production of ethanol, oil, protein, and glucosamine. It can ferment different pentoses and hexoses; however, the yields of products highly depend on the nutrients and cultivation conditions. In this study, the effects of different morphologic forms, cultivation time and temperature, presence or absence of oxygen, carbon sources, and concentration of nitrogen source on the products of *M. indicus* were investigated.
Results: The fungus with all morphologies produced high yields of ethanol, in the range of 0.32–0.43 g/g, on glucose. However, the fungus with filamentous morphology produced higher amounts of oil, protein, phosphate, and glucosamine together with ethanol, compared with other morphologies. A higher amount of oil (0.145 g/g biomass) was produced at 28°C, while the best temperature for protein and glucosamine production was 32 and 37°C, respectively. Although ethanol was produced at a higher yield (0.44 g/g) under anaerobic conditions compared with aerobic conditions (yield of 0.41 g/g), aerobic cultivation resulted in higher yields of protein (0.51 g/g biomass), glucosamine (0.16 g/g AIM), and phosphate (0.11 g/g AIM).
Conclusions: It is not possible to have the maximum amounts of the products simultaneously. The fermentation conditions and composition of culture media determine the product yields. Carbon source type and the addition of nitrogen source are among the most influencing factors on the product yields. Moreover, all measured products were made with higher yields in cultivation on glucose, except glucosamine, which was produced with higher yields on xylose.

1. Introduction

Mucor indicus, also as known as *Mucor rouxii*, *Amylomyces rouxii, Chlamydomucor rouxii,* and *Mucor rouxianus*, is a Zygomycetes fungus, recognized in 1665 by Robert Hooke [1]. This dimorphic fungus was originally separated from traditional foods, e.g., tempeh and beers, such as channg (or jnard) from millet and barley in India and Nepal. It can grow on a variety of lignocellulosic sugars, including hexoses and pentoses. *M. indicus* is also capable of producing valuable products such as bioethanol, glucosamine, and polyunsaturated fatty acids, especially gamma-linolenic acid (omega 6) [1,2].

In terms of volume, market, and sustainability, ethanol is the most important product of biotechnology and considered the best substitute for fossil fuels. It can be produced with high yield and productivity by *M. indicus* [3,4]. In addition, the fungus cell wall contains a relatively high concentration of chitosan. Chitosan is a biocompatible, antimicrobial, non-toxic natural polymer. This linear polysaccharide is produced through the chemical deacetylation of *N*-acetyl glucosamine in the fungus. This low-cost biodegradable polymer has many applications, especially in the pharmaceutical, food, and agriculture industries [1,2,5,6,7]. Moreover, the main building block of chitosan is a highly valuable chemical, glucosamine. Glucosamine is required for healing skin injuries and osteoarthritis therapy and nowadays is widely used as a dietary supplement and for medical purposes [8,9].

Another important aspect of *M. indicus* is essential lipids production, which has commercial, pharmaceutical, and nutraceutical importance. These fatty acids include linolenic, linoleic, and oleic acids, which cannot be made in the human body and must be provided by food. Moreover, these lipids have a high potential for biodiesel synthesis [10,11,12]. Furthermore, linolenic acid has an important role in the pharmaceutical industry, especially in making drugs for treating illnesses such as atopic eczema, rheumatoid arthritis, fatty liver, kidney diseases, and multiple sclerosis [10,13].

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Bioethanol, glucosamine, and oil production have been the subject of several separate studies in recent years by using different microorganisms. *M. indicus* showed a number of advantages, one of which is production of glucosamine and oil in addition to ethanol [2,14,15,16]. It was shown that ethanol and glucosamine yields by this fungus depend highly on the medium's composition [3,17]. However, limited information is available on oil production by this fungus [18,19]. To our knowledge, no data are available in the literature on the improvement of *M. indicus* cultivation, despite its many valuable products.

This study deals with efficient production of major metabolites (i.e., ethanol and glycerol), glucosamine, protein, and oil by *M. indicus*. Effects of different morphologies of *M. indicus*, cultivation time and temperature, presence or absence of oxygen, carbon sources and nitrogen sources of the products were investigated. Moreover, possible improvement in oil extraction was investigated by changing the number of extraction steps, period of sonication, and solvent volume.

2. Materials and methods

2.1. Organisms and growth conditions

M. indicus CCUG 22424 from the Culture Collection, University of Gotëborg, Sweden, was used as the fermenting microorganism in all experiments. The fungal spores were grown on slants containing (g/L) glucose, 40; agar, 16; and peptone, 10, at 32°C for 5 d. Then, the spore suspension was prepared by adding 10 mL of sterile distilled water to each slant and vigorously shaking with a tube shaker. Next, the fungal spores were germinated and grown using a specific amount of spores in 500-mL Erlenmeyer flasks with 250 mL of a liquid mixture containing (g/L) glucose, 50; $CaCl_2$, 1; $MgSO_4.7H_2O$, 0.75; $(NH_4)_2SO_4$, 7.5; and KH_2PO_4, 3.5. The flasks were incubated in a shaking incubator at 32°C and 130 rpm for 48 h. Adding a large amount of spores ($6\ (\pm 3) \times 10^6$ spores/mL) resulted in growing the fungus as purely yeast-like and mostly yeast-like morphology under anaerobic and aerobic conditions, respectively. Lower inoculum levels ($3\ (\pm 1) \times 10^4$ spores/mL) under aerobic conditions caused filamentous growth.

