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INULINASE PRODUCTION BY *ASPERGILLUS NIGER* MYCELIUM IMMOBILIZED ON POLYURETHANE FOAM IN A BIOREACTOR WITH ALTERNATIVE OXYGENATION

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Abstract- A system for the production of extracellular inulinase with the use of *Aspergillus niger* 20 Osm mycelium immobilized on several polyurethane foam disks in a 2-liter bioreactor was developed. Because the application of the disks reduced the efficiency of the conventional aeration system initially employed in the study and caused difficulties in maintaining sufficient concentrations of dissolved oxygen, an alternative culture oxygenation method based on O_2 generation by decomposition of sequential doses of hydrogen peroxide introduced into a bioreactor medium was used. The application of the alternative oxygenation system during repeated batch culture made it possible to maintain adequate concentrations of dissolved oxygen (50 – 99%) and a proper level of inulinase biosynthesis for a long time (up to 22 days). In the optimized conditions, the immobilized mycelium produced 86 321 units of inulinase activity during a 7-cycle process.
Key words: inulinase, hydrogen peroxide, oxygenation, immobilization, mycelium, polyurethane foam

INTRODUCTION

Immobilization of whole cells has, in the last few decades, become an important tool in biotechnology. The process involves attachment of cells in the distinct solid phase which permits exchange of substrates, products, inhibitors, etc., but at the same time separates the catalytic cell biomass from the liquid phase containing substrates and products. Immobilization offers the possibility of reuse of cells and their simple separation from the fermentation broth, as well as facilitating isolation and purification of reaction products and reducing the costs of bioprocesses [1-3]. Moreover, immobilization often improves the operational and storage stability of the enzymes present in immobilized whole cells and, particularly in the case of plant cells, stimulates the production of secondary metabolites and enhances secretion of intracellular metabolites [4]. There are a wide variety of methods for the immobilization of microbial cells, which use processes such as adsorption, covalent bonding, crosslinking, encapsulation, and entrapment to bind microbial cells into various carriers [4]. Among carrier materials, polyurethane foam is particularly convenient for use in bioprocesses due to its chemical stability and mechanical plasticity. Polyurethane foam has been used for the immobilization of some fungal strains producing glucose oxidase [5], pectinase [6], proteinase [7], and acid phosphatase [8]. Use of immobilized mycelium may help to extend the time of a bioprocess, because most immobilization techniques allow multiple replacement of the medium during a single culture.

Microbial inulinases, a group of enzymes which play a crucial role in the hydrolysis of inulin for commercial applications, have so far been relatively rarely produced using immobilized cells [9-11]. In the majority of reported applications of inulinases, e.g., production of fructose syrups from inulin-containing sources [12-13], partial hydrolysis of inulin to obtain oligosaccharides [14-16], and production of ethanol from inulin [17-18], enzymes obtained from non-immobilized cells have been used.
In this study we developed an efficient system for semi-continuous production of inulinase with the use of mycelium immobilized on polyurethane foam. To overcome the problem of an insufficient gas-liquid oxygen transfer rate encountered in conventionally aerated cultures, an alternative oxygenation system, with sequential dosing of hydrogen peroxide was used.

MATERIALS AND METHODS

Strain and media: *A. niger* 20 Osm was selected in our previous experiments as having high inulinase activity in submerged cultures and as being resistant to some abiotic stresses (among others to oxidative stress) [19]. The strain was maintained on malt agar slants at 4°C. The basal culture medium used for inulinase production contained (g/L): sucrose (15), yeast extract (10), K_2HPO_4 (5), $NaNO_3$ (2), and $MgSO_4 \cdot 7H_2O$ (0.25).

Preparation of spores of *A. niger* 20 Osm for immobilization: After 5 days of growth on malt agar slants at 30°C, the spores of *A. niger* 20 Osm were harvested, washed twice with sterile 0.1 M McIlvaine

buffer, pH 5.0, and filtered through glass wool to remove hyphal fragments. A bioreactor was inoculated with 10 ml of the spore suspension containing about 5 x 10^7 spores/ml.

Immobilization procedure: A bioreactor with fixed polyurethane foam disks (9 cm in diameter, 5 or 8 mm thick, average pore diameter of 0.3 mm; installed on an agitation drive shaft) filled with 1.6 l of 0.1 M McIlvaine buffer, pH 5.0, was sterilized for 30 min. in an autoclave at 0.075 MPa. After cooling the buffer, a dense spore suspension from three agar slants was poured into the bioreactor. The suspension was incubated at 30°C for 24 h, after which the non-adsorbed spores were poured off from the supports (under sterile conditions). The disks were then washed twice with sterile distilled water, and 1.6 l of the basal medium was poured into the bioreactor.

