# Optimization of growth conditions and continuous production of inulinase using immobilized *Aspergillus niger* cells

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**Aim**
The aim of the study was the optimization of growth conditions for the production of inulinase as well as the continuous production of the enzyme in an airlift bioreactor using *Aspergillus niger* cells.

**Methods**
First, inulinase production by *A. niger* cells, using different carbon and nitrogen sources, was studied on a shake flask level. Second, the cells were adsorbed onto different carriers, and their production over several successive batches was tested. Finally, the economically-favorable continuous production of inulinase by *A. niger* cells immobilized onto linen fibers was carried out in an airlift bioreactor using crude inulin juice as the fermentation medium.

**Results**
Although all tested substances resulted in the biosynthesis of certain amounts of inulinase enzyme, the highest titer of 163.5 U/ml was obtained when the producing cells were incubated for 96 h at 27°C and 180 rpm in a fermentation medium containing both inulin and peptone as sole carbon and nitrogen sources, respectively. Moreover, when the cells of the tested microorganism were adsorbed onto different carriers, especially linen fibers, their productivity was also successfully maintained, to different extents, for five successive batches. However, as commercially pure inulin is very expensive and available in only small quantities, the fermentation medium was later substituted by a crude inulin extract obtained by mechanical crushing and filtration of Jerusalem artichoke tubers. The crude inulin juice was able to sustain inulinase production during the second batch cultivation of *A. niger* cells that were immobilized by their adsorption onto linen fibers to a satisfactory level of about 122 U/ml. Furthermore, the use of the previously mentioned crude inulin preparation was also compared with the use of either complete or minimal media, composed solely of 1% pure inulin, for the continuous production of inulinase enzyme by *A. niger* cells that were immobilized in their maximum production phase and packed inside an external loop airlift bioreactor. The results of this experiment were very encouraging as, using this technique, an inulinase production of about 838 U/ml over an incubation period of 48 h was obtained compared with a production of about 996 and 1013 U/ml, which resulted from the use of either minimized or complete media, respectively, for the same incubation period.

**Conclusion**
The method adopted in this study for inulinase production is simple, economic, time saving, and nontoxic to the microorganism. Moreover, the loaded linen fiber pads are reusable.

**Keywords:**
airlift bioreactor, *Aspergillus niger*, inulin, inulinase

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## Introduction

Inulin is a widespread polyfructan, naturally occurring in more than 30 000 edible plant species [1]. It consists of linear chains of β(2,1)-linked fructose residues attached to a terminal sucrose molecule [2]. Apart from its role as a food component, inulin has also received great importance as a raw material for the production of fructose syrup [3,4] and inulooligosaccharides [5]. Fructose is a safe alternative to sucrose, which is known to be the cause of many health problems including corpulence, carcinogenicity, atherosclerosis, and diabetes [2]. In addition, fructose also increases the absorption of iron, as it forms an iron–fructose complex whose absorption was found to be much better than that of inorganic

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iron [6]. Fructose can be produced from inulin either enzymatically or chemically through acid hydrolysis. The latter method is not recommended because of the undesirable coloring of inulin hydrolysate and the formation of difructose anhydride, which has practically no sweetening properties [2]. Moreover, the enzymatic production of organic products, especially those used in food and pharmaceutical industries, has many advantages over chemical processes: The productivity is generally higher, because of the high specificity of the enzymes for their substrates; the production cost is relatively lower; and most importantly it creates less pollution. Therefore, many efforts have been made to replace chemical processes with enzymatic ones [7]. Unfortunately, the conventional enzymatic production of fructose from inulin involves many steps and results in only a 45% fructose yield. In contrast, the almost complete hydrolysis of inulin (90–95%) into fructose can be performed in a single step using inulinase enzyme [β(2,1)-fructan fructanohydrolase] [6,8]. Inulinase is produced by many microorganisms, including filamentous fungi, yeasts, and bacteria. The fermentation of inulinase by these microorganisms can be greatly improved by modifying some parameters, including the physiochemical and nutritional conditions of growth required by the producing cells. In this study, Jerusalem artichoke (*Helianthus tuberosus*) was used as a cheap source of inulin, as about 80% w/w (dry weight basis) of the tuber acts as a store for carbohydrates [7]. Moreover, in comparison with conventional fermentations, immobilization of living cells provides several important advantages such as a faster production rate, easier purification of products, and a higher productivity over a certain period of time [9]. One of the most reliable, safe, and easy methods of immobilization is the adsorption of the producing cells onto an inert, suitable support [10–12]. Therefore, the present study was carried out to examine the inulinase productivity of *Aspergillus niger* cells under different cultivation conditions and to study the effect of adsorption immobilization of these cells onto different carriers on the productivity. Finally, the continuous production of inulinase by the producing cells that were immobilized onto linen fibers and packed inside an external loop airlift bioreactor was also investigated over a number of successive batches, using either complete or minimal media as well as crude inulin juice.

