Original Article

NEW ISOLATE FOR ENHANCEMENT PRODUCTION OF MICROBIAL INULINASE

NOVO ISOLADO PARA REFORÇO DE PRODUÇÃO MICROBIANA INULINASE

Enas N. DANIAL; Widad K. W. Al-Bishri
Department of Biochemistry, Faculty of Girls Science, King Abdulaziz University Jeddah, Saudi Arabia.
Egypt. enas_mahdy@yahoo.com

ABSTRACT The optimization of growth conditions for the production of inulinase by *Penicillium funiculosum* cells were studied as well as the continuous production of the enzyme using immobilized cells. The highest amount of enzyme (163.5U/mL) was obtained when the producing cells were incubated for 96 hours at 27°C and 200 rpm in a fermentation medium containing both inulin and peptone as sole carbon and nitrogen sources respectively. However, when the cells of the tested microorganism were adsorbed on different carriers, especially linen fibers, their production ability was also successfully maintained, to different extends, for seven successive batches. Moreover, commercially pure inulin is very expensive in only small quantities, this fermentation medium was later substituted by a crude inulin solution obtained from Jerusalem artichoke tubers (*Helianthus tuberosus).* The crude inulin juice was able to sustain inulinase production during the second batch cultivation of the *P. funiculosum*, immobilized by their adsorption on linen fibers, in a satisfactory level of about 122U/mL. Moreover, the use of the previously mentioned crude inulin preparation was also compared to the use of either complete or minimal media, composed solely of 1% pure inulin. The method, adopted in this study for inulinase production, is simple, economic, time saving, non-toxic to the microorganism and the loaded linen pads are reusable.

KEYWORDS: *Penicillium funiculosum.* Biochemical inulinase. Inulin. Continuous production.

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. Inulin consists of linearb-2,1-linked polyfructose chains displaying a terminal glucose unit (SINGH et al 2007). A part from being historically consumed by human, inulin has received a great importance as a raw material for the production of inulo-oligosaccharides (ZHENGYU et al 2005) and fructose syrup (ZHANG et al 2004 and GILL et al 2006). 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; DERYCKE 1983). In addition to that, fructose also causes the increases the absorption of iron as it forms an iron-fructose complex which absorption was found to be 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 the formation of difructose anhydride which has practically no sweetening properties (JUN et al 2007). Moreover, 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 the 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) (GILL et al 2003; KALIL et al 2001; SELVAKURMAR; PANDEY 1999). The fermentation production of inulinase by microorganisms can be greatly improved through modifying some parameters including physicochemical and nutritional conditions of growth of the producing cells (ENAS et al 2010). Generally, the carbon source has been estimated as an important factor in enzyme production. Industrial application of inulinase, moreover, 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

Received: 13/11/14
Accepted: 20/01/15

inulin is only available at high cost. In comparison with conventional fermentations, immobilization of living 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; CABRAL 1983). One of the most reliable, safe and easy method of immobilization is the adsorption of the producing cells on an inert, suitable support (CABRAL; KENNEDY 1991; FARID et al 1996; ATWA 2003).

Therefore the present study was performed in order to explore the inulinase production ability of *P. funiculosum* cells under different cultivation conditions as well as the effect of the adsorption immobilization of these cells using different carriers, on their production rate. Finally, the continuous production of the producing cells, immobilized on linen fibers and also investigated over a number of successive batches, using either complete or minimal media as well as 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 7 days then preserved at -80°C in glycerol.

Preparation of crude inulin solution

15 g of Jerusalem artichoke tubers (*Helianthus tuberosus*), locally collected, were washed, sliced and grinded with a blender along with 100ml of distilled water, then filtered through a fine gauze. The pH of the solution was adjusted at 6.2 by the addition of concentrated sodium hydroxide solution (1M). The resulting juice was sterilized, at 121°C and 1.5 atmospheric pressure, for 15 min (ABOO BAKER et al 2009). The raw inulin extract was analyzed and its inulin content concentration was estimated, by the method of ASHWELL (1957) to be approximately 1.00 % (w/v).

Culture Medium and Cultivation Conditions. Shake flask fermentation of free cells

Inulinase production was carried out in 250mL flask contain 50ml/flask of basal Czapek's Dox (CD) medium of SHARMA et al (2006) containing (g/L): Inulin, 10; $NaNO_3$, 3; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5 and $Fe_2SO_4.7H_2O$, 0.01 (pH 6.5). The flasks were then sterilized, inoculated with about $2x10^9$ spore/ml of the producing microorganism and incubated for 96hr at 120rpm 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, in the concentration of 10g/L either individually, or in combination with inulin which was then supplemented as either 1 or 5g/L.

The effect of various organic and inorganic nitrogen sources, were individually supplemented in 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 as well as yeast, beef and meat extracts) were added in the concentration of 50g/L. On the other hand, the inorganic nitrogen sources under study (NH_4SO_4 and NH_4Cl) were added according to their nitrogen content so that the latter was equivalent to that of the $NaNO_3$ which was omitted from the medium.