Growth conditions: All fermentations were carried out in a 2-l Biostat B glass fermentor (B. Braun Biotech International GmbH, Melsungen, Germany) initially filled with 1.6 l of the basal medium. Fermentations were performed for 4-22 days at 30°C and 200 rpm. The pH was kept at 6.0 by the addition of 0.1 M NaOH. During the culture, the medium was replaced with fresh sterile medium every 48 or 72 h with the use of an UltraPump II peristaltic pump (Cole-Parmer Instrument Co., Barrington, Illinois, USA). Every time after medium removal fermentor vessel was rinsed twice with sterile water before adding fresh medium.

The dissolved oxygen (DO) concentration of the fermentation broth was measured by an Ingold electrode (Mettler-Toledo Inc., Columbus, Ohio, USA). The values of the readings were expressed as percentage of the initial level of O_2 concentration. The DO level in the alternatively oxygenated culture media was controlled by a device which automatically introduced appropriate amounts of 10% hydrogen peroxide solution into the media (Fig. 1) [20]. As a result of this treatment, the total volume of each medium increased to 1.9-2.3 l at the end of every cycle. The cultures with conventional aeration were aerated at 1 l air/min, and occasionally antifoam A emulsion (Sigma-Aldrich, St. Louis, Missouri, USA) was added to break the foam.

Analytical procedures: For inulinase assay, a reaction mixture containing 0.05 ml of an appropriately diluted culture filtrate and 0.95 ml of 0.5% inulin dissolved in 0.1M acetate buffer (pH 5.0) was incubated at 50°C. After 20 minutes of incubation, the increase in reducing sugars was estimated with the 3,5-dinitrosalicylic acid method [21]. Absorbance was measured at 550 nm. One unit (U) of inulinase activity was defined as the amount of the enzyme which produced 1 µmole of reducing sugars per min under the above conditions. Catalase activity was measured spectrophotometrically by following the decrease in light absorption at 525 nm resulting from the decomposition of H_2O_2 by the enzyme [22]. One unit (U) of catalase activity was defined as the amount of the enzyme catalyzing the decomposition of 1 µmole of hydrogen peroxide per minute at 30°C. The final enzyme activities were calculated by taking into consideration the cyclic changes in the volume of the medium caused by the continuous addition of hydrogen peroxide solution. To determine dry weight, polyurethane foam disks were weighed before mounting on the agitation drive shaft. After culture, whole polyurethane foam disks with immobilized mycelium were dried at 105°C to obtain a constant weight. The biomass dry weight was determined from the difference between the mycelium plus carrier disks and the disks alone.

RESULTS

The experiments were carried out to investigate inulinase biosynthesis using mycelium of *A. niger* 20 Osm immobilized on polyurethane foam in bioreactor cultures with conventional and alternative oxygenation. Mycelium grown from spores inside polyurethane foam disks formed a dense thick layer on the surface of the support. There were no free pellets in the medium. In the first stage of the studies, a culture with conventional aeration was carried out. In these conditions, a rapid growth of the mycelium was observed, which was accompanied by a parallel increase in inulinase activity and a decrease in the DO concentration in the medium (Table 1). The initial DO concentration (100%) dropped to 55% after 24 h of growth and subsequently to zero after 48 h. At that time, the culture medium was replaced with a fresh one. After 72 hours of cultivation, the mycelia growing on the individual disks merged, forming one large cylinder. Fermentation was stopped 24 hours later when the mycelium became unstable and many fragments were released from it. The level of inulinase activity in the medium was increasing till the last day of the process (Table 1).

The rapid decrease in oxygen in the culture medium was probably caused by the very fast increase in mycelium weight correlated with enhanced oxygen consumption. When additional disks were installed on the agitation drive shaft, standard aeration became less effective because the gas bubbles were not dispersed uniformly throughout the entire volume of the medium, which affected the overall transfer of oxygen.