## Materials and methods

### Microorganism

The production of inulinase was carried out using a locally isolated strain of *A. niger*. The microorganism was maintained on Czapek's Dox (CD) agar medium [13] at 30°C for 7 days and then stored in the refrigerator until use.

### Authentic enzyme and chemicals

Inulinase (EC.3.2.1.7) was supplied by NOVO Industry (A/S, Seoul, Korea). Pure inulin and the remaining chemicals used were obtained from Sigma (St. Louis, Missouri, USA). All solvents (analytical grade) were obtained from Merck (Darmstadt, Germany).

### Supports tested for cell immobilization

The immobilization of *Aspergillus* cells, and eventually their inulinase productivity, was tested by the adsorption method using three different carriers: glass wool (Pyrex fiber glass, Sliver 8 μm; Corning Glass Work, Corning, New York, USA), linen, and synthetic fibers (the latter two were locally provided).

### Preparation of crude inulin solution

Twenty grams of Jerusalem artichoke tubers (*H. tuberosus*), collected locally, were washed, sliced, and grinded using a blender along with 100 ml of distilled water, then filtered through a fine gauze. The pH of the solution was adjusted to 6.2 by addition of concentrated sodium hydroxide. The resulting juice was sterilized at 121°C and 1.5 atmospheric pressure for 15 min [14]. The raw inulin extract was analyzed, and its inulin content concentration was estimated, according to the method described by Ashwell [15], to be ~1.5% (w/v).

### Recovery and activity assay of inulinase

Inulinase activity was assayed by measuring the amount of reducing sugars released from inulin [16]. The fermentation broth was centrifuged at 3000*g* and 4°C for 5 min. The obtained supernatant was used as the crude enzyme. A reaction mixture of 0.1 ml of the enzyme sample and 0.9 ml of acetate buffer (0.1 mol/l, pH 5.0) containing 2% inulin was incubated at 50°C in a water bath for 15 min. The mixture was then kept at 100°C for 10 min to inactivate the enzyme. The same mixture to which the same amount of inactivated crude enzyme (heated at 100°C for 10 min) was added before the reaction was used as a control. The reaction mixture was assayed for reducing sugars according to the method of Nelson–Somogyi cited by Spiro [17]. The calibrating curve was drawn with fructose (10–100 mg). One unit of inulinase was defined as the amount of enzyme that released one micromole of fructose from inulin per min under assay conditions.

### Media and cultivation conditions

#### *Shake flask fermentation of free cells*

Unless otherwise mentioned, inulinase production was carried out in 250 ml Erlenmeyer flasks, each containing 50 ml of basal CD medium [13] comprising (g/l): inulin, 10; $NaNO_3$, 3; $K_2HPO_4$, 1; $MgSO_4 0.7H_2O$, 0.5; KCl, 0.5; and $Fe_2SO_4 0.7H_2O$, 0.01 (pH 6.5). The flasks were then sterilized, inoculated with about $2 \times 10^9$ spores/ml of the producing microorganism, and incubated for 96 h at 120 rpm and 30°C. The effect of various carbon sources, such as fructose, glucose, maltose, starch, and lactose, was investigated. Each carbon source was added to the basal medium (without inulin) at a concentration of 10 g/l either individually or in combination with inulin, which was then supplemented at a concentration of either 1 or 5 g/l. Similarly, various organic and inorganic nitrogen sources were individually added to the basal medium as a substitute for $NaNO_3$ in order to study their effect on

inulinase production. The tested organic nitrogen sources (peptone, urea, and yeast, beef, and meat extracts) were added at a concentration of 50 g/l. The inorganic nitrogen sources under study ($NH_4SO_4$ and $NH_4Cl$) were added according to their nitrogen content such that it was equivalent to that of $NaNO_3$, which was omitted from the medium.