Shake flask fermentation of immobilized cells

The immobilization of the microorganism under test was studied using the adsorption method (FARID et al 1996 and ATWA 2003). The immobilization of *P. funiculosum*, and eventually their inulinase production ability by adding 1.5g of each different carriers tested support glass wool (Pyrex fiber glass, Sliver 8 micron, Corning glass work, Corning New York), synthetic fibers (locally provided) and linen fibers, were added to a 250mL flask containing 50mL of the optimized medium composed of (g/L): Inulin, 10; Peptone, 50; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5 and $Fe_2SO_4.7H_2O$, 0.01 (pH 6.5). The flasks were then sterilized, inoculated with about $2x10^9$ spore/mL of the producing microorganism and incubated for 96hr at 120rpm and 30°C.

In order to assess the production ability of the immobilized cells for another batch, the loaded pads were washed thoroughly with normal saline, carefully squeezed and used to inoculate 50mL of a fresh sterile medium which was then re-incubated, under the former conditions, but for a shorter incubation period of 72hr.

Effect of different constituents of the fermentation medium

Moreover, an experiment was performed as an attempt to reduce the constituents of the fermentation medium used in the repeated batch

cultivation of the cells previously adsorbed on linen fibers and in their maximum inulinase production phase. This was achieved in order to decrease the growth of the escaped cells as well as to produce inulinase using the cheapest possible medium. Therefore, as described previously, *P. funiculosum* cells were inoculated in 50mL of sterile medium along with 1.5g of linen fibers in each flask. After about 96hr of incubation at 30°C and 120rpm, the linen fiber pads saturated with the cells in their maximum production phase, were washed thoroughly with normal saline solution then carefully squeezed using previously sterilized forceps. These pads were then transferred to new flasks containing different ratios of the main medium's constituents.

Pure inulin, obtained from Sigma products (St. Lois, MO). Solvents (analytical grade) were obtained from Merck, Darmstadt, Germany. Crude inulin, obtained by the 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 72hr. At the end of this incubation period, the inulinase production and the dry weight of unadsorbed 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 3000xg and 4°C for 5 minutes. The obtained supernatant was taken as the crude enzyme. A reaction mixture of 0.1mL of the enzyme sample and 0.9mL of acetate buffer (0.1M, pH5.0) containing 2% inulin was incubated at 40°C in a water bath for 20 minutes. Then the mixture was kept at 100°C for 10 minutes to inactivate the enzyme. The same mixture to which the same amount of inactivated crude enzyme (heated at 100°C for 10 minutes) was added before the reaction, 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 (10-100mg). One unit of the inulinase was defined as the amount of enzyme that released 1 µmol of fructose from inulin per minute under assay conditions.

RESULTS AND DISCUSSION

Optimization of growth and inulinase production using free cells

Effect of different incubation periods

The production of inulinase enzyme by *P.funiculosum* cells on inulin basal CD fermentation medium was monitored over a period of 120 hours under the previously mentioned shaking cultivation condition of 120rpm and 30°C. The results, illustrated graphically in Figure 1, showed that the activity of inulinase was detected in the fermentation medium after about 6hr of incubation as 4.73U/mL. This recorded enzyme activity was found to increase linearly with time by a production rate, Qp, of 0.8U/mL/hr and reached a maximum volumetric production of 122.3U/mL after about 96 hr of incubation.

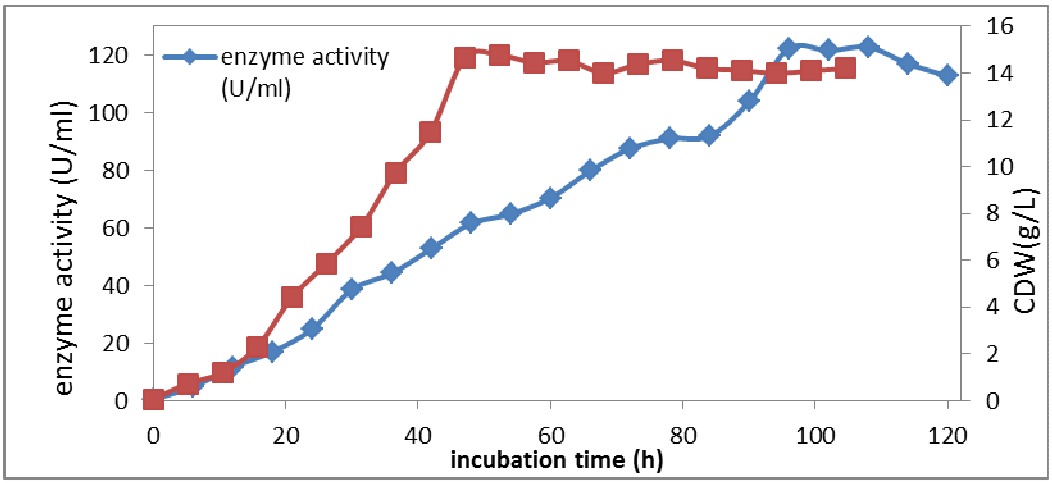

Figure 1. Effect of different incubation periods on growth and inulinase production of free *Penicillium funiculosum* cells cultivated in basal Czapek's Dox medium.