Because this type of aeration did not provide sufficient amounts of oxygen to the growing mycelium, an alternative method for increasing the DO concentration in the culture was used in subsequent experiments. The method involved adding hydrogen peroxide (H_2O_2) to the culture medium, which was then decomposed to oxygen and water by extracellular catalase secreted into the medium by *A. niger*. First, some test cultures were conducted to choose the appropriate arrangement of foam disks on the agitation drive shaft and to optimize the operating parameters of the pump and the concentration of hydrogen peroxide introduced into the media. It was found that the proper configuration of the pump's work cycles and a gradual increment in H_2O_2 concentration were two key factors for successful long-term enzyme production. On the first day of culture, the

growing mycelium was very sensitive to residual hydrogen peroxide in the medium, and so, during that period, the maximal rate of growth and the highest inulinase activity were obtained when the culture was aerated conventionally. Between 24 and 48 hours of the process, the best results were obtained when the bioreactor was both aerated and oxygenated with H_2O_2 (the pump working cycle was: work – 2s; pause – at least 30s). From the third to the last day, only oxygenation without aeration was used (the pump working cycle was: work – 2s; pause – at least 20 s) as the mycelium became resistant to the hydrogen peroxide doses used in this process. A single dose contained 0.127 mM of the oxidant. Because such amounts were decomposed quickly, the successive pulses did not lead to unfavorable accumulation of this compound in the culture media. It was found that 72-hour medium-replacement cycles were more favorable than 48-hour ones. The average daily inulinase production was similar in both cases but the process with 72-hour cycles was easier to conduct and consumed less culture medium (data not shown).

After optimizing the key parameters of the process during test cultures, two long-term processes were conducted. In the first one, the unconventionally oxygenated mycelium immobilized on polyurethane foam produced inulinases efficiently for 22 days (7 cycles). Under alternative oxygenation mycelium disks remained separate throughout the period of the culture (Fig. 2). The extracellular catalase secreted by the mycelium of *A. niger* decomposed the successive doses of hydrogen peroxide continuously, which made it possible to maintain the DO concentration within the range of 50% to 99% (Fig. 3). Inulinases were secreted in considerable amounts up to the last day of the process, and reached the highest values in the medium after the first and the seventh cycle (Fig. 4). This was probably due to the prolonged time of the first cycle (96 hours) and partial lysis of the mycelium during the last cycle resulting in facilitated release of the enzymes to the medium. Total inulinase activity produced by the immobilized mycelium during this culture was 63 457 U. The whole amount of 10% H_2O_2 introduced into the medium was 4730 ml.

In the next experiment, thinner (5 mm) foam disks were used, which made it possible to increase their number from 5 to 6. The alternative oxygenation system was effective in these conditions (Fig. 5). Inulinase production was considerably higher as compared to the previous culture (Fig. 6). Total inulinase activity produced by the immobilized mycelium during this culture was 86 321 U. A total amount of 4845 ml of 10% H_2O_2 was introduced into the medium during the 7 culture cycles. The average daily intake of 10% H_2O_2 was 238.5 ml. Decomposition of that amount of hydrogen peroxide generated 11.22 g of oxygen. This indicates that an average of 7.79 mg of O_2 was introduced every minute into the culture media.

It was observed that growth of *A. niger* mycelium in the alternative oxygenation conditions was possible even on the first and second days of culture if some catalase was added into the culture medium. When 16 000 units of this enzyme were introduced into the bioreactor (to obtain 10 U/ml catalase activity) at the beginning of the process, the mycelial growth rate was comparable to the value obtained for the culture with conventional aeration (Table 2).

DISCUSSION

One of the most important objectives of designing a bioprocess with immobilized mycelium is to provide sufficient amounts of oxygen to the culture. Oxygen solubility in media used for cultivation of microorganisms is very low, usually between 5 and 7 mg/L, and such amounts of this gas can be used up by bacteria or fungi within less than one minute. As a result of the rapid consumption of oxygen, cellular respiration and protein synthesis is partially or completely inhibited [23]. Additionally, fermentation broths are often viscous, causing reduction in the oxygen diffusion rate. Typically, oxygen concentration in bioreactor cultures is regulated by controlling the rate of aeration or the speed of agitation, but these methods have some limitations due to extended foaming, high energy consumption, cell damage, etc. In our experiments with immobilized mycelium, conventional aeration was not effective and so an alternative method of medium oxygenation had to be designed.

Owing to the use of the alternative oxygenation system during repeated batch culture (RBC), the time of efficient inulinase production by *A. niger* was extended to 22 days as compared to the 4-day culture under conventional aeration. The choice of RBC, in which the volume of the medium returns to its initial value at the beginning of each cycle, prevented overloading of the bioreactor caused by the continuous dosing of H_2O_2 solution.