*Shake flask fermentation of immobilized cells*

The immobilization of *A. niger* was studied using the adsorption method [11,12]. A total of 1.4 g of each tested support (glass wool and synthetic and linen fibers) was added to a 250 ml flask containing 50 ml of the optimized medium composed of (g/l): inulin, 10; peptone, 50; $K_2HPO_4$, 1; $MgSO_40.7H_2O$, 0.5; KCl, 0.5; and $Fe_2SO_40.7H_2O$, 0.01 (pH 6.5). The flasks were then sterilized, inoculated with about $2 \times 10^9$ spores/ml of the producing microorganism, and incubated for 96 h at 120 rpm and 30°C. To assess the productivity of the immobilized cells for another batch, the loaded pads were washed thoroughly with normal saline, carefully squeezed under sterile conditions, and used to inoculate 50 ml of a fresh sterile medium, which was then reincubated under the former conditions but for a shorter incubation period of 72 h.

An experiment was carried out as an attempt to reduce the quantity of the constituents of the fermentation medium used during repeated batch cultivation of the cells previously adsorbed onto linen fibers in their maximum inulinase production phase. This was achieved to decrease the growth of the escaped cells and to produce inulinase using the cheapest possible medium. Therefore, as described previously, *A. niger* cells were inoculated in 50 ml of sterile medium along with 1.4 g of linen fibers in each flask. After 96 h of incubation at 30°C and 120 rpm, the linen fiber pads saturated with the cells in their maximum production phase were washed thoroughly with normal saline solution and carefully squeezed using sterilized forceps. These pads were then transferred to new flasks containing different ratios of the constituents of the main medium as shown in Table 1. Crude inulin, obtained by mechanical crushing and filtration of Jerusalem artichoke tubers, as previously explained, was also tested. These flasks were then reincubated at 30°C and 120 rpm for another 72 h. At the end of this incubation period, inulinase production and the cell dry weight of unadsorbed cells in each flask were estimated.

*Airlift bioreactor fermentation of immobilized cells*

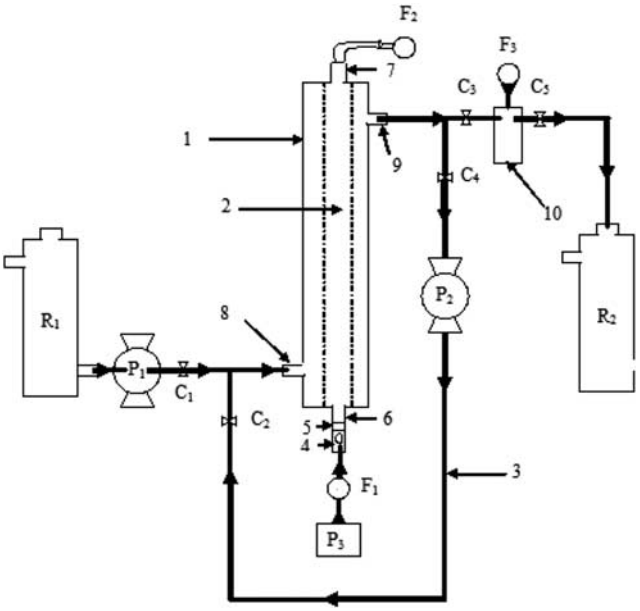
The production of inulinase enzyme by *A. niger* cells immobilized onto linen fibers was investigated in an external loop airlift bioreactor [12], using either complete or minimized fermentation media as well as raw inulin juice. A schematic diagram of the designed apparatus is illustrated in Fig. 1. The bioreactor consists mainly of a riser column with a height of 40 cm and a downcomer tube with a diameter of 1 cm. The riser column is composed of an inner perforated column with an internal diameter of 3 cm jacketed by an outer column that has an internal diameter of 4 cm. The inner column was designed to hold up the linen fiber pads, on which the producing cells were previously immobilized, and to prevent their fluidization. The perforation of the column allowed the fermentation broth to come in contact with the immobilized cells in many parts and also helped achieve a good oxygen transfer to the packed fibers.

