Production of Inulinase by Free and Immobilized Cells of Penicillium funiculosum p.36

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\textbf{ABSTRACT}

The aim of this work was to optimize the growth conditions and continuous production of the enzyme using free and immobilized cells of inulinase by Penicillium funiculosum. The highest yield of enzyme (163.5U/mL) was obtained when the culture was incubated at 27°C and 200 rpm for 96h in a fermentation medium containing both inulin and peptone as sole carbon and nitrogen source, respectively. When the cells of the P. funiculosum were immobilized on different carriers, especially linen fibers, their production ability was successfully maintained for seven successive batches. When the fermentation was carried out using inulin juice prepared from Jerusalem artichoke tubers (in place of pure inulin), inulinase production could be sustained till the second cultivation batch of the P. funiculosum immobilized on linen fibers, yielding 122 U/mL enzyme. Results proved the feasibility of using crude inulin juice as a simple and economic carbon source for the production of inulinase.

\textbf{Key words:} Penicillium funiculosum, biochemical inulinase, inulin, repeated batch cultivation

\textbf{INTRODUCTION}

Inulin, a poly-fructan, occurs as a reserve carbohydrate in plant families representing more than 30,000 species. Most of them are dicotyledonous plants belonging to the Compositae and Campanulaceae families. Inulin is widely accumulated in the underground organs of Jerusalem artichoke, dahlia, and chicory and consists of linear-2,1-linked polyfructose chains displaying a terminal glucose unit (Singh et al. 2007). Apart from being historically consumed by the humans, inulin has received a great importance as a raw material for the production of inulooligosaccharides (Zhengyu et al. 2005) and fructose syrup (Zhang et al. 2004; Missao et al. 2015). Fructose is a safe alternate to sucrose, which is known to be the cause of many health problems including corpulence, carcinogenicity, diabetes and atherosclerosis (Vandamme and Derycke 1983). Fructose increases the absorption of iron as it forms an iron-fructose complex, which was much better than that of inorganic iron (Gupta et al. 1994). Fructose can be produced from inulin either enzymatically or chemically through acid hydrolysis. The latter method is not recommended due to the undesirable coloring of inulin hydrolysate and formation of difructose anhydride, which has practically no sweetening properties (Jun et al. 2007). The enzymatic production of organic products, especially those used in food and pharmaceutical industries, has many advantages over chemical processes. The best method for produce fructose in high yield is via enzymatic reaction (inulinase), where 95% pure fructose could be produced after one step of enzymatic hydrolysis of inulin.

Inulinase is produced by many microorganisms, including bacteria, yeasts and filamentous fungi. Traditionally, inulinase has been produced by...
submerged fermentation (SmF) (Selvakurmar and Pandey 1999; Gill et al. 2003; Kalil et al. 2011). The fermentation production of inulinase by such microorganisms can be greatly improved by modifying some parameters, including physiochemical and nutritional conditions of growth of the producing cells (Danial et al. 2010). Generally, the carbon source has been estimated as an important factor in enzyme production. Industrial application of inulinase would only be feasible if the carbon sources were available in large quantities at competitive price (Arun et al. 2006). A reduction in the production cost can be achieved by the usage of inexpensive inulin-containing substrates such as dahlia, chicory, garlic, onion, wheat, rye, barley and banana, which are often abundant, whereas pure inulin is available at high cost.

In comparison with the conventional fermentations, immobilization of microbial cells provide several important advantages such as faster production rate, easier purification of products as well as higher productivity over a certain period of time (Kennedy and Cabral 1983; Tarek et al. 2014). One of the most reliable, safe and easy methods of immobilization is the adsorption of the cells on an inert suitable support (Cabral and Kennedy 1991; Farid et al. 1996; Atwa 2003). Therefore, the present study aimed to explore the inulinase production ability of Penicillium funiculosum under different cultivation conditions as well as the effect of the immobilization of these cells using different carriers on their production rate. Finally, the repeated batch cultivation using immobilized cells on linen fibers was investigated over a number of successive batches, using complete minimal medium and crude inulin juice.

MATERIAL AND METHODS

Microorganism
Among 30 different fungal strains isolated from Red sea sediment, a potent strain, which gave a high yield of inulinase was chosen for further study and kindly identified by Assuit University Mycological Center AUMC as P. funiculosum and designated as P. funiculosum P36. The fungus was routinely grown on Czapek’s Dox agar medium (Sharma et al. 2006) at 30°C for seven days then preserved at -80°C in glycerol.

Preparation of crude inulin solution
Fifteen grams of Jerusalem artichoke tubers (Helianthus tuberosis), locally collected, were washed, sliced and grinded with 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 the addition of concentrated sodium hydroxide solution. The resulting juice was sterilized at 121°C for 15 min (Abboo-Baker et al. 2009). The raw inulin extract was analyzed and its inulin concentration was estimated to be approximately 1.00 % (w/v) by the method of Ashwell (1957).