It is well known that hydrogen peroxide, as a reactive oxygen species, is responsible for the induction of oxidative stress conditions in a culture [24-26]. H_2O_2 is toxic to microbial cells because of its ability to oxidize lipid membranes, proteins, and DNA [27]. Our experiments showed that the mycelium of *A. niger* is very sensitive to hydrogen peroxide during the first day of growth, when the fungus is in the exponential phase of growth and catalase production is low. This is in agreement with some results obtained for filamentous fungi and yeast. Relatively low concentrations of H_2O_2 (30 mM) in the medium have caused a significant slowdown in the growth and accumulation of oxidative-modified proteins in the cells of several fungal strains (e.g., from the genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Cladosporium*) in the exponential growth phase [24]. Also exponentially growing yeast cells (*Saccharomyces cerevisiae* and *Yarrowia lipolytica*) have been more sensitive to hydrogen peroxide than stationary phase cells [27-29].

As hydrogen peroxide is able to oxidize proteins, it could also affect extracellular inulinase activity. However, no such effect was observed in the experiments described here, in which the concentration of H_2O_2 in the medium was kept at a very low level (maximally 80 μM/L). Moreover, it was verified that 24-hour incubation of *A.*

niger 20 Osm inulinase at an even 100-fold higher concentration (8 mM of H_2O_2) did not decrease the activity of this enzyme (data not shown).

So far, immobilization of mycelium in polyurethane foam using the alternative oxygenation method based on catalase-mediated decomposition of H_2O_2 has only been used for the production of inulinases. However, several papers concerning alternative oxygenation of free mycelium cultures [25,30,31], mycelium immobilized in pumice stones [32], and non-immobilized bacterial cells [33,34] have been published. Oxygen supply based on the addition of H_2O_2 has been used for the production of xanthan gum by *Xanthomonas campestris*. The application of this method made it possible to maintain DO levels above the setpoint of 50% throughout the cultivation (72 hours), while under conventional aeration the DO level dropped to less than 20% in about 6 hours [33]. Similar results were obtained in our experiments with *A. niger*, in which the DO levels in the medium during culture with alternative oxygenation were maintained within the range of 52–99%, whereas after 48 hours of conventionally aerated culture, they dropped to 0%. It was found that the unconventional oxygenation slows down mycelium growth. A test culture was performed to establish the dry mass of the mycelium after 4 days of growth under conditions identical to those used during the first 96 hours of the long-term 7-cycle process. The dry mass of the mycelium was only 9.85 g, which was about 2.6-fold less than the dry mass of the mycelium obtained after 4 days of conventionally aerated culture. The slowdown in the growth of the immobilized mycelium during culture with unconventional oxygenation should be considered as an advantage. Under these conditions, the individual mycelium disks remained separate, improving in this way oxygen and substrate diffusion to the fungal cells and enhancing enzyme secretion. *Xanthomonas campestris* has also exhibited a slowdown in its growth when cultivated under alternative oxygenation conditions, but in this case, the negative effect of hydrogen peroxide was not so significant - the maximum specific growth rate was 89% of the value obtained in a conventional cultivation system [33].

The mycelium of *Aspergillus niger* 20 Osm was very sensitive to the presence of hydrogen peroxide in the medium on the first day of culture, making it necessary to use conventional aeration for the first 24 hours. This was probably caused by the insufficient level of catalase in the medium. Similar observations have been made by Rosenberg *et al*., [30] with reference to an *Aspergillus niger* mutant used for the production of gluconic acid. Because hydrogen peroxide inhibited the growth of the mutant, during the first 12 hours of the experiment oxygen was supplied by classical aeration, and only afterwards was the phase of unconventionally oxygenated production started, which was continued up to 41 hours. Those authors did not detect extracellular catalase in the culture medium. By contrast, *Aspergillus niger* 20 Osm produced this form of the enzyme, which was very beneficial in the case when a very reactive oxidant such as hydrogen peroxide was added to the medium.