After this incubation period, a gradual decrease in inulinase activity was observed. The reported production decrease rate, -Qp, was about 1.3U/mL/hr. The cell growth was also studied

during the course of fermentation and was found to increase gradually with time by a specific growth rate u of about 0.75g/L/hr. A maximum CDW (Xmax) of about 14.5g/L was recorded after 96 hr of incubation. This result is in agreement with the reports of inulinase production by *A. niger* NK-126 grown on Jerusalem artichoke, time course of inulinase production showed the maximum inulinase production in 96h (CRUZ et al 1998).

After that a slight cell lyses was observed with a specific degradation rate, –u, of about 0.007g/L/hr resulting in a CDW of 14.1g/L after 120 hours of incubation. This result showed that inulinase production was growth associated and that the maximum inulinase productivity of the producing organism was just before the onset of its stationary phase of growth (SINGH et al 2007). Moreover, the yield coefficient calculations revealed that a maximum yield coefficient (units of inulinase per gram of cell mass formed) of 8434.5U/g was recorded after 96 hr of incubation.

Effect of different carbon sources

Different carbon sources of different categories were tested for their ability to sustain substantial amounts of inulinase enzyme production (Figure 2). Among them, inulin resulted in a maximum enzyme production of about 122U/mL followed by sucrose which resulted in approximately 97 U/ml of the enzyme.

Lower enzyme titers ranging between 51 and 89U/mL were recorded upon using other carbon sources including (in descending order of enzyme activity obtained): glucose, fructose, maltose, starch and finally lactose. 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, in small percentages of 0.1 and 0.5%, as an attempt to initiate higher inulinase production (KUSHI et al., 2000; GAO et al 2012).This is in agreement with present results. The enzymes seem this goal was achieved since the addition of inulin in these percentages resulted in significant increases in the enzyme production results ranging from 5.1 to 15%. However, none of these enzyme titers could exceed that obtained when inulin was added as a sole carbon source in the fermentation medium this result was agreement with the result obtained by. Kim and Kim (1992) and SINGH et al (2007).

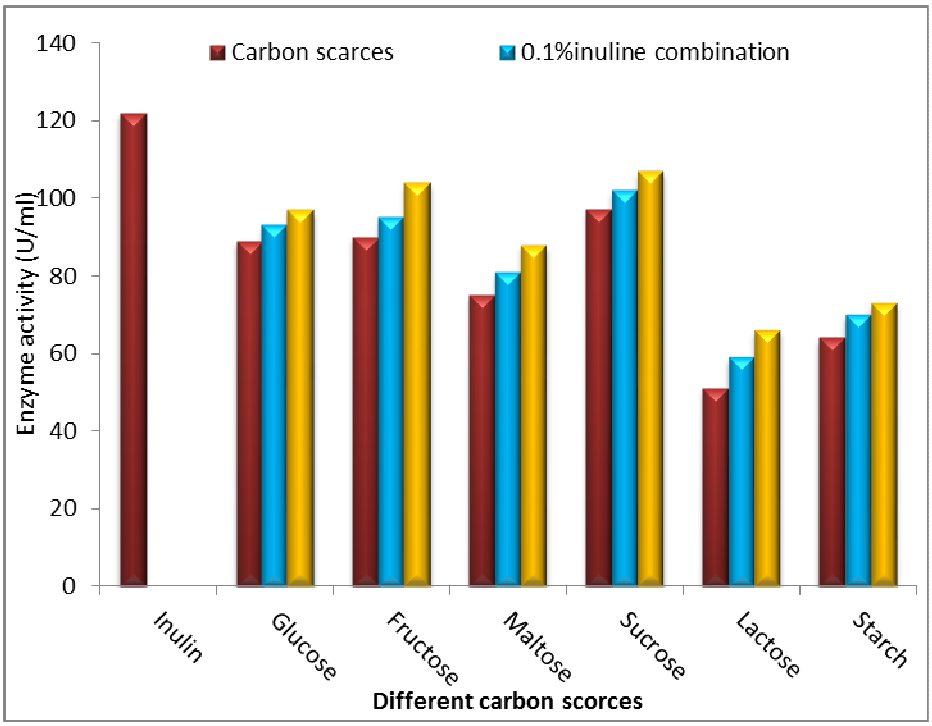

Figure 2. Effect of different carbon sources , added either individually or in addition to 0.1 or 0.5% inulin, on inulinase production by free *P. funiculosum* cells.