**Table 1 Optimization of the fermentation medium used in the second batch production of inulinase by *Aspergillus niger* cells immobilized onto linen fibers**

| | Inulin (g/l) | Peptone (g/l) | Salt content (%) |
|---|---|---|---|
| Control medium number | | | |
| 1 | 10 | 50 | 100 |
| 2 | 10 | 50 | 100 |
| Second batch medium number | | | |
| 1 | 10 | 50 | 100 |
| 2 | 10 | 25 | 100 |
| 3 | 10 | 5 | 100 |
| 4 | 10 | 1 | 100 |
| 5 | 10 | 0 | 100 |
| 6 | 10 | 50 | 50 |
| 7 | 10 | 50 | 25 |
| 8 | 10 | 50 | 0 |
| 9 | 8 | 50 | 100 |
| 10 | 5 | 50 | 100 |
| 11 | 2 | 50 | 100 |
| 12 | 7.5 | 37.5 | 75 |
| 13 | 5 | 25 | 50 |
| 14 | 2.5 | 12.5 | 25 |
| 15 | 10 | 0 | 0 |
| 16 | 10 | 0 | 0 |

Controls 1 and 2, inulinase production by free cells and by the first batch of cultivated immobilized cells, respectively.

**Figure 1**




Schematic diagram of the airlift bioreactor and its accessories used for the production of inulinase by immobilized *Aspergillus niger* cells. 1, riser column; 2, inner perforated column; 3, downcomer tube; 4, one-way valve; 5, air sparger; 6, air inlet; 7, air outlet; 8, medium inlet; 9, medium outlet; $C_1$–$C_5$, clamps; $F_1$, $F_2$, $F_3$, air filters; $P_1$, $P_2$, peristaltic pump; $P_3$, air pump; $R_1$, medium feeding reservoir; $R_2$, product collection reservoir.

The whole system was mounted inside an incubator adjusted at 30°C.

The inoculum was in the form of eight firmly squeezed linen pads supporting *Aspergillus* cells that were previously immobilized by their cultivation in the optimized medium for 96 h at 120 rpm and 30°C. The linen pads loaded with the immobilized cells were packed, under aseptic conditions, inside the inner column of the bioreactor. A working volume of 360 ml of each tested medium was fed one at a time. The aeration rate of the bioreactor was adjusted at 0.5 v/v/m. The fermentation medium once introduced by a peristaltic pump P1 into the riser column was left without circulation for 30 h in contact with the immobilized cells. This phase was performed to reinitiate the inulinase production of the immobilized cells. Thereafter, peristaltic pump P2 was adjusted such that the medium could circulate at a rate of 30 ml/h. During the experiment, 20 ml aliquots of the culture were systematically withdrawn with a syringe through an inline air filter. These samples were assayed to monitor inulinase production and cell escapement.