It has been observed that the addition of H_2O_2 to an *A. niger* culture may induce the key defensive enzymes, intracellular [24,26] and extracellular [25] catalases. We did not investigate in any detail whether the production of extracellular catalase by *A. niger* 20 Osm was inducible by hydrogen peroxide, but the results presented in Table 1 and Figures 4 and 6 show clearly that the activities of this enzyme assayed after 48 hours of culture in an unconventionally oxygenated medium were considerably higher than the activities obtained during conventionally aerated culture (about 1.6-fold for the first and 1.2-fold for the second alternatively oxygenated culture).

Up to now, the unconventional oxygenation method has only been used for the production of a few substances such as gluconic acid [30,32], xanthan [33,34], perillyl alcohol, and perillyl aldehyde [31]. The use of this method with the immobilized mycelium of *A. niger* enabled prolonged semi-continuous production of inulinases. There are only a few publications reporting production of inulinases by immobilized microorganisms. *Kluyveromyces marxianus* cells with inulinase activity have been immobilized in open pore gelatin pellets [9]. Mycelium of *A. niger* immobilized on pumice stones produced high amounts of inulinases (up to 15.1 U/ml) during six-day repeated-batch culture [10]. Cells of *Bacillus* sp. 11 immobilized on formaldehyde-activated polysulfone membranes produced thermostable exo-inulinase during 10 days of semicontinuous cultivation, and at the end of this period the residual enzyme activities of the biocatalysts were 60-90% of their initial values [11]. The method described in this paper made it possible to extend the time of inulinase production up to 22 days (and potentially longer), thus providing an interesting alternative to other immobilization and oxygenation techniques used for bioreactor cultures.

The alternative oxygenation system utilizing hydrogen peroxide for oxygen generation turns out to be an efficient solution for bioreactor culture of immobilized mycelium. This type of oxygen delivery system has many advantages, including higher DO concentrations, reduced power consumption, low risk of microbial contamination, and the ability to work at low agitation rates, which reduces shear forces and foam formation [35]. The process described in this paper permits production of more than one enzyme in one step, which significantly increases the efficiency and improves the economy of the process. It is worth noting that DO concentration in the culture medium can be efficiently increased by the addition of H_2O_2 in cultures of strains such as *A. niger* 20 Osm, which show resistance to abiotic stresses (especially oxidative stress) [19]. In contrast to conventional aeration, the DO levels obtained under alternative oxygenation of the culture were relatively stable and sufficient throughout the entire 22-day process. In this work it was confirmed that the proposed method can be applied for cultures of immobilized mycelium of *A. niger* and could probably be

used in a wider range of bioprocesses using microorganisms with catalase activity.

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Table 1- *Enzyme production and DO concentration during conventionally aerated Aspergillus niger 20 Osm culture*

Cultivation time [h]	Inulinase activity [U/ml]	Catalase activity [U/ml]	DO concentration [%]
Cycle 1			
24	2.31	0.65	55
48	3.91	0.79	0
Cycle 2			
72	5.93	0.64	0
96	7.71	0.72	0

Table 2- The effect of the type of oxygen delivery system on mycelium dry weight after 48 hours of culture

Type of aeration/oxygenation	Mycelial dry weight after 48 hours [g]
Alternative oxygenation without external catalase	0,52
Alternative oxygenation with external catalase	4,11
Classical aeration	4,62

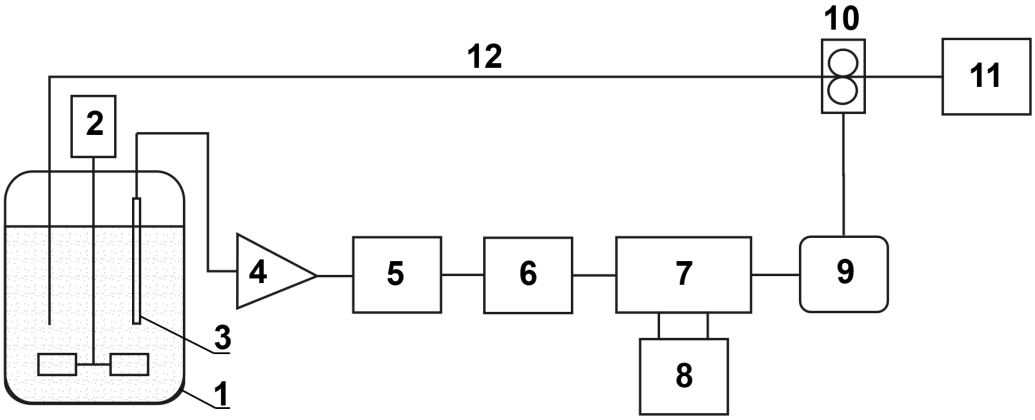

Fig. 1- Scheme of alternative oxygenation system
1 – fermentor vessel, 2 – agitation system, 3 – O_2 electrode, 4 – amplifier, 5 – analog-to-digital converter, 6 – filter, 7 – control system, 8 – programmable O_2 concentration regulator
9 – pump power supply, 10 – peristaltic pump, 11 – H_2O_2 container, 12 – H_2O_2 supply tube

