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Production of an Endoinulinase from *Aspergillus niger* AUMC 9375, by Solid State Fermentation of Agricultural Wastes, with Purification and Characterization of the Free and Immobilized Enzyme

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Two different substrates, sunflower (*Helianthus annuus* L.) tubers and lettuce (*Lactuca sativa*) roots, were tested. Using a mixture of both wastes resulted in higher production of endoinulinase than either waste alone. Also, ten fungal species grown on these substrates as inexpensive, carbon sources were screened for the best production of endoinulinase activities. Of these, *Aspergillus niger* AUMC 9375 was the most productive, when grown on the mixture using a 6:1 w/w ratio of sun flower: lettuce, and yielded the highest levels of inulinase at 50% moisture, 30°C, pH 5.0, with seven days of incubation, and with yeast extract as the best nitrogen source. Inulinase was purified to homogeneity by ion-exchange chromatography and gel-filtration giving a 51.11 fold purification. The mixture of sunflower tubers and lettuce roots has potential to be an effective and economical substrate for inulinase production. Inulinase was successfully immobilized with an immobilization yield of 71.28%. After incubation for 2 h at 60°C, the free enzyme activity decreased markedly to 10%, whereas that of the immobilized form decreased only to 87%. A reusability test demonstrated the durability of the immobilized inulinase for 10 cycles and in addition, that it could be stored for 32 days at 4°C. These results indicate that this inulinase, in the immobilized form, is a potential candidate for large-scale production of high purity fructose syrups.

***Keywords*:** inulinase production, solid state fermentation, *Aspergillus niger*, immobilization

Introduction

Inulinase (2,1-β-D-fructan fructanohydrolase, EC 3.2.1.7) is an important, commercially used enzyme that is usually extracellular, inducible and versatile. Fructose can be produced by processing starch with α-amylase, amyloglucosidase and glucose isomerase, but with a yield of fructose of only 45% in contrast, fructose production with inulinase can yield 95% (Cruz *et al*., 1998). Inulin can be hydrolyzed by two different types of inulinases: exoinulinase (β-Dfructan fructohydrolase, EC 3.2.1.80), which liberates fructose and endoinulinase (2-1-β-D-fructan fructanohydrolase, EC 3.2.1.7), which produces fructooligosaccharides (Skowronek and Fiedurek, 2004).

Fructose is considered as a safe alternative sweetener to sucrose because it has beneficial effects in diabetic patients, increases iron absorption in children and has a higher sweetening capacity (Pawan, 1973). Sucrose, on the other hand, is known to cause problems related to corpulence, cariogenicity and artherosclerosis (Vandamme and Derycke, 1983). In addition, fructose has higher solubility than sucrose, is less viscous, and in low levels, can be metabolized without a need for insulin (Fleming and GrootWassink, 1979). Fructo-oligosaccharides have good functional and nutritional properties such as having low calorie content, being a Bifidus stimulating factor and being a source of dietary fiber in food preparations (Roberfroid *et al*., 1998). These oligosaccharides, therefore, are now widely used to replace sugars in many food applications such as in chocolate and dairy products (Vandamme and Derycke, 1983).

Inulinase is reported to be produced in both submerged and solid state fermentation. Despite the fact that industrial enzymes, including inulinases, are widely produced in submerged fermentation, solid state fermentation (SSF) still has potential for economical production of enzymes in small-scale units at a relatively higher volumetric productivity (Pandey *et al*., 1999). The choice of microorganism is important when using SSF for the production of enzymes. It is desirable that the organism grows at low water activity, thereby discouraging growth of contaminating organisms and thus allowing the product to be classified as GRAS (Generally Recognized As Safe).

Inulin is a potential source of fructose, also known as fruit sugar (Mazutti *et al*., 2010). It is present as a reserve carbohydrate in the roots and tubers of composite plants such as Jerusalem artichoke (*Helianthus tuberosus* L.), Chicory (*Cichorium intybus* L.), Sunflower (*Helianthus annuus* L.), and Dahlia (*Dahlia pinnata* Cav.), also in small amounts in onion and garlic (Chi *et al*., 2011). Among the plants that store fructans are many of significant economic importance, such as cereals (e.g., barley, wheat, and oat), vegetables (e.g., chicory, onion, and lettuce) and forage grasses (e.g., *Lolium* and *Festuca*) (Hendry and Wallace, 1993).

Inulinases can be derived from many microorganisms. In the last three decades, significant efforts have been made to find the best microbial source for the extraction of inulinase (Vijayaraghavan *et al*., 2009). Microorganisms that have been

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Table 1. **Screening of the potentiality of some fungal species to produce endo-inulinase using Sun flower tubers and Lettuce roots as carbon sources by solid state fermentation**