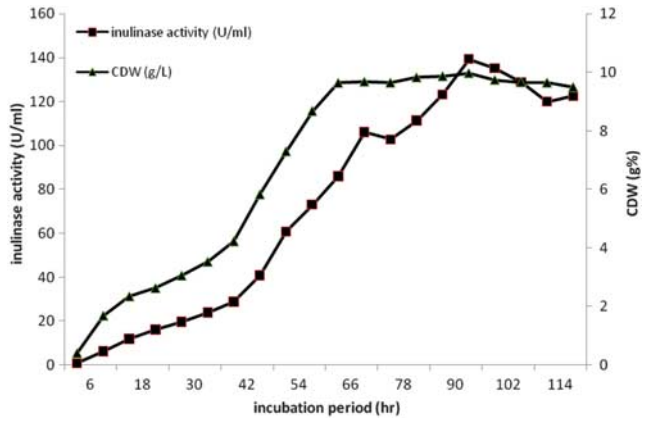
## Results and discussion

### Optimization of growth and inulinase production parameters of free cells on shake flask level

*Effect of different incubation periods*

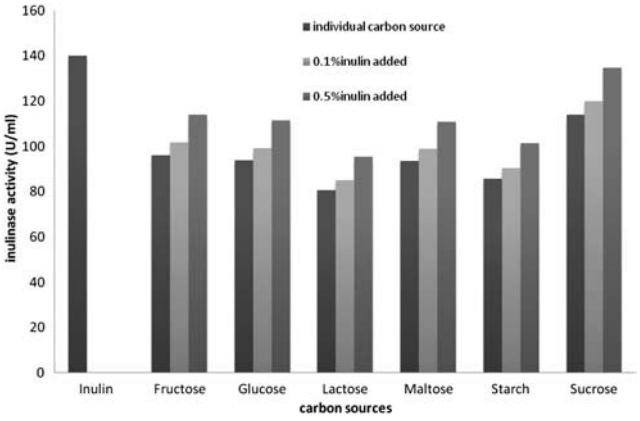
The production of inulinase by *A. niger* cells on inulin basal CD fermentation medium was monitored over a period of 120 h under the previously mentioned shaking cultivation conditions of 120 rpm and 30°C. The results illustrated graphically in Fig. 2 show that the activity of inulinase was 0.88 U/ml in the fermentation medium after 6 h of incubation. This recorded enzyme activity was found to increase linearly with time by a production rate (Qp) of 1.6 U/ml/h and reached a maximum volumetric production of 139.349 U/ml after about 96 h of incubation. After this incubation period, a gradual decrease in inulinase activity was observed. The reported production decrease rate (−Qp) was about 0.69 U/ml/h. Cell growth was also studied during the course of fermentation and was found to increase gradually with time by a specific growth rate (μ) of about 0.35 g/l/h. A maximum cell dry weight ($X_{max}$) of about 9.98 g% was recorded after 96 h of incubation. Thereafter, a slight cell lysis was observed with a specific degradation rate (−μ) of about 0.02 g/l/h, resulting in a cell dry weight of 9.51 g/l after 120 h of incubation. This result showed that inulinase production was growth-dependent and that the maximum inulinase productivity of the producing organism was just before the onset of its stationary phase of growth. Moreover, a maximum yield coefficient (units of inulinase per gram of cell mass formed) of 1396.3 U/g cells was recorded after 96 h of incubation.

**Figure 2**




Effect of different incubation periods on the growth of and inulinase production by free *Aspergillus niger* cells cultivated in basal Czapek's Dox medium.

**Figure 3**

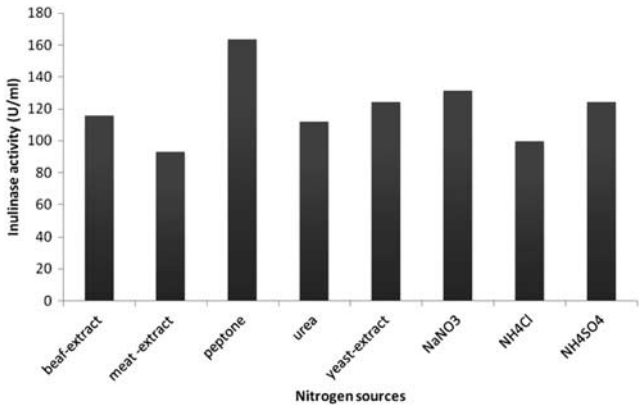



Effect of different carbon sources, added either individually or in addition to 0.1 or 0.5% inulin, on inulinase production by free *Aspergillus niger* cells.

*Effect of different carbon sources*

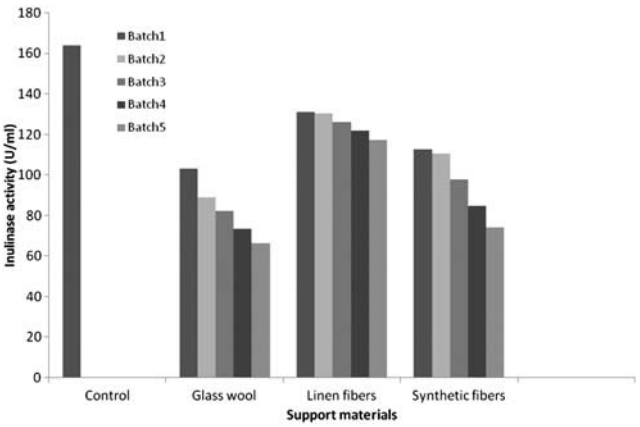
Different carbon sources were tested for their ability to sustain substantial amounts of inulinase enzyme production (Fig. 3). Among them, inulin resulted in a maximum enzyme production of about 140 U/ml, followed by sucrose, which resulted in ~114 U/ml of the enzyme. Lower enzyme titers ranging between 96 and 80 U/ml were recorded upon using other carbon sources including (in descending order of enzyme activity recorded): fructose, glucose, maltose, starch, and finally lactose. However, because the use of inulin as a sole carbon source in the fermentation medium was inconvenient owing to its high cost, it was therefore added to the medium containing each individual carbon source, in small percentages of 0.1 and 0.5%, as an attempt to initiate higher inulinase production. This goal was achieved as the addition of inulin in these percentages resulted in significant increases in enzyme production (results ranging from 5.6 to 12%). However, none of these enzyme titers could exceed the level obtained when inulin was added as a sole carbon source in the fermentation medium.