Effect of different nitrogen sources

Different nitrogen sources, either organic or inorganic, were also tested for inulinase production. The results in Figure 3 showed that the latter reached a maximum production of about 154 U/mL when peptone was used as a sole nitrogen source in the fermentation medium.

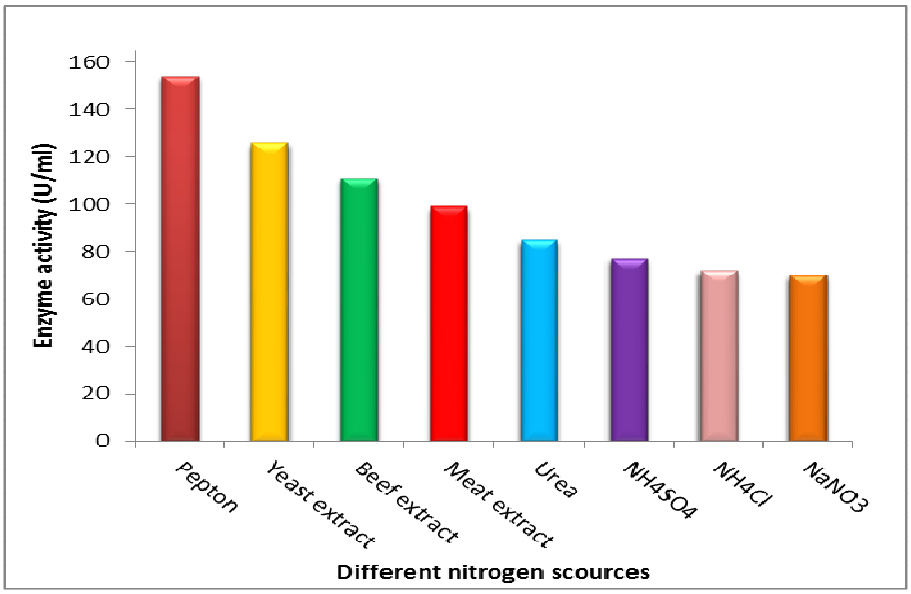

Figure 3. Effect of different nitrogen sources on inulinase production by free *P. funiculosum* cells .

Much lower results ranging between 126 and 99U/mL were recorded upon using other organic nitrogen sources including (in descending order of enzyme production results): yeast and beef extracts, urea then finally meat extract. On the other and, the use of inorganic nitrogen sources, such as $NaNO_3$, NH_4Cl and NH_4SO_4 resulted in enzyme titers of approximately 70, 72 and 77 U/mL respectively. Yeast extract was found to be the best nitrogen source to be used in conjunction with dandelion root extract for inulinase production followed by corn steep liquor (KANGO, 2008). VISWANATHAN and KULKARNI (1995) found CSL to be the best N-source in media containing kuth root powder as source of inulin while CRUZ et al. (1998) have found *A.niger*-245 to produce maximum amount of inulinase 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

P. funiculosum cells were tested for their ability to produce inulinase while immobilized by their adsorption on different carriers including glass wool, synthetic and linen fibers. The enzyme production results were compared to those obtained when the free cells of the latter fungus were cultivated on the same optimized culture medium under similar cultivation conditions. The results alliterated in Figure 4 showed that the linen fibers was the best adsorptive carrier for the production of inulinase by the cells of *P. funiculosum.*

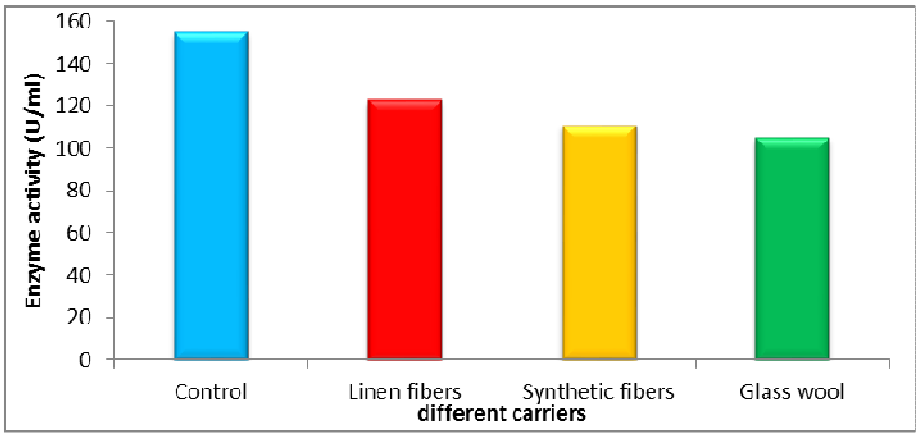

Figure 4. Effect of immobilizing *Penicillium funiculosum* cells on different carriers

Effect of different incubation periods on the production of inulinase of *P. funiculosum* cultivated as immobilized cells in linen fibers

In this experiment the effect of different incubation periods on the production of inulinase was studied to select the optimum production period by using immobilized cells of *P. funiculosum* on linen fibers compared by the free cells. The results in Figure 5 showed that the inulinase production by the immobilized and free cells reach to the maximum level 155 and 123 U/mL after 72 and 120 h of incubation respectively. This result was very promising since this previously mentioned inulinase titer was, more or less, attained after a much shorter incubation time, as the cells were inoculated in their maximum production phase. This is in agreement with earlier studies of SKOWRONEK and FIEDUREK (2006) which found optimal inulinase production by immobilized mycelium of *A. niger*.