Waste	Sun flower tubers			Lettuce roots		
Fungal isolate	Inulinase activity (Units/ml)	Protein (mg/ml)	Specific activity (Units/mg protein)	Inulinase activity (Units/ml)	Protein (mg/ml)	Specific activity Units/mg protein
Aspergillus terreus	200±0.4 [a]	3.5±0.7 [a]	57.1 [b]	240±0.6 [a]	3.0±0.3 [a]	80.0 [a]
A. niveus	230±0.4 [a]	3.8±0.7 [a]	60.5 [c]	265±0.6 [a]	3.2±0.3 [a]	82.8 [a]
A. niger	**300±0.4 [c]**	**3.0±0.7 [a]**	**100.0 [d]**	**290±0.6 [a]**	**3.3±0.3 [a]**	**87.9 [a]**
Penicillium chrysogenum	100±0.4 [b]	2.8±0.7 [b]	35.7 [a]	140±0.6 [b]	3.0±0.3 [a]	46.6 [b]
P. citrinum	120±0.4 [b]	2.5±0.7 [b]	48.0 [a]	180±0.6 [b]	3.0±0.3 [a]	60.0 [b]
P. olsonii	140±0.4 [b]	3.1±0.7 [a]	45.2 [a]	190±0.6 [b]	3.2±0.3 [a]	59.4 [b]
Trichoderma viride	180±0.4 [b]	3.4±0.7 [a]	52.9 [b]	170±0.6 [b]	2.9±0.3 [b]	58.6 [b]
T. harzianum	160±0.4 [b]	3.5±0.7 [a]	45.7 [a]	140±0.6 [b]	3.4±0.3 [a]	41.2 [b]
T. longibrachiatum	180±0.4 [b]	3.2±0.7 [a]	56.3 [b]	180±0.6 [b]	3.5±0.3 [a]	51.4 [b]
Ulocladium botrytis	210±0.4 [a]	3.0±0.7 [a]	70.0 [c]	220±0.6 [a]	2.9±0.3 [b]	75.8 [a]

The same letters within the same column are nonsignificant (P<0.05)
*Values are expressed as mean±SD.

reported to produce a high level of inulinase include various fungal, yeast and bacterial strains such are *Aspergillus aureus*, *A. oryzae*, *A. awamori*, *A. ficcum*, *A. niger*, *Fusarium oxysporum*, *Penicillium purpurogenum*, *Rhizopus* sp., *Streptomyces* sp., *Acetobacter* sp., *Arthrobacter* sp., *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus* sp., and *Schizosaccharomyces alluvius* (Pandey *et al*., 1999). Production of microbial inulinases has recently drawn attention as these enzymes offer several industrial advantages. Inulinases of many microorganisms, especially of filamentous fungi, are used to optimize the process of inulin hydrolysis in the food industries and for the production of alcohol, acetone and butanol (Pandey *et al*., 1999). They are also used in medicine as a tool for the diagnosis of renal problems (Kuehnle *et al*., 1992).

There are many techniques to immobilize enzymes, such as adsorption, covalent linkage, encapsulation, entrapment and crosslinking (Betancor *et al*., 2008). Each technique has it pros and cons; however, covalent linkage has the advantage of keeping the enzymes well bound to the carrier, thereby avoiding enzyme diffusion (Tanriseven and Dogan, 2001), which is why it is widely preferred by industry. Covalent immobilization of enzymes allows reuse of the immobilized enzymes many times, reducing their cost and hence the cost of the products. Unfortunately, efficient commercial carriers suitable for immobilization of enzymes used by industry are few and relatively expensive (Bickerstaff, 1995). Biopolymers, such alginates, carrageenans and chitosans are commercially available, have diverse features and are available at a reasonable cost, which could make them good candidates for immobilizing enzymes (Hugerth *et al*., 1997).

The objectives of the present study were the screening of various agricultural wastes as substrates for high inulinase production from *Aspergillus niger* AUMC 9375, optimization of the fermentation conditions to obtain high enzyme activity, purification and immobilization of the enzyme, and complete characterization of the enzyme before and after immobilization. *Aspergillus niger* AUMC 9375 was chosen after performing an initial screening of 10 fungal species belonging to 4 genera for their capability to produce inulinases by solid state fermentation, when grown on a mixture of sunflower tubers and lettuce roots as carbon sources. To our knowledge, there is not any report about inulinase production by *A. niger* AUMC 9375 using a mixture of sunflower tubers and lettuce roots.

Materials and Methods

Chemicals

Inulin, bovine serum albumin, 3, 5-dinitrosalicylic acid and sodium alginate were purchased from Sigma Aldrich. All the other chemicals used were also analytical grade.

Fungal inoculum (growth enhancement) medium (g/L)

Peptone, 5; Yeast extract, 1; glucose, 10; $MgSO_4·7H_2O$, 0.5; KH_2PO_4, 0.5 and distilled H_2O.

Fermentative media (g/L)

The fermentative medium was prepared using Yeast extract, 10; $NaNO_3$, 10; KH_2PO_4, 5; $MgSO_4·7H_2O$, 1 and distilled H_2O, pH 5.

Identification of fungal isolates

The microbial isolates screened were (*Aspergillus terreus, A. niveus*, *A. niger*, *Penicillium chrysogenum*, *P. citrinum*, *P. olsonii*, *Trichoderma viride*, *T. harzianum*, *T. longibrachiatum* and *Ulocladium botrytis*). These fungal isolates were isolated from soil in Cairo governorate and identified by the Taxonomy Department, Ain-Shams University, Cairo city, Egypt. The fungal isolates were maintained as single spore isolations on potato-dextrose-agar (PDA) medium, subcultured on PDA slopes and incubated at 30°C for 7 days. Among the ten fungal isolates, a strain that gave a high yield of inulinase was chosen for further study. It was kindly identified by the Assuit University Mycological Center (AUMC) and designated as *A. niger* AUMC 9375. The fungus was routinely grown on potato dextrose agar medium at 28°C for 5 days and then preserved at -80°C in glycerol.