Culture Medium and Cultivation Conditions
Shake flask fermentation of free cells
Inulinase production was carried out in 250 mL flask by taking 50 mL/flask of basal Czapek’s Dox (CD) medium (Sharma et al. 2006) containing (g/L): inulin, 10; NaNO₃, 3; K₂HPO₄, 1; MgSO₄.7H₂O, 0.5; KCl, 0.5 and Fe₂SO₄.7H₂O, 0.01 (pH 6.5). The flasks were then sterilized, inoculated with about 2x10⁵ spore/ml of the producing microorganism and incubated for at 120 rpm and 30°C for 96 h. The effect of various carbon sources, such as fructose, glucose, maltose, starch and lactose was investigated by adding them to the basal medium, without inulin, at a concentration of 10g/L either individually, or supplemented with inulin at 1 or 5g/L.

Various organic and inorganic nitrogen sources (peptone, urea, yeast extract, beef extract and meat extracts) were individually supplemented in the basal medium as a substitute for NaNO₃ in order to study their effect on inulinase production at 50 g/L. The inorganic nitrogen sources under study (NH₄SO₄ and NH₄Cl) were added according to their nitrogen content so that the latter was equivalent to that of the NaNO₃ which was omitted from the medium.

Shake flask fermentation of immobilized cells
The immobilization of P. funiculosum was studied using the adsorption method (Farid et al. 1996; Atwa 2003) by adding 1.5 g of each different carriers such as glass wool (Pyrex fiber glass, sliver 8 micron, corning glass work, Corning New York), synthetic fibers (locally provided) and linen fiber to 50 mL of the medium composed of (g/L): inulin, 10; peptone, 50; K₂HPO₄, 1; MgSO₄.7H₂O, 0.5; KCl, 0.5 and Fe₂SO₄.7H₂O, 0.01 (pH 6.5) in 250 mL flask. The flasks were then sterilized,
Inoculated with 2x10⁸ spore/mL of the culture and incubated at 120 rpm and 30°C for 96 h.
In order to assess the production ability of the immobilized cells for another batch, the loaded pads were washed thoroughly with normal saline, squeezed and used to inoculate 50 mL of a fresh sterile medium, which was then re-incubated under the conditions as above but for 72 h.

Effect of different constituents of the fermentation medium
An experiment was performed as an attempt to reduce the constituents of the fermentation medium used in the repeated batch cultivation of the cells immobilized on linen fibers and to determine their maximum inulinase production phase. *P. funiculosum* cells were inoculated in 50 mL of sterile medium along with 1.5 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 then squeezed using previously sterilized forceps. These pads were then transferred to new flasks containing different ratios of the main medium’s constituents as shown in Table 1.

Inulin was obtained from Sigma products (St. Lois, MO). Solvents (analytical grade) were obtained from Merck, Darmstadt, Germany. Crude inulin was obtained by the mechanical crushing and filtration of Jerusalem artichoke tubers. These flasks were then incubated at 30°C and 120 rpm for another 72 h. Then, the inulinase production and dry weight of unabsorbed cells in each flask were determined.

**Inulinase activity**
Inulinase activity was assayed by measuring the amount of reducing sugar released from inulin (Jun et al. 2007). The fermentation broth was centrifuged at 3000 xg and 4°C for 5 min. The supernatant was taken as the crude enzyme. A reaction mixture of 0.1 mL of the enzyme sample and 0.9 mL of acetate buffer (0.1 M, pH5.0) containing 2% inulin was incubated at 40°C in a water bath for 20 min. Then the mixture was incubated at 100°C for 10 min to inactivate the enzyme. The reaction mixture with inactive enzyme was used as a blank. The mixture reaction was assayed for reducing sugars by the method of Nelson–Somogyi (Nelson 1944). The calibrating curve was drawn with fructose (10-100 mg). One unit of the inulinase was defined as the amount of enzyme that released 1 μm of fructose from inulin per minute under assay conditions.

**Table 1** - Optimization of the fermentation medium used in the second batch production of inulinase by *Penicillium funiculosum* cells immobilized on linen fibers.