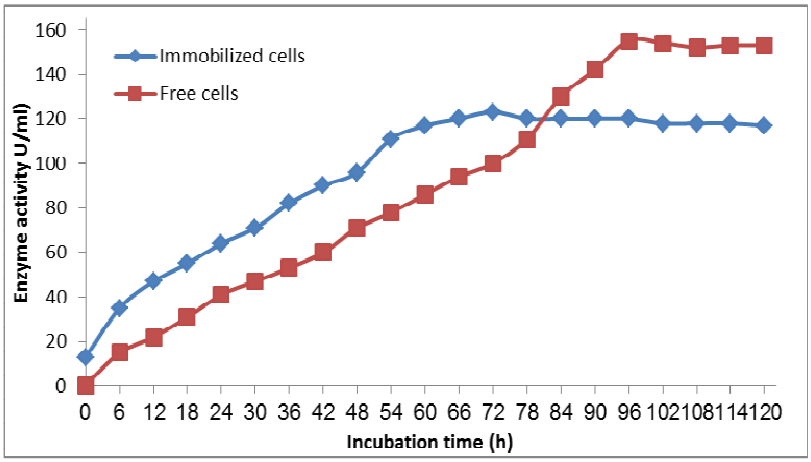

Figure 5. Effect of different incubation periods on the production of inulinase of *P. funiculosum* cultivated as immobilized cells in linen fibers

Continuous batch fermentation of *P. funiculosum* cells immobilized on linen fibers

The results in Figure 6 indicated that the cells immobilized on linen fibers were only slightly affected by the immobilization process since they were able to produce a satisfactory level of enzyme production of about 123U/mL compared to 155U/mL produced by the cultivation of the free cells for the same incubation period of 96hr. The successive batch cultivation of the immobilized cells was performed in order to test their ability to produce inulinase. The obtained inulinase production level could also be more or less sustained in appropriate ranges for five consecutive batches resulting in a total enzyme production of 580U/mL in a combined serial incubation period of 360hr. On the other hand, 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 105U/mL respectively. It was noticed that these titers were maintained, with only a slight decrease, during the experiment. 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 inulinase,

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 (SKOWRONEK et al 2011)

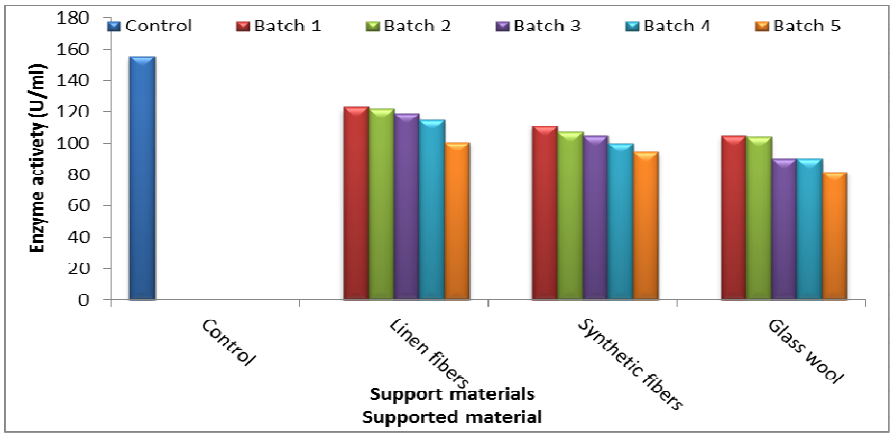

Figure 6. Inulinase production during repeated batch cultivation of *P. funiculosum* cells immobilized on different support materials. (control: inulinase production by free cells)

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

The results, illustrated in Figure 7, showed that a maximum inulinase production of about 155 U/mL was obtained when free cells were cultivated for 96hr (control 1). Also, the first batch cultivation of immobilized cells (control 2) resulted in a satisfactory inulinase production of 133U/mL. However, the results showed no significant differences between the inulinase titers estimated in the fermentation broths of media n° 1 to 8 (used in second batch cultivation) which ranged between 131 and 111U/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 the salts 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 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, ranging between 75 and 25%, of the medium's composition were used (media n° 12 to 14) since the production of inulinase decreased to 91 and 60 U/mL respectively.