Inoculum preparation

Spores from the agar slants were suspended in sterile saline solution (0.85% NaCl) containing 0.01% Tween 80 to obtain 2.0×10^6 spore/ml. For all the experiments, 1 ml of this suspension was inoculated into the enhancement medium and the culture was incubated in a bench-top shaker at 30°C for 48 h, then 5 ml of inoculum pellets were used for inoculation of the fermentative medium.

Solid state fermentation (SSF) and screening of the plant residues for the best inulinase production

Fermentation was carried out in 250 ml Erlenmeyer flasks containing either 10 g of oven-dried sunflower tubers, lettuce roots or a mixture of both (Mixture, SL) in different ratios, each in three flasks. Sunflower tubers were obtained from the Agricultural Research Center, Egypt. These plant residues were washed with cold water, ground and blended using a mixer and then each was used as a carbon source throughout the study). Each flask was supplemented with fermentative medium giving a moisture content of 50%, plugged with non-absorbent cotton and autoclaved at 121°C for 20 min. Fungal pellets (5 ml) of each fungal culture were transferred to the flasks containing fermentative medium, and incubated for 7 days at 30°C, with the pH adjusted to 6.0 (Fawzi, 2011). At the end of the fermentation process, the contents of each flask were rapidly filtered using Whatman No.1 filter paper. The filtrate was then subjected to an enzyme assay for the determination of inulinase activity in order to determine the best fungus and plant residue type, which were then chosen for further fermentation studies.

Enzyme assays

Inulinase activity was estimated using inulin as a substrate according to the method described by Singh *et al.* (2007) as follows: 0.1 ml of crude enzyme was added to 0.9 ml of 0.5% (w/v) inulin (Sigma) in 0.2 M citrate phosphate buffer (pH 6.0) and incubated at 50°C for 20 min. The reaction was terminated by boiling for 5 min (Kochhar *et al.*, 1999). The reducing sugars were subsequently analyzed by 3, 5 dinitrosalicylic acid (DNS) reagent (Miller, 1959). Absorbance was determined using a spectrophotometer at 540 nm. A distilled water blank was run in parallel. One unit of inulinase (U) was defined as the amount of enzyme that produced 1 μmol/ min of fructose under the assay conditions described above.

Protein determination

The protein content of the enzyme preparations was determined by the method of Lowry *et al.* (1951). All determinations were carried out in triplicate.

Optimization of cultural conditions

A mixture of sunflower tubers and lettuce roots (10 g) in different ratios (w/w) (1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 2:1, 3:1, 4:1, 5:1 and 6:1 sunflower: lettuce roots) was added to the fermentative medium to determine the best ratio. Using this ratio, the flasks were then supplemented with 1% (w/w) organic nitrogen sources (casein, peptone, yeast extract) or inorganic nitrogen sources ($NaNO_3$, NH_4SO_4, NH_4NO_3, NH_4Cl). Various physical parameters such as incubation period (3–8 days), pH (3, 4, 5, 6, 7, and 8), temperature (30, 40, 50, 60°C) and moisture level (30, 40, 50, 60, and 70%) were also optimized by conventional methods for maximal enzyme production. All the experiments were conducted in triplicate. For all these assays, pellets (5 ml) of the selected fungus were inoculated into the fermentative medium and incubated as described above, then the inulinase activities were determined.

Enzyme purification

Aliquots of cell-free dialysate (CFD) were separately treated with either $(NH_4)_2SO_4$ using the range of saturation from 0.5 to 0.9, methanol, acetone, ethanol or iso-propanol in a ratio of 1:1, 2:1, 3:1, 4:1 or 5:1 (alcohol : CFD). All samples were left overnight at 4°C. The precipitates were collected by centrifugation at 12×10^3 g for 15 min dissolved in 5 ml acetate buffer (0.1 mol/L, pH 4.8) and dialyzed overnight against the same buffer. The dialyzed sample was then passed through a DEAE-sepharose (2.5 × 25 cm) column (Diethylaminoethyl sepharose, fast flow, fibrous form-Sigma product) that had been pretreated with distilled water followed by washing with 1 N HCl and water until the pH of the suspension was about 6.0 (Peterson and Sober, 1962; Palmer, 1991). It was then washed several times with 0.5 N NaOH until no more colour was removed then equilibrated with 0.01 mol/L sodium acetate buffer (pH 4.8) at a flow rate of 1.5 ml/min. Proteins were eluted with a linear gradient of NaCl (0–1 M). Active fractions were pooled, lyophilized and subjected to gel filtration using a Sephadex G-100 column (2.0 × 20 cm) equilibrated with 0.01 mol/L sodium acetate buffer (pH 4.8). The protein concentration of each fraction was estimated. Inulinase activity of each fraction was determined as described in the enzyme assay section, then active fractions were collected and dialyzed once again against acetate buffer to remove Na^+ and Cl^- ions. This enzyme preparation was lyophilized and stored at 0°C for further investigations.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out according to Laemmli (1970), using 10% polyacrylamide. Proteins were detected by Coomassie Brilliant Blue R250 staining.