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<tr>
<th>Inulin (g/L)</th>
<th>Peptone (g/L)</th>
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<td>Control medium n°</td>
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<td>15</td>
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1: Control and 2: inulinase production by free cells and first batch cultivated immobilized cell respectively

**RESULTS AND DISCUSSION**

**Optimization of growth and inulinase production using free cells**

**Effect of different incubation periods**
The studies on the production of inulinase enzyme by P36 cells on inulin basal CD fermentation medium over a period of 120 h showed the activity of inulinase in the fermentation medium after about 6 h (4.73 U/mL). The enzyme activity increased linearly with time by a production rate (Qp) of 0.8 U/mL/h and reached a maximum volumetric production of 122.3 U/mL after about 96 h. Then, a gradual decrease in the activity was observed. The production decrease rate (-Qp) was about 1.3 U/mL/h. The cell growth during the course of fermentation increased gradually with time by a specific growth rate u of about 0.75 h⁻¹. A maximum CDW (X_max) of about 14.5 g/L was recorded after 96 h of incubation. This was in agreement with Cruz et al. (1998), where a maximum inulinase production in 96h was obtained by *A. niger* NK-126 when grown on Jerusalem artichoke. After that a slight cell lyases...
of growth. The maximum inulinase productivity of the producing organism was just before the onset of its stationary phase of growth. The maximum yield coefficient (units of inulinase per gram of cell mass formed) was 8434.5 U/g after 96 h of incubation (Singh et al. 2007).

Effect of different carbon sources
Results showed that, inulin resulted in a maximum enzyme production of about 122 U/mL, followed by sucrose (97 U/mL). Lower enzyme titers ranging between 51 and 89 U/mL were recorded upon using other carbon sources, including glucose, fructose, maltose, starch and lactose respectively. However, since the use of inulin as a sole carbon source in the fermentation medium was inconvenient due to its important factor as inducer for inulinase the latter was, therefore, added to the medium containing each individual carbon source (0.1 and 0.5%) as an attempt to initiate higher inulinase production (Kushi et al. 2000; Gao et al. 2012). This was in agreement with present results, in which the addition of inulin in these percentages resulted in significant increases in the enzyme production, ranging from 5.1 to 15%. However, none of the enzyme titers exceeded that obtained when inulin was added as a sole carbon source in the fermentation medium; this result was agreement with Kim and Kim (1992) and (Singh et al. 2007).

Effect of different nitrogen sources
Studies on the effect of different nitrogen sources showed that inulinase production reached a maximum production about 154 U/mL when peptone was used as a sole nitrogen source in the fermentation medium. Lower results ranging between 99-126 U/mL were recorded upon using yeast extract and beef extracts, urea and meat extract. The use of inorganic nitrogen sources, such as NaNO₃, NH₄Cl and (NH₄)₂SO₄ resulted in enzyme titers 70, 72 and 77 U/mL, respectively. Yeast extract was the best nitrogen source used in conjunction with dandelion root extract for inulinase production, followed by corn steep liquor by Kango (2008). Viswanathan and Kulkarni (1995) found corn steep liquor (CSL) as the best N-source in media containing kuth root powder as source of inulin. Cruz et al. (1998) found maximum inulinase by A. niger-245 on medium containing casein and dahlia extract.

Optimization of growth and inulinase production using immobilized cells
Effect of immobilizing P. funiculosum cells on different carriers
Enzyme production by P. funiculosum cells immobilized by their adsorption on glass wool, synthetic and linen fibers was compared with free cells. Results showed that the linen fiber was the best adsorptive carrier for the production of inulinase by P. funiculosum. The decrease in enzyme activity was due to the effect of immobilization on the production in terms of decreased productivity as a result of the limited oxygen diffusion and substrate availability inside the immobilization matrix (Farid et al 1996).

Effect of different incubation periods on the production of inulinase by P. funiculosum immobilized cells on linen fibers
Studies on the effect of different incubation periods on the production of inulinase to select the optimum production period showed that the inulinase production by the immobilized and free cells reached maximum as 155 and 123 U/mL after 72 and 120 h of incubation, respectively (Fig. 1). This result was very promising since this was attained in a much shorter incubation time as the cells were inoculated in their maximum production phase. This was in agreement with earlier studies of Skowronek and Fiedurek (2006), which found optimal inulinase production by immobilized mycelium of A. niger.

Continuous batch fermentation of P. funiculosum cells immobilized on linen fibers
The results in Figure 2 indicated that the cells immobilized on linen fibers were slightly affected by the immobilization process since they were able to produce a satisfactory level of enzyme production of about 123 U/mL compared to 155 U/mL produced by the free cells for the same incubation period of 96 h. The successive batch cultivation of the immobilized cells was performed in order to test their ability showed that inulinase production was more or less sustained in appropriate ranges for five consecutive batches, resulting in a total enzyme production of 580 U/mL in a combined serial incubation period of 360 h. The first batch cultivation of the P. funiculosum cells adsorbed on either synthetic
fibers or glass wool resulted in lower inulinase results of 111 and 105 U/mL respectively and these titers were maintained, with only a slight decrease, during the experiment. Use of the immobilized mycelium might help to extend the time of a bioprocess because most immobilization techniques allow multiple replacement of the medium during a single culture. Microbial inulinase have so far been relatively rarely produced using immobilized cells (Skowronek et al. 2011).