2.2. Biomass determination and lipid extraction

The fungal biomass was centrifuged (4000 rpm, 10 min), washed three times with distilled water, and freeze-dried (Alpha 1-2 LD., Christ, Osterode am Harz, Germany). Then, 1 g of dried biomass was mixed with 20 mL of hexane-ethanol (1:1) and sonicated in an ice ultrasonic bath (SONICA3200L ETH S3, Soltec, Milano, Italy) for 3 min. The solution was then incubated at 32°C with shaking speed of 130 rpm for 2 h. Afterward, the mixture was filtered (Whatman no. 40), 20 mL of fresh solvent was added, and then it was sonicated again in an ice bath. Next, all filtered solutions were mixed together and evaporated at 65°C by a vacuum evaporator. Finally, the oil's weight was determined gravimetrically [19]. Oil extraction of the samples was performed at the optimum conditions of extraction, a 3-stage process with 14-min sonication time and 80-mL solvent. Obtaining the optimum condition was based on preliminary experiments explained in detail in the **Supplementary material**.

2.3. Analytical method

Ethanol and glycerol concentrations were analyzed by high-performance liquid chromatography (HPLC) equipped with UV and RI detectors (Jasco International Co., Tokyo, Japan) using an ion-exchange column (HPX-87H, Bio-Rad Laboratories, Hercules, USA) at 60°C with 0.6 mL/min eluent (5 mM H_2SO_4).

The protein content of the fungal biomass was determined by the Biuret method [20]. The amounts of glucosamine (GlcN) and *N*-acetyl glucosamine (GlcNAc) were analyzed using colorimetric and HPLC methods, as reported by Zamani et al. [17]. This method was based on a combination of a two-step sulfuric acid hydrolysis and nitrous acid degradation that produces acetic acid and 2,5-anhydromannose. The concentrations of acetic acid and 2,5-anhydromannose were measured by HPLC with an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, USA) at 60°C with 0.6 = mL/min eluent of 5-mM H_2SO_4. The acetic acid was monitored by UV detector (Jasco International CO., Tokyo, Japan), while anhydromannose was measured by the chromatogram provided by RI detector (Jasco International CO., Tokyo, Japan).

The amount of phosphate was also measured by a spectrometric method according to European Standard ISO 6878 [21]. The method was based on measuring the absorbance of the blue complex obtained by mixing the biomass hydrolysate with ammonium molybdate reagent and ascorbic acid at 880 nm.

All experiments in this work were duplicated, and the averages of the two replications are presented. To analyze the data statistically, the analysis of variance (ANOVA) was performed at 5% level of significance ($P < 0.05$) to compare the means of two replications. The statistical analysis results are presented in Supplementary material (**Table S1-S11**).

3. Results

3.1. Effects of morphology

M. indicus was cultured in filamentous, mostly filamentous, mostly yeast-like, and purely yeast-like morphologies. Its morphology was induced by changing the size of the inoculum and the presence of oxygen in the medium. Ethanol, glycerol, and oil extraction yields of various morphologies of this fungus are presented in Fig. 1, while protein, glucosamine, and *N*-acetyl glucosamine contents are shown in Table 1.

M. indicus with filamentous morphology after 48-h cultivation produced 0.32 (g/g glucose consumed) ethanol and 0.032 (g/g glucose consumed) glycerol. In this morphology, the biomass contained 0.51 and 0.14 (g/g biomass) protein and oil, respectively. Furthermore, *N*-acetyl glucosamine, glucosamine and phosphate accounted for 0.21, 0.16, and 0.11 (g/g AIM), respectively.

After 48 h, compared to the filamentous morphology, the amounts of ethanol and glycerol increased in other morphologies, particularly in the purely yeast morphology (**Table S1**). On the other hand, the filamentous morphology contained the highest amounts of protein, *N*-acetyl glucosamine, and glucosamine (**Table S2**). Furthermore, phosphate yield was almost the same in filamentous and mostly filamentous morphologies, while it was higher in yeast-like and mostly yeast-like morphologies (**Table S2**). Moreover, changing the morphology showed significant effects on oil production yield (**Table S1**). The oil yield was considerably lower in the yeast-like and mostly yeast-like morphologies.

As the fungus with filamentous morphology under aerobic conditions produced higher yields of oil, protein, and glucosamine and comparable amounts of ethanol, this morphology was selected for further investigation.

3.2. Effects of cultivation time

The products of *M. indicus* after 24, 48, and 72 h were established (Table 2, Fig. 2). Increasing cultivation time resulted in the production of lower amounts of ethanol and glycerol (**Table S3**). Reduced yields were observed after consuming glucose in the media. Moreover, the amounts of protein, *N*-acetyl glucosamine, glucosamine, and phosphate did not significantly change with fermentation time (**Table S4** and Table 1b) and reached a maximum at 48 h. In addition,

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