Immobilization of purified inulinase covalent linkage to glutaraldehyde-alginate beds

Sodium alginate was purchased from Sigma and used as organic carrier (natural polysaccharide polymer) to immobilize purified inulinase via entrapment by cross-linking. Encapsulation: 2% sodium alginate solution was used for the preparation of the cationic solution; xanthan was dissolved in a 4% calcium chloride solution to give a final xanthan concentration of 0.4%. About 2.2 ml of the cationic solution was then added drop wise to 50 ml of the sodium alginate solution. A gelation time of 30 min was observed. The capsules were rinsed with 100 mM acetate buffer pH 4.8 and hardened in 2% calcium chloride for 2 h, at 4°C. The immobilization beads were rinsed again with acetate buffer

and incubated in 1.25% glutaraldehyde for 30 min at 4°C to activate the alginate beads. Beads were rinsed again with acetate buffer and stored in glycerol overnight. Prior to use for bioconversion trials, the immobilized enzyme was thoroughly rinsed with acetate buffer. The glutaraldehyde-activated alginate beads were placed into contact with inulinase solution (1 ml, 140.43 Units/mg carrier) and the residual inulinase activity in the solution was assayed. The reaction solution contained 2 mg/ml of inulin. Immobilized enzymes were kept under refrigeration. Then, they were sequentially with distilled water and acetate buffer. After these washes, the enzymes were immersed in 0.05 mol/L calcium chloride solution. Samples were taken periodically for protein content assay.

The immobilization yield was calculated by the following equation:

% imm yield = immobilized enzyme/total enzyme added

Catalytic properties of the free and immobilized inulinase

In order to validate the efficiency of the carrier for immobilization of inulinase for use by industry, the following experiments were conducted.

pH and pH stability: To determine the optimum pH for the activities of free and immobilized enzyme, the reaction mixture was incubated at various pHs at 50°C for 20 min. pH stability for the free and immobilized inulinase was investigated under the same conditions as described above, with the residual activity measured after incubating the reaction mixture for 1 h at 50°C.

Temperature optimum and thermal stability: The optimum temperatures for the free and immobilized inulinase were examined. 0.1 ml enzyme solution was incubated for 20 min in 0.9 ml of 0.5% (w/v) inulin at pH 5.0 and temperatures from 25 to 70°C. The optimum temperature outcome was then taken as 100% activity and the relative activity at each temperature was expressed as a percentage of that activity. To determine the stability of the immobilized enzyme at high temperatures, the enzymes were incubated in the enzyme buffer solution for a period of 2 h at 50 and 60°C and then they were examined for enzyme activity as above. The optimum temperature outcome was again taken as 100% activity and the relative activity after incubation at each temperature was expressed as a percentage of that activity.

Operational stability of the immobilized enzyme: The operational stability of the immobilized enzyme was determined according to the following procedures. The enzyme was immersed in 0.05 mol/L of calcium chloride solution overnight. After the mixture was incubated at 50°C for 20 min, the reaction was started by adding 0.5% inulin and then the reaction mixture was analyzed as above. Afterward, the solid phase was filtered out and washed thoroughly with distilled water, then the above reaction was repeated under the same conditions.

Determination of the action type of the *A. niger* inulinase: Thin layer chromatographic analysis: The inulin hydrolysis products produced by *A. niger* inulinase were determined by thin layer chromatography (TLC) using 20 cm plates (Merk, TLC aluminium sheets 20 × 20 cm, silica gel-60F). one ml of enzyme solution was incubated with 1.0 ml of 1.0% inulin dissolved in 0.1 M acetate buffer (pH 5.0). After 15 min, the reaction was stopped by boiling. Samples (3 μl) were spotted onto silica gel-60 aluminum sheets, and 1% (w/v) inulin, fructose, glucose and sucrose standards (3 μl) were also applied to the chromatograms. The chromatography was carried out using as solvent acetic acid:chloroform:water (35:30:5, v/v/v). Carbohydrates were detected by staining the air dried plates with aniline-diphenylamine reagent. Fructose and fructooligomers were visualized as brown spots, glucose as a blue spot and a sucrose as a dark green spot. The colors are clearly visible for up to 6 h (Yun *et al*., 1997).

Statistical validation of treatment effects: The mean, standard deviation, T-score and probability "P" values of 3 replicates of the investigated factors and the control were computed according to the mathematical principles described by Glantz (1992). The obtained data were exposed to analysis of variance tests at the ($P \leq 0.05$) level for comparison of means. The analyses were carried out using IMB SPSS Statistics (14.0) software.