Among various substrates (carbon source) employed for inulinase production, inulin-containing plant materials offer advantages in comparison to pure substrates. Complex substrates from agro industrial wastes or vegetal extract are showing very interesting results for inulinase production (Park and Yun 2001 and Sharma et al., 2006).

However, medium 15, composed of only pure inulin as 10g/l, as well as medium 16, composed of crude inulin solution as 10g/L, both resulted in satisfactory inulinase levels of 131 and 122U/mL 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 is in agreement with the reports of inulinase production by other *A. niger* (KANGO, 2008), due to the catabolic repression of enzyme synthesis by high concentration of simple sugars.

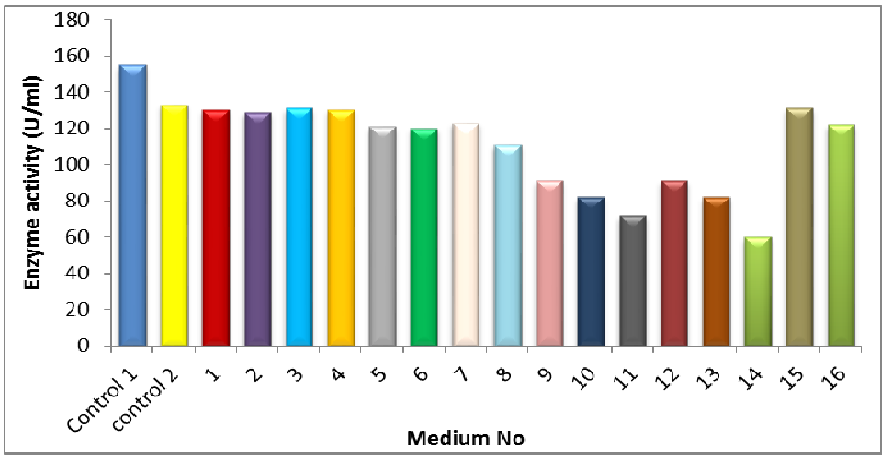

Figure 7. Optimization of the fermentation medium used in the second batch production of inulinase by *P. funiculosum* cells immobilized on linen fibers. (control 1 and 2: inulinase production by free cells and first batch cultivated immobilized cells respectively)

Optimization of fermentation medium used for immobilized cells

The results, illustrated in Figure 8 showed that inulinase production increased gradually at the rate of 2.5 U/mL/hr and reached a volumetric production of 123 U/mL after only 48 hr of incubation. This maximum inulinase production level was maintained till 78hr of incubation by using complete medium.

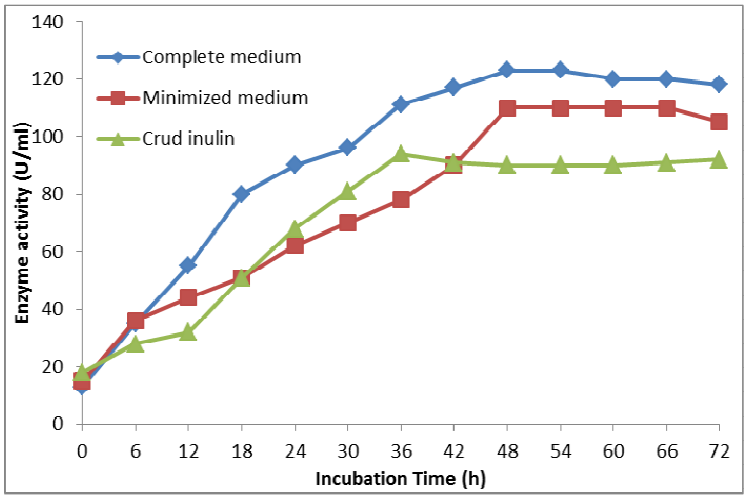

Figure 8. Continuous production of inulinase by *P. funiculosum* 36 cells, immobilized on linen fibers using either complete or minimized media as well as crude inulin juice.

The using of the minimal medium, previously deduced on the flask level. Although the latter medium was only composed of 10g/L pure inulin, without any other content of the medium, the inulinase production ability of the cells of was satisfactory restored to about 112U/mL after only 48 hr of incubation in which the recorded productivity rate was 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 need nutrients that will only maintain their inulinase production ability on the expense of their growth. This resulted also in the reduction of the unwanted growth of the escaping cells and that favors the latter recovery of the produced enzyme.

Finally performed using the crude inulin solution, prepared as previously mentioned in the material and method section. The latter resulted in a slight decrease in the inulinase production level compared to that obtained using either complete or minimal media. The recorded productivity rate under these conditions was 2.8 g/L/h for the first 24 hours of the incubation period when, the activity was 68 U/mL. The production level of enzyme was reached to 94U/mL after 36 hr. to be 2.6 g/L/h. 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 secretion of intracellular metabolites (SKOWRONEK ET al 2012).