**Figure 4**




Effect of different organic and inorganic nitrogen sources on inulinase production by free *Aspergillus niger* cells.

**Figure 5**




Inulinase production during repeated batch cultivation of *Aspergillus niger* cells immobilized onto different support materials. Control, inulinase production by free cells.

*Effect of different nitrogen sources*

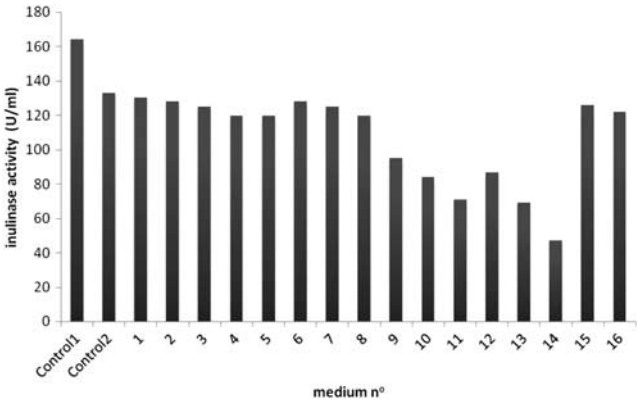
Different nitrogen sources, either organic or inorganic, were also tested for inulinase productivity. The results in Fig. 4 show that a maximum production of about 163.5 U/ml could be achieved when peptone was used as a sole nitrogen source in the fermentation medium. Much lower yields ranging between 124 and 93 U/ml were recorded upon using other organic nitrogen sources including (in descending order of enzyme productivity): yeast and beef extracts, urea, and then finally meat extract. In contrast, the use of inorganic nitrogen sources such as $NaNO_3$, $NH_4SO_4$, and $NH_4Cl$ resulted in enzyme titers of approximately 131, 125, and 99.5 U/ml, respectively.

## Optimization of growth and inulinase production parameters of immobilized cells on shake flask level

*Effect of immobilizing Aspergillus niger cells on different carriers*

*A. niger* cells were tested for their ability to produce inulinase while immobilized by adsorption onto different carriers including glass wool and synthetic and linen fibers. The enzyme production results were compared with those obtained when free cells of the fungus were cultivated on the same optimized culture medium under similar cultivation conditions. The results in Fig. 5 indicate that the cells immobilized onto linen fibers were only slightly affected by the immobilization process, as they were able to produce a satisfactory enzyme concentration of about 131 U/ml compared with 164 U/ml produced by cultivation of free cells for the same incubation period of 96 h. Successive batch cultivation of the immobilized cells was performed to test their inulinase productivity. This result was very promising because this previously mentioned inulinase titer was, more or less, attained after a much shorter incubation time of 72 h, as the cells were inoculated in their maximum production phase. The obtained yield of inulinase could also be more or less sustained within appropriate ranges for five consecutive batches, resulting in a total enzyme yield of 630 U/ml within a combined serial incubation period of 384 h. In contrast, the first batch cultivation of the *A. niger* cells adsorbed onto either synthetic fibers or glass wool resulted in lower inulinase yields of 112 and 103 U/ml, respectively. It was observed that these titers were maintained, with only a slight decrease, during the experiment.

**Figure 6**




Optimization of the fermentation medium used in the second batch production of inulinase by *Aspergillus niger* cells immobilized onto linen fibers. Controls 1 and 2, inulinase production by free cells and by the first batch of cultivated immobilized cells, respectively.