Results and Discussion

Screening of the plant residues and fungal strains

It is well known that there has been a growing interest in microorganisms, from various sources, that have the potential for substantial industrial enzyme production. Therefore, the exploration of new strains for the production of an enzyme such as inulinase is a worthy undertaking. Various substrates can be used for inulinase production, but carbohydrate-rich feedstocks are more advantageous than pure substrates. In this study, two inulin-rich substrates (sunflower tubers, lettuce roots) and ten fungal isolates belonging to four genera were screened for fructofuranosidase production. The highest inulinase-specific activities (100 and 87.9 Units/mg protein) were obtained by solid state fermentation with *A. niger* AUMC 9375, using sunflower tubers and lettuce roots as carbon sources, respectively. When *A. niger* AUMC 9375 was grown on a mixture of sunflower tubers and lettuce roots at different ratios under the condi-

Table 2. Potentiality of *A. niger* AUMC 9375 to produce endo-inulinase on a mixture of Sunflower tubers and Lettuce roots at different ratios by solid state fermentation

Ratios of Sun flower tubers: Lettuce roots (w:w)	Inulinase activity (Units/ml)	Protein (mg)	Specific activity (Units/mg protein)
1:1	307±0.8 [b]	2.5±0.5 [a]	122.8 [b]
1:2	310±0.9 [b]	2.5±0.9 [a]	124.1 [b]
1:3	313±0.5 [b]	2.5±0.3 [a]	125.2 [b]
1:4	321±0.6 [b]	2.5±0.4 [a]	128.4 [b]
1:5	350±0.4 [c]	2.0±0.1 [b]	175.0 [c]
1:6	300±0.6 [a]	3.0±0.6 [c]	107.0 [a]
2:1	400±0.9 [d]	2.0±0.6 [b]	200.0 [d]
3:1	390±0.6 [c]	2.5±0.4 [a]	156.0 [c]
4:1	350±0.3 [c]	2.6±0.7 [a]	134.6 [c]
5:1	310±0.2 [b]	2.9±0.9 [c]	106.9 [a]
6:1	**440±0.4 [d]**	**1.9±0.8 [b]**	**232.5 [d]**

The same letters within the same column are nonsignificant ($P<0.05$)
*Values are expressed as mean±SD.

Table 3. Optimization of cultural conditions for the best inulinase production

Property	Parameters
Optimum ratios of sun flower tubers and lettuce roots in the waste mixture	6:1 (w/w)
Optimum nitrogen source	Yeast extract
Optimum incubation period (days)	7
Optimum initial pH	5.5
Optimum temperature (°C)	30
Optimum moisture level (%)	50

tions previously described, it was found that the highest inulinase specific activity (232.5 Units/mg protein) after 7 days of incubation at 30°C (Table 2) was achieved using a 6:1 ratio of sunflower tubers: lettuce roots (w/w).

Optimization of culture conditions for the best inulinase production

In developing a fermentation process, the optimization of cultivation conditions and selection of appropriate substrates in the most favorable concentrations have primary importance due to their impact on the economic feasibility of the process. Many existing studies have sought to obtain high-yield production through optimization of processes at low cost. These studies have generally used classical optimization methods based on varying one factor at a time (Nyanhongo *et al*., 2002; Bankar *et al*., 2009).

In this work, some experiments were performed to evaluate the effect of different concentrations of the mixture components as the sole carbon source, different nitrogen sources, different incubation periods and differences in initial pH value, temperature and moisture level on inulinase activity.

Effect of different ratios of sunflower tubers and lettuce roots in the reaction mixture

The best ratio of the mixture components in the fermentative medium for elevating the inulinase activity was 6:1 (sunflower tubers: lettuce roots w/w) (Table 3). In this respect, other authors have reported different optimum concentrations for composite plants extracts: 3% for Jerusalem artichoke (Barta, 1993) and 5% for sunflower (Gill *et al*., 2003) as carbon sources for inulinase production. The best concentration varied according to the type of plant used (Sanal *et al*., 2005).

Effect of different nitrogen sources

When the effect of organic and inorganic nitrogen sources on inulinase production from *A. niger* AUMC 9375 was investigated, it was observed that yeast extract at 6.5 g/L was the best nitrogen source for the production of inulinase, giving a specific activity of 230 Units/mg protein (Table 3), followed by peptone, giving 200 Units/mg protein. These results were also reported by Chen *et al*. (2009). This also is in accord with Yu *et al*. (2009) who obtained maximum inulinase activity at 5 g/L yeast extract and Kango (2008) who obtained the maximum inulinase activity from *A. niger* with yeast extract.

Effect of different incubation periods

Seven days was the ideal incubation period, giving an inulinase specific activity of 228 Units/mg protein (Table 3). This finding was in accord with observations made by Ettalibi and Baratti (2001) and Gill *et al*. (2006). The enzyme activity started decreasing gradually after this time. The reduction in enzyme activity probably was caused by depletion of nutrients in the medium (substrate limitation) or accumulation of autotoxic products of the organism in the medium (Stanbury *et al*., 1997).

Initial pH

It is well known that the initial pH of the fermentation medium and incubation temperature are very important factors for high-yield production of a microbial substance. In recent studies, the optimum pH values for inulinase production by various microorganisms were reported to vary between 4.8–7.5 (Chen *et al*., 2009; Mughal *et al*., 2009). The present experiments showed that optimal pH for inulinase specific activity was 5.5, giving 235 Units/mg protein (Table 3).

Temperature

In the current study, *A. niger* was cultivated at different temperatures and a maximum inulinase specific activity of 234 Units/mg protein was obtained at 30°C (Table 3). Similar results were obtained by Pessoni *et al*. (1999) and Shady *et al*. (2000) who stated that the optimum temperatures for inulinase production from *Penicillium citrinum* and *Kluyveromyces marxianus* were 28°C and 30°C, respectively. This differs from results reported by Selvakumar and Pandey (1999), when cultivating *Kluyveromyces marxianus* in solid-state fermentations.