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 hours i.e. 68000U/L/day compared to a production of 56000 and 61500 U/L/day which were obtained when the immobilized producing cells were cultivated using pure inulin in either minimized or complete media respectively.

CONCLUSIONS

The production of inulinase enzyme by *P. funiculosum* cells immobilized by their adsorption on the surfaces of linen fibers, using crude inulin extraction, is a very promising method that could be performed on large scales for the economic, industrial production of the enzyme.

The main advantage of this method is the higher productivity of the immobilized cells compared to the free ones considering the possibility of their repeated batch cultivation. It was also noticed that the production time in the immobilized reduced to more than the half. Moreover, owing to the use of crude inulin juice, the low percent of cell growth, and eventually cell escapement, was attained. The latter made the recovery and purification of the enzyme much easier.

This method is simple, economic, and time saving, non-toxic to the microorganism and the loaded linen pads are reusable.

RESUMO: A otimização das condições de crescimento para a produção de inulinase por células de *Penicillium funiculosum* foram estudados, bem como a produção contínua da enzima utilizando células imobilizadas. A maior quantidade de enzima (163.5U / mL) foi obtida quando as células produtoras foram incubadas durante 96 horas a 27 ° C e 200 rpm num meio de fermentação contendo ambos inulina e peptona como fontes de carbono e nitrogênio, respectivamente. No entanto, quando as células do microorganismo testado foram adsorvidas em diferentes suportes, especialmente fibras de linho, a sua capacidade de produção foi também mantida com sucesso, por diferentes extensões, e por sete lotes sucessivos. Por outro lado, a inulina comercialmente pura é muito dispendiosa em apenas pequenas quantidades. Este meio de fermentação foi depois substituído por uma solução de inulina bruta obtida a partir de tubérculos de alcachofra-girassol (*Helianthus tuberosus*). A inulina bruta foi capaz de sustentar a produção de inulinase durante o segundo lote de cultura de *P. funiculosum*, imobilizado pela sua adsorção nas fibras de linho, em um nível satisfatório de aproximadamente 122U / mL. Além disso, a utilização da preparação de inulina bruta anteriormente mencionada foi também comparada com o uso de meios completos ou mínimos, compostos unicamente de 1% de inulina pura. O método, adotada neste estudo para produção da enzima, é simples, de baixo custo e com economia de tempo. Além disso, não apresenta toxicidade para o microorganismo e os suportes de linho são reutilizáveis.

PALAVRAS-CHAVE: *Penicillium funiculosum. Inulinaase bioquímica*. Inulina. Produção continua.

REFERENCES

ABOO BAKER, D. H. A.; EL-GENAIHI, S. E.; ABOUL ENEIN, A. M.; DANIAL, E. N. Comparative study of inulin in two Asteraceae plants Chicory and *Jerusalem Artichoke*. **Ain Shams University Research Bulletin**; v. 22, p. 1-11, 2009.

ARUN, D. S.; SANDEEP, K.; PRABHJO, T. K. G. Inulinase production using garlic (Allium sativum) powder as a potential substrate *in Streptomyces* sp. **Journal of Food Engineering.,** v. 77, p. 486–491, 2006.

ASHWELL, G. Colorimetric analysis of sugars. **Methods in Enzymology,** v. 3, p. 73-107.

ATWA, N. A. Microbial and Biochemical studies on the production of antibiotics for veterinary uses. PhD thesis, Microbiology Department, **Faculty of sciences, Cairo University,** 1957. 2003.

CABRAL, M. S.; KENNEDY, J. F. Covalent and coordination immobilization of proteins. In Protein immobilization. **Marcel Dekker.** NY. p. 73-138, 1991.

CRUZ, V. A.; BELOTE, J. G.; BELLINE; M. Z.; CRUZ, R. Production and action pattern of inulinase from *Aspergillus niger*-245:Hydrolysis of inulin from several sources. **Revista de Microbiologia**, v. 29, p. 301–306, 1998.

ENAS, N. D.; GHADA, E. A.; ELNASHAR, M. M. Immobilized Inulinase on Grafted Alginate Beads Prepared by the One-Step and the Two-Steps Methods **Ind. Eng. Chem. Res**., v. 49, p. 3120–3125, 2010.

FARID, M. A.; EL-BATAL, A. I.; EL-DIWANY, A. I.; EL-ANSHASY, H. A. Optimization of glutamic acid production with immobilized cells of *Corynebacterium glutamicum.* **Adv Food Sci**.; v. 18, p. 34-38, 1996.

GAO, J.; CHEN, L.; YUAN, W. Effects of carbon sources, oxygenation and ethanol on the production of inulinase by *Kluyveromyces marxianus* YX01 **J. BioSci. Biotech.,** v. 1, n. 2, p. 155-161, 2012.