*Optimization of the fermentation medium used for the second batch production of inulinase by Aspergillus niger cells immobilized onto linen fibers*

The results illustrated in Fig. 6 show that a maximum inulinase production of about 162 U/ml was obtained when free cells were cultivated for 96 h (control 1). Moreover, the first batch cultivation of immobilized cells (control 2) resulted in a satisfactory inulinase production of 134.5 U/ml for the same incubation period. However, the results showed no significant differences between the inulinase titers estimated in the fermentation broths of media no. 1 to 8 (used in the second batch cultivation of 72 h), which ranged between 130.28 and 120.44 U/ml.

This means that the inulinase productivity of the cells was more or less maintained in the second batch even when the peptone or salt content of the medium was reduced or even eliminated. However, it was found that the inulin content of the medium was critical for both the growth of the producing organism and its productivity, as its reduction, keeping the percentage of the other constituents constant, affected the inulinase titer and cell growth greatly (media no. 9 to 11). The critical effect of inulin on inulinase production was also revealed when different percentages ranging between 75 and 25% were used in the media (media no. 12 to 14), as the production of inulinase decreased to 87.16 and 47.21 U/ml, respectively. However, medium 15, composed of only pure inulin (10 g/l), and medium 16, composed of crude inulin solution (15 g/l), resulted in satisfactory inulinase levels of 126 and 122 U/ml, respectively. Relying on these results, the complete medium could be substituted by either medium 15 (minimal medium) or medium 16 (raw inulin extract) for the production of inulinase during the repeated batch cultivation of *A. niger* cells immobilized onto linen fibers.

### Optimization of fermentation medium used for the continuous production of inulinase by immobilized cells in an airlift bioreactor

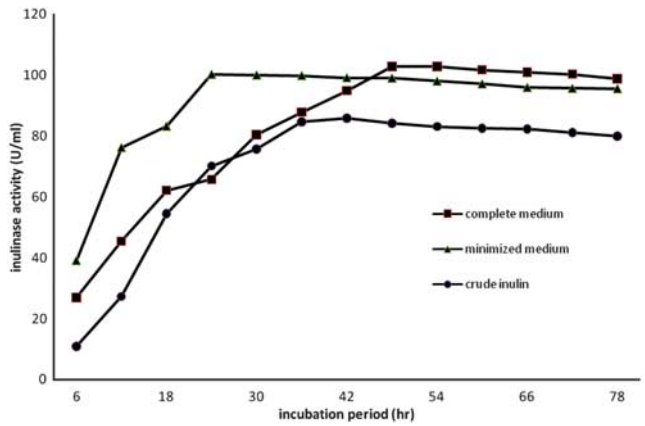
*Inulinase production using complete medium*

The results illustrated in Fig. 7 show that inulinase production increased gradually at the rate of 1.56 U/ml/hr and reached a volumetric production of 102.6 U/ml after only 48 h of incubation. This maximum inulinase production level was maintained until 78 h of incubation.

*Inulinase production using minimal medium*

The experiment was repeated using minimal medium as previously described. Although this medium was only composed of 10 g/l pure inulin, lacking any other media component, the inulinase productivity of the cells, of about 100 U/ml, was satisfactorily restored after only 24 h of incubation wherein the recorded productivity rate was 2.54 U/ml/h. This titer was more or less maintained until the end of the fermentation time (Fig. 7). The obtained results could be attributed to the fact that immobilized cells need nutrients that will only maintain their inulinase productivity on the expense of their growth. It was also observed that the use of a minimal medium resulted in a reduction in unwanted growth of escaping cells, which favors recovery of the produced enzyme.

**Figure 7**




Continuous production of inulinase by *Aspergillus niger* cells immobilized onto linen fibers in an airlift bioreactor, using either complete or minimized media as well as crude inulin juice.

*Inulinase production using crude inulin solution*

The experiment was finally performed using the crude inulin solution, prepared as previously mentioned in the Material and methods section. The latter resulted in a slightly reduced inulinase yield compared with that obtained using either complete or minimal media (Fig. 7). The recorded productivity rate under these conditions was 2.03 g/l/h for the first 36 h of the incubation period. However, a satisfactory production level ranging between 85.9 and 79.9 U/ml was then reached and approximately sustained for another 42 h. These results were very encouraging as, using this technique, a combined production of about 838 U/ml of inulinase was obtained from a very economic crude extract of inulin in only 48 h, which is comparable with yields of 996 and 1013 U/ml that were obtained when immobilized cells were cultivated using pure inulin in either minimized or complete media, respectively, for the same incubation period.