Effect of moisture level

The highest inulinase specific activity, 235 Units/mg protein, was obtained at 50% moisture (Table 3). Similar results were obtained by Mahesh *et al.* (2013), who found that 40% moisture was the optimum for inulinase production from *A. niger* using banana peel as a carbon source.

Table 4. Purification steps of inulinase from *A. niger* AUMC 9375 grown on a mixture of Sun flower tubers and Lettuce roots as a carbon source

Purification step	Total activity (Units/ml)	Total protein (mg)	Specific activity (Units/mg protein)	Yield (%)	Purification fold
Cell free filtrate	440.116	1.893	232.49	100.00	1.00
Cell free precipitate Acetone : filtrate (1:1 v/v)	374.58	0.172	2177.79	85.12	9.37
Ion exchange DEAE-Sepharose	166.66	0.053	3144.53	37.87	13.52
Gel filtration Sephadex G-100	142.60	0.012	11883.33	32.40	51.11

Table 5. **The overall performance of the free and immobilized endolinulinase**

Carrier	Form of enzyme	Protein (mg/ml)	Activity (U/ml)	Specific activity (U/mg protein)	Retained activity (%)	Amount of glucose (mg/ml)	Immobilization yield (%)
Alginate beads	Free inulinase	0.012	140.43	11702.50	-	0.68	-
	Immobilized inulinase	0.011/g carrier	129.57	11779.09	92.27	1.54	71.28

Enzyme purification

The first step of inulinase purification was precipitation of the protein from the cell-free dialysate. Acetone (1:1 v/v) was found to be effective, yielding a protein fraction having the highest total activity of inulinase. This resulted in 9.37 fold purification (Table 4) with a yield of 85.12% of the original activity.

A summary of the purification steps for inulinase is presented in Table 4. The precipitated enzyme was purified by ion exchange through DEAE-sepharose. In this step, inulinase was purified 13.52 fold with a yield of 37.87% and specific activity of 3144.53 U/mg protein (Table 4). The purification procedure was completed by gel filtration using a Sephadex G-100 column. In this step inulinase was further purified 51.11 fold with a yield of 32.4% and a final specific activity of 11883.33 U/mg protein (Table 4).

Concerning the purification steps, the superiority of acetone in obtaining a protein fraction having the highest total enzyme activity confirmed that inulinase has a particular structure that makes it resistant to the denaturing effect of organic solvents. Suitability of organic solvents in this regard was previously recorded by several authors (Viswanathan and Kulkarni, 1995; Hamdy, 2002).

Concerning the purification steps, the superiority of acetone in obtaining a protein fraction having the highest total enzyme activity confirmed that inulinase has a particular structure that makes it resistant to the denaturing effect of organic solvents. Suitability of organic solvents in this regard was previously recorded by several authors (Viswanathan and Kulkarni, 1995; Hamdy, 2002).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The homogeneity of inulinase obtained from *A. niger* AUMC was confirmed by SDS-PAGE. The single protein band visualized as inulinase is shown in Fig. 1. The relative molecular mass of the enzyme determined by this method was 75 kDa. This value is in accordance with Ettabili and Baratti (1990); Balayan *et al.* (1996) and Uhm *et al.* (1999) who found that the relative molecular mass of inulinases from *Aspergillus* and *Penicillium*, as determined by SDS-PAGE, was in the range of 54–78 kDa. Also, this molecular weight is similar to that obtained by Fawzi (2011).

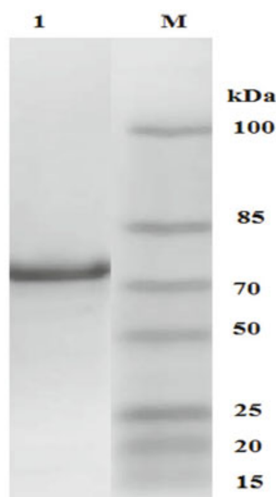

Fig. 1. **SDS-PAGE analysis of the purified inulinase.** Lane 1, Inulinase sample. Lane M, molecular weight standards (Marker).

The overall performance of free inulinase and inulinase immobilized by encapsulation

As shown in Table 5, inulinase was successfully immobilized with an immobilization yield of 71.28%, high specific activity of 11779.09 U/mg protein and retained activity of 92.27% compared to specific activity of 11702.50 U/mg protein for the free inulinase. This suggests the immobilized enzyme has interesting catalytic properties, different from