GILL, P. K.; Manhas, R. K.; SINGH, P. Hydrolysis of inulin by immobilized thermostable extracellular exoinulinase from *Aspergillus fumigatus*. **Journal of Food Engineering.,** v. 76, p. 369-375, 2006.

GILL, P. K.; SHARMA, A. D.; HARCHAND, R. K.; SINGH, P. Effect of media supplements and culture conditions on inulinase production by an actinomycetes strain, **Biores. Technol**., v. 87, p. 359–362, 2003.

GUPTA, A. K.; SINGH, D. P.; KAUR, N.; SINGH, R. Production, purification and immobilization of inulinase from *Kluyveromyces fragilis.* **Journal of Chemical Technology and Biotechnology,** v. 59, p. 377-385, 1994.

JUN, S.; ZHENMING, C.; JING, L.; LINGMEI, G.; FANG, G. Inulinase production by the marine yeast *Cryptococcus aureus* G7a and inulin hydrolysis by the crude inulinase. **Process Biochemistry,** v. 42, p. 805–811, 2007.

KALIL, S. J.; SUZAN, R.; MAUGERI, F.; Rodrigues, M. I. Optimization of inulinase production by *Kluyveromyces marxianus* using factorial design. **Appl. Biochem. Biotechnol**., v. 94, p. 257–264, 2001.

KANGO, N. Production of inulinase using tap roots of dandelion (Taraxacum officinale) by *Aspergillus niger*. **Journal of Food Engineering.,** v. 85, p. 473–478, 2008.

KENNEDY, J. F.; CABRAL, M. S. Immobilized living cells and their applications. Applied Biochemistry and Bioengineering, Vol. 4. In: Immobilized microbial cells. pp 189-280, **Academic Press** NY:1983.

KIM, D. M.; KIM, H. K. Continuous production of gluconic acid and sorbitol from Jerusalem artichoke and glucose using an oxidoreductase of *Zymomonus mobilis* and inulinase. **Biotechnology and bioengineering.,** v. 39, p. 336-342, 1992.

KUSHI, R. T., MONTI, R., CONTIERO, J. Production, purification and characterization of an extracellular inulinase from *Kluyveromyces marxianus* var. *bulgaricus.* **Journal of Industrial Microbiology and Biotechnology,** v. 25, p. 63–69, 2000.

NELSON, N. A photometric adaptation of somogyi method for the determination of glucose. **Journal of Biological Chemistry**, v. 153, p. 375–380, 1944.

PARK, J. P.; YUN, J. W. Utilization of chicory roots for microbial endoinulinase production. **Letters in Applied Microbiology**, v. 33, p. 183–187, 2007.

SELVAKURMAR, P.; PANDEY, A. Comparative studies on inulinase synthesis by Staphylococcus sp. and *Kluyveromyces marxianus* in submerged culture, **Biores. Technol**., v. 69, p. 123–127, 1999.

SHARMA, A. D.; KAINTH, S.; GILL, P. K. Inulinase production using garlic (*Allium sativum*) powder as a potential substrate in *Streptomyces* sp. **Journal of Food Engineering,** v. 77, p. 486-491, 2006.

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 Biochemistry,** v. 42, p. 805-811, 2007.

SINGH, R. S.; BALWINDER, S. S.; MUNISH, P. Optimization of medium and process parameters for the production of inulinase from a newly isolated *Kluyveromyces marxianus* YS-1. **Bioresource Technology,** v. 98, p. 2518–2525, 2007

SKOWRONEK, M.; FIEDUREK, J.; TRYTEK, M. Inulinase production by *Aspergillus niger*. mycelium immobilized on polyurethane foam in a bioreactor with alternative oxygenation. **International Journal of Biotechnology Applications**, v. 3, n. 2, p. 80-88, 2011.

SKOWRONEK, M.; FIEDUREK, J. Inulinase biosynthesis using immobilized mycelium of *Aspergillus niger* **Enzyme and Microbial Technology,** v. 38, p. 162–167, 2006.

SPIRO, R. G. Analysis of sugars found in glycoproteins. **Meth Enzymol**, v. 8, p. 3-26, 1966.

VANDAMME, E. J.; DERYCKE, D. G. Microbial inulinases: fermentation process, properties, and applications. **Advances in Applied Microbiology,** v. 29, p. 139-176,1983.

VISWANATHAN, P., KULKARNI, P. R. *Saussurea lappa* (kuth) as a new source of inulin for fermentative production of inulinase in a laboratory stirred fermenter. **Bioresource Technology**, v. 52, p. 181–184, 1995.

ZHANG, L. WANG, Y. Gene engineering of producing fructose by inulnase hydrolyzing *Helianthus tuberosus*. **Chinese Patent** 1465699, 2004.

ZHENGYU, J.; JING, W.; BO, J.; XUEMING, X. Production of inulo-oligosaccharides by endoinulinases from *Aspergillus funiculum*. **Food Research International**; v. 3, p. 301-308, 2005.