Fig. 2- Mycelium of *Aspergillus niger* 20 Osm immobilized on polyurethane foam disks installed on the agitation drive shaft of a Biostat B bioreactor.

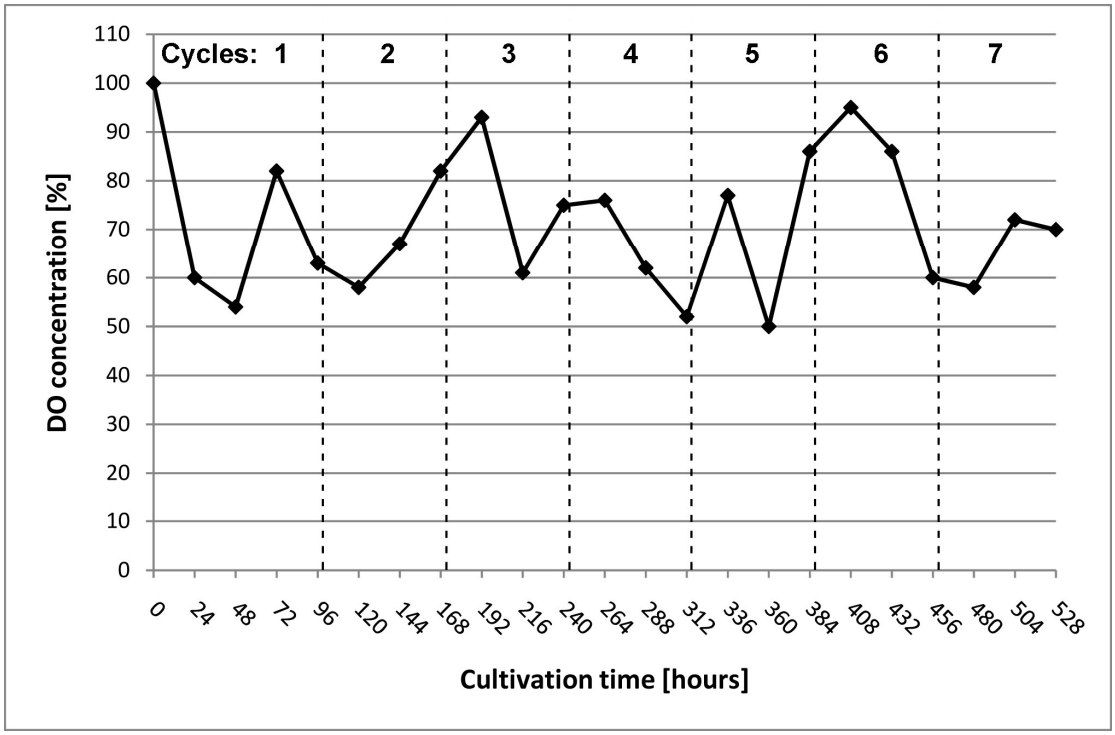

Fig. 3-DO concentration during alternatively oxygenated *Aspergillus niger* 20 Osm culture on 5 polyurethane foam disks