## Conclusion

From these experiments, we can conclude that the production of inulinase by *A. niger* cells immobilized by their adsorption onto the surface of linen fibers, using crude inulin extraction, is a very promising method that could be performed on large scales for economic, industrial production of the enzyme. The main advantage of this method is the higher productivity of the immobilized cells compared with that of the free cells, considering the possibility of their repeated batch cultivation. It was also observed that the production time during the repeated batch cultivations reduced by more than half. Moreover, with the use of crude inulin juice, a low percentage of cell growth, and eventually cell escapement, was attained. The latter made the recovery and purification of the enzyme much easier. As a final conclusion, this method is simple, economic, time saving, and nontoxic to the microorganism. In addition, the loaded linen pads are reusable.

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### Conflicts of interest

There are no conflicts of interest.

## References


1 Singh RS, Sooch BS, Puri M. Optimization of medium and process parameters for the production of inulinase from a newly isolated *Kluyveromyces marxianus* YS-1. Bioresour Technol 2007; 98:2518–2525.

2 Vandamme EJ, Derycke DG. Microbial inulinases: fermentation process, properties, and applications. Adv Appl Microbiol 1983; 29 (C):139–176.

3 Zhang L, Wang Y. Gene engineering of producing fructose by inulase hydrolyzing *Helianthus tuberosus*. Chinese Patent 02132446; 2002.

4 Gill PK, Manhas RK, Singh P. Hydrolysis of inulin by immobilized thermostable extracellular exoinulinase from *Aspergillus fumigates.* J Food Eng 2006; 76:369–375.

5 Zhengyu J, Jing W, Bo J, Xueming X. Production of inulooligosaccharides by endoinulinases from *Aspergillus ficuum.* Food Res Int 2005; 38: 301–308.

6 Gupta AK, Singh DP, Kaur N, Singh R. Production, purification and immobilisation of inulinase from *Kluyveromyces fragilis.* J Chem Technol Biotechnol 1994; 59:377–385.

7 Kim D-M, Kim H-S. Continuous production of gluconic acid and sorbitol from Jerusalem artichoke and glucose using an oxidoreductase of *Zymomonas mobilis* and inulinase. Biotechnol Bioeng 1992; 39:336–342.

8 Vranesic D, Kurtanjek Z, Santos AMP, Maugeri F. Optimisation of inulinase production by *Kluyveromyces bulgaricus.* Food Technol Biotechnol 2002; 40:67–73.

9 Kennedy JF, Cabral MS. *Immobilized living cells and their applications. Applied biochemistry and bioengineering*. In: Chibata I, Wingard LB, editors. *Immobilized microbial cells*. Vol. 4 NY: Academic Press; 1983. pp. 189–280.

10 Cabral MS, Kennedy JF. Covalent and coordination immobilization of proteins. In: Taylor RF, editor. *Protein immobilization*. NY: Marcel Dekker; 1991. pp. 73–138.

11 Farid MA, El-Batal AI, El-Diwany AI, El-Anshasy HA. Optimization of glutamic acid production with immobilized cells of *Corynebacterium glutamicum.* Adv Food Sci 1996; 18:34–38.

12 Atwa NA. Microbial and biochemical studies on the production of antibiotics for veterinary uses [PhD thesis]. Microbiology Department, Faculty of Sciences, Cairo University; 2003..

13 Sharma AD, Kainth S, Gill PK. Inulinase production using garlic (*Allium sativum*) powder as a potential substrate in Streptomyces sp. J Food Eng 2006; 77:486–491.

14 Aboo Baker DHA, El-Genaihi SE, Aboul Enein AM, Danial EN. Comparative study of inulin in two Asteraceae plants Chicory and Jerusalem artichoke. Ain Shams Univ Res bull 2009; 22:1–11.

15 Ashwell G. Colorimetric analysis of sugars. Methods Enzymol 1957; 3 (C):73–105.

16 Sheng J, Chi Z, Li J, Gao L, Gong F. Inulinase production by the marine yeast *Cryptococcus aureus* G7a and inulin hydrolysis by the crude inulinase. Process Biochem 2007; 42:805–811.

17 Spiro RG. Analysis of sugars found in glycoproteins. Methods Enzymol 1966; 8 (C):3–26.