![Graph](image)

**Figure 1** - Effect of different incubation periods on the production of inulinase by *P. funiculo*sum immobilized cells in linen fibers.

**Figure 2** - Inulinase production during repeated batch cultivation of *P. funiculosum* cells immobilized on different support materials. (control: inulinase production by free cells).

**Effect of different constituents of the fermentation medium used in the production of inulinase by *P. funiculosum* immobilized on linen fibers**

The results showed that a maximum inulinase production of about 155 U/mL was obtained when free cells were cultivated for 96 h (control1); the first batch cultivation of immobilized cells (control 2) resulted in 133 U/mL (Fig. 3). However, there was no significant difference between the inulinase titers in the fermentation broths of media n° 1 to 8 (used in second batch cultivation), which ranged between 131 and 111 U/mL. This meant that the inulinase production ability of the cells were more or less maintained in the second batch even when the peptone, or salts content of the medium was reduced or even eliminated. However, inulin content of the medium was critical for both the growth of the producing organism and its production ability since its reduction, while keeping the percent of the other constituent’s constant, affected greatly the inulinase titer and the cells growth (media n° 9 to 11). The critical effect of inulin for inulinase production was also revealed when different percentages, from 25 to 75%, in the medium were used (media n° 12 to 14) since the production of inulinase decreased to 60-91 U/mL. Among various carbon sources employed for inulinase production, inulin-containing plant materials offer over advantages in comparison to pure substrates (Park and Yun 2001; Sharma et al. 2006). However, medium 15, comprising only pure inulin (10 g/L) as well as medium 16 comprising crude inulin solution (10 g/L) resulted 131 and 122U/mL enzyme activity, respectively. Relying on these results, the complete medium could be substituted by either medium 15 (minimal medium) or 16 (raw inulin extract) for the production of inulinase during the repeated batch cultivation of *P. funiculosum* cells immobilized on linen fibers. This was in agreement with the reports of inulinase production by other *A. niger* (Kango et al. 2008) due to the catabolic repression of enzyme synthesis by high concentration of simple sugars.

**Optimization of fermentation medium used for immobilized cells**

The results in Figure 3 showed that inulinase production increased gradually at the rate of 2.5 U/mL/h and reached a volumetric production of 123 U/mL after 48 h of incubation. This maximum inulinase production level was maintained till 78 h of incubation by using complete medium. Although the minimal medium was only composed of 10 g/L pure inulin, without any other content of the medium, the inulinase production reached to about 112 U/mL after only 48 h with the productivity rate of 2.3 U/mL/h. This titer was
more or less maintained till the end of the fermentation time. This result could be attributed to the fact that the immobilized cells needed nutrients and would maintain their inulinase production ability on the expense of their growth.

**Figure 3** - Optimization of the fermentation medium used in the second batch production of inulinase by *P. funiculosum* cells immobilized on linen fibers. (control 1 and 2: inulinase production by free cells and first batch cultivated immobilized cells, respectively).

The crude inulin solution resulted in a slight decrease in the inulinase production level compared to that either complete or minimal media. The productivity rate under these conditions was 2.8 g/l/h for the first 24 h of the incubation, when, the activity was 68 U/mL. The production level of enzyme reached to 94 U/mL after 36 h. with 2.6 g/L/h productivity rate. The 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 the secretion of intracellular metabolites (Skowronek et al. 2011).

These results were very encouraging since using this technique, a combined production of about 68 U/mL of inulinase was obtained from a very economic crude extract of inulin in only 24 h, i.e., 68,000 U/L/day compared to a production of 56,000 and 61,500U/L/day, which were obtained when the immobilized cells were cultivated using pure inulin in either minimal or complete media, respectively.

**Figure 4** - Continuous production of inulinase by *Penicillium funiculosum* P36 cells, immobilized on linen fibers using either complete or minimized media as well as crude inulin juice.

**CONCLUSION**

From the results it was concluded that the production of inulinase enzyme by *P. funiculosum* 36 cells immobilized by their adsorption on the surfaces of linen fibers using crude inulin extraction, could be a very promising method that could be performed on large-scale for the economic, industrial production of the enzyme. The main advantage of this method was the higher productivity by the immobilized cells compared to the free cells considering the possibility of their repeated batch cultivation. It was also noticed that the production time in the immobilized state reduced to more than the half. Thus, this method would be a simple process economic with time saving and non-toxic to the microorganism.

**REFERENCES**


Gill PK, Sharma AD, Harchand RK, Singh P. Effect of media supplements and culture conditions on inulinase production by an actinomycetes strain. **Bioresource Technol.** 2003; 87: 359-362.


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.


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