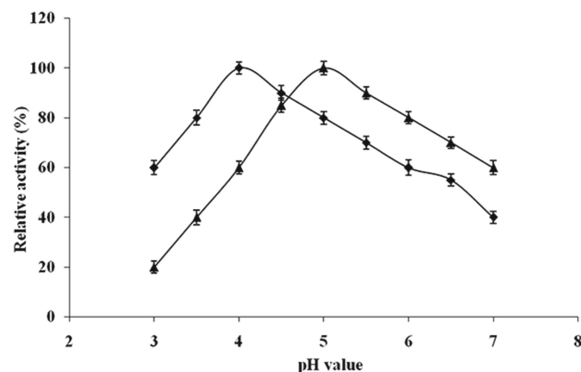

Fig. 2. **Optimum pH profile of free (-▲-) and immobilized (-◆-) inulinase.**

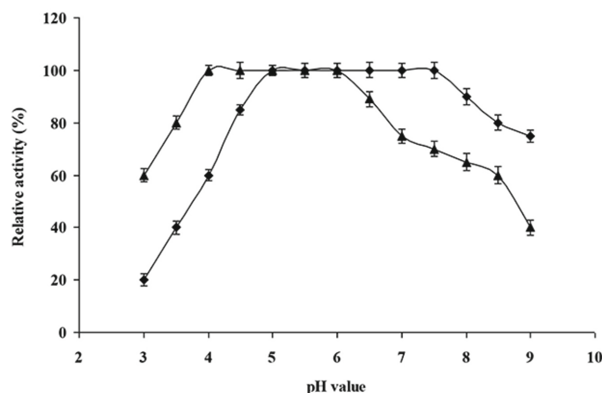

Fig. 3. **pH stability profile of free (-▲-) and immobilized (-◆-) inulinase.**

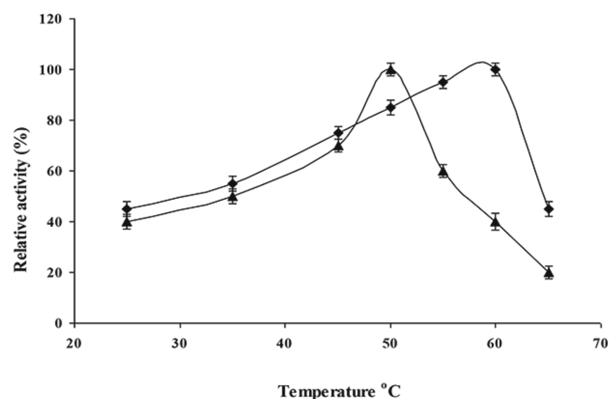

Fig. 4. **Optimum temperature profile of free (-▲-) and immobilized (-◆-) inulinase.**

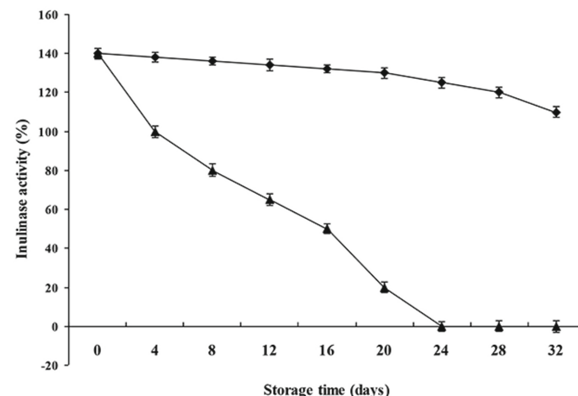

Fig. 6. **Changes in activity of free and immobilized inulinase stored under refrigeration at 4°C for 32 days.**

the free enzyme. These catalytic differences could be related to mass diffusion coefficients and conformational changes of the protein as well as the interaction of the substrate with the polymer matrix.

Catalytic properties of the free and immobilized inulinase

pH and pH stability: Free inulinase showed its maximum activity at pH 5.0 (Fig. 2), whereas the optimum pH of the immobilized enzyme was shifted to a more acidic value of pH 4.0. This pH shift appears partly because there was a micro-environmental difference such as surface charge of the carrier material. Free enzyme was quite stable in the pH ranges from 4 to 6.0 (Fig. 3), while the immobilized one was stable from 5 to 7.5. These results are in agreement with Yun *et al.* (2000).

Temperature and thermal stability: In this study, the optimum temperature for the immobilized enzyme increased to 60°C compared to 50°C for the free enzyme (Fig. 4). This result is consistent with reports by many other authors (Wenling *et al.*, 1999; Yun *et al.*, 2000). This temperature shift could be due to the formation of a molecular cage around the enzyme, which protected the enzyme molecules from the temperature (Tor *et al.*, 1989). For industrial application, a relatively high temperature of 60°C is preferably used to avoid microbial contamination, and it permits a greater concentration of the sugars, an ideal condition for fructooligosaccharide production by inulinases (Santos and Maugeri, 2002).

Immobilization usually improves the enzyme's thermal stability (Betancor *et al.*, 2008) and a new thermostable inulinolytic enzyme could play an important role in the fructose food industries (Wang *et al.*, 2008). After 2 h at 60°C, the enzyme activity of the free enzyme decreased greatly to 10%, whereas that of the immobilized enzyme retained 87% of its activity (Fig. 5). These results make this immobilized enzyme form very promising for industrial use.