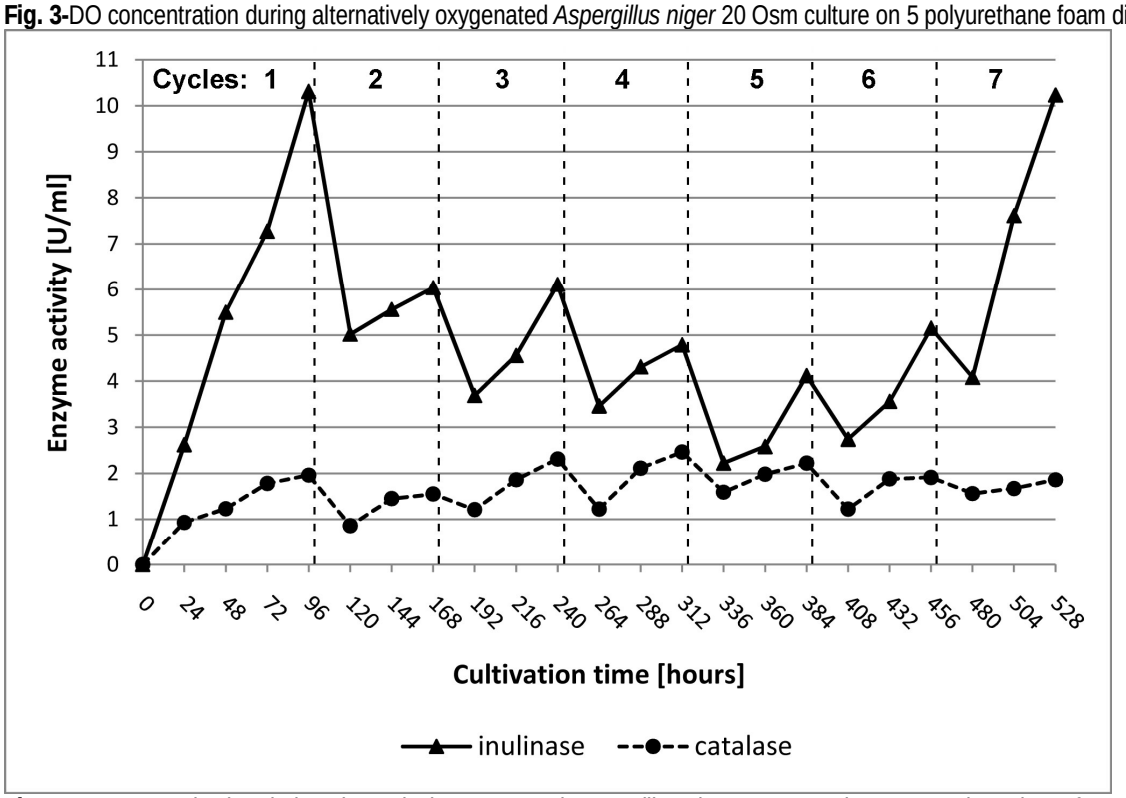

Fig. 4-Enzyme production during alternatively oxygenated *Aspergillus niger* 20 Osm culture on 5 polyurethane foam disks

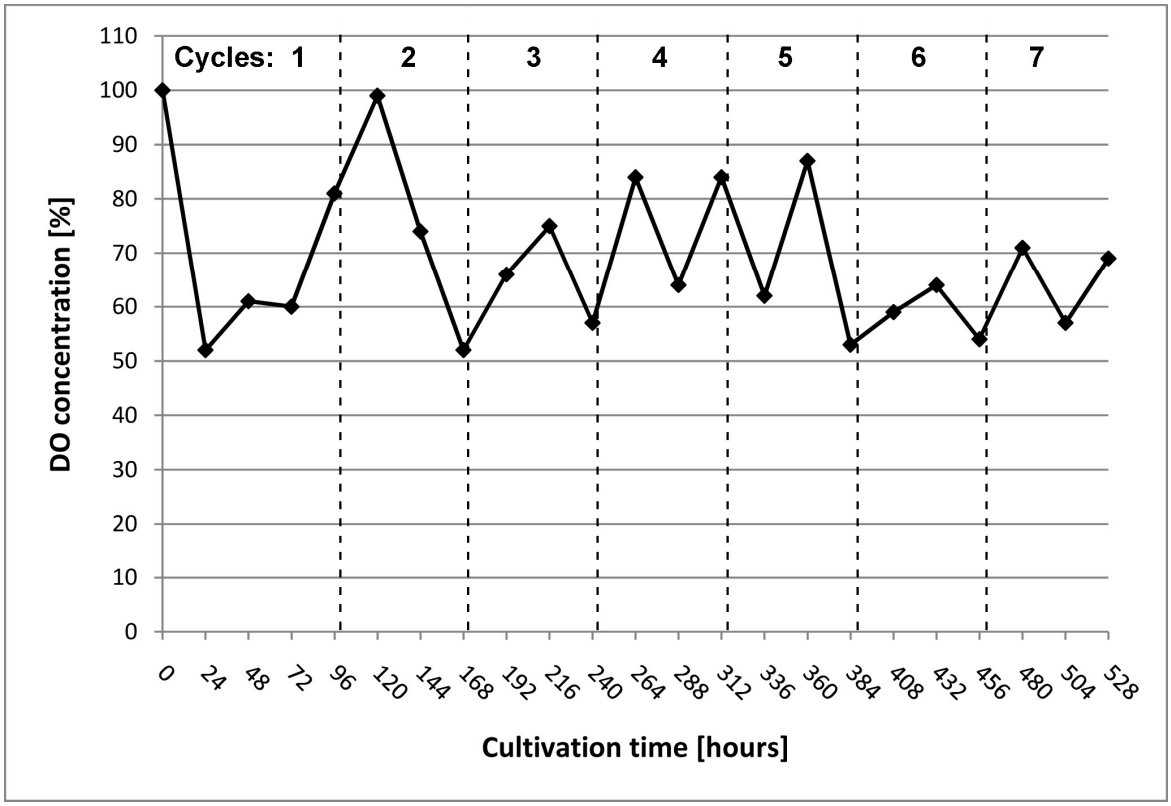

Fig. 5-DO concentration during alternatively oxygenated *Aspergillus niger* 20 Osm culture on 6 polyurethane foam disks

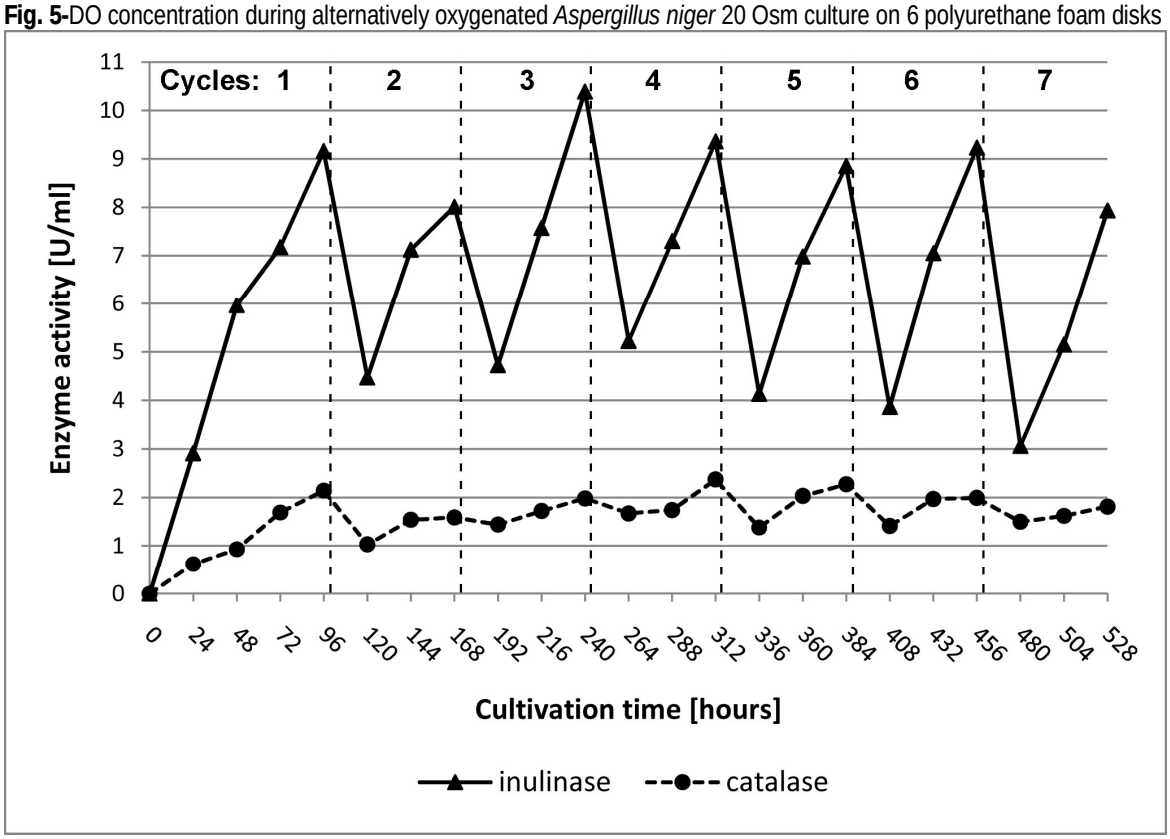

Fig. 6-Enzyme production during alternatively oxygenated *Aspergillus niger* 20 Osm culture on 6 polyurethane foam disks