Storage stability of inulinase under refrigeration (4°C): After 32 days of storage under refrigeration at 4°C, the activity of the immobilized inulinase decreased slightly from 140 to 110 Units, thus retaining over 78.5% of its activity (Fig. 6) compared to the complete loss of activity for the free enzyme after 24 days. These results may be attributed to the formation of ionic interactions (enzyme-gel polyelectrolyte complexes) that improved the stability of the immobilized enzyme (Severian and Esteban, 1998). Because there has been no ideal or easy storage method for free inulinase in the factory, the operators avoid early purchasing of all the inulinase that will be needed. Furthermore, this instability problem highlights the need for the factory staff to be able

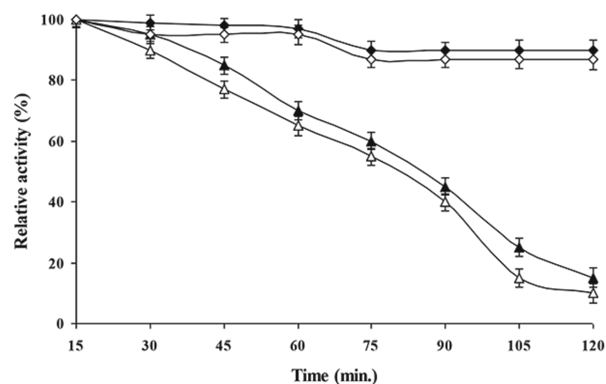

Fig. 5. **Thermal stability profile of free enzyme at 50°C (-▲-), 60°C (-△-) and immobilized inulinase at 50°C (-◆-), 60°C (-◇-).**

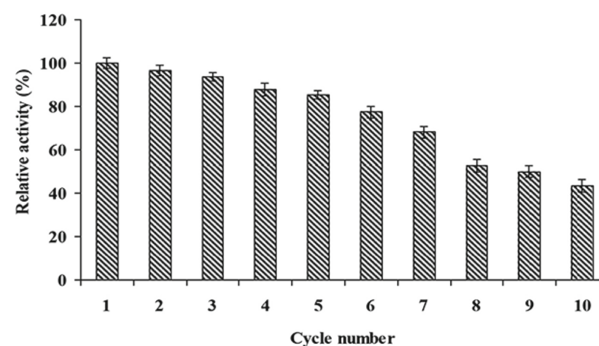

Fig. 7. **Operational stability of immobilized *A. niger* AUMC 9375 inulinase.**

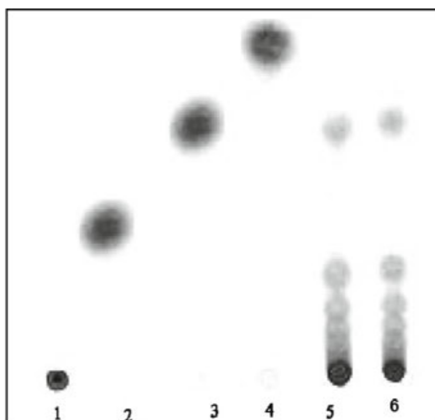

Fig. 8. **TLC determination of hydrolysis products generated by inulinase from *A. niger*. 1, Inulin; 2, Glucose; 3, Fructose; 4, Sucrose (1-4 as standards); 5, 30 min hydrolysis and 6, 60 min hydrolysis.**

to monitor the activity of their inulinase supply throughout the season.

Operational stability of the immobilized enzyme: Enzyme stability issues are always of high significance in the production of reproducibly stable biocatalysts. The main advantage of enzyme immobilization is the easy separation and reusability of the enzyme (operational stability). Another advantage is the stabilization of the immobilized enzyme, which was confirmed by the shelf-life stability test. The operational stability of the immobilized *A. niger* AUMC 9375 inulinase was evaluated in a repeated batch process (Fig. 7). After the fifth cycle, there was a drop in the concentration of released reducing sugars. This drop in activity was possibly due to the loss of loosely bound enzyme from the carrier. A small decrease in inulin hydrolysis (by only 2.6% over four cycles) was recorded, the loss in activity could be attributed to the enzyme inactivation due to continuous use (Nakane *et al*., 2001). Later, the hydrolysis efficiency declined distinctly and in the tenth cycle dropped to about 43.4%. The immobilized inulinase was able to maintain a good yield of reducing sugars (50.1%), equivalent to 49.9% of the initial activity after the ninth cycle. These results indicate that the bound enzyme could be used repeatedly for 9 cycles without any appreciable loss in sugar yield, which averaged 50.1%.

The analysis of hydrolysis products

The inulinase isolated here from *A. niger* hydrolyzed inulin internally by endo-cleavage (Fig. 8). Fructooligosaccharides, glucose and fructose were identified as products of inulinase action. These results indicate that this inulinase of *A. niger* is an endoinulinase. Inulin hydrolysis by an exoinulinase would have produced only fructose (Yun *et al*., 1997). Endoinulinases are potentially useful for large-scale production of fructooligosaccharides from inulin-containing agricultural crops. Because they hydrolyze fructane which type inulin by endo-action mode by producing a series of fructooligosaccharides.

This work consists of two major sections; the first deals with the rapid, economical production of highly active inulinase, a versatile enzyme used in many fields. It was concluded that solid state fermentation is efficient for the production of inulinase when using a mixture of sunflower and lettuce, yielding (440 U/ml) at 50% moisture with 6.5 g/L of yeast extract, and with an incubation period of 7 days. The sunflower-lettuce mixture is a rich source of inulin and has application in industrial production of fructooligosaccharides due to its easy and inexpensive availability when compared with usual substrates. The mixture can be used in various studies as fermentation medium components due to its high inulin content. Furthermore, by limiting costs, this research paves the way for commercialization and scale-up of inulinase production by *A. niger* through SSF, yielding products that have diverse applications in the industrial sector.

The second section consists of the immobilization process, which improved the enzyme's optimum temperature as well the enzyme's thermal stability from 50 to 60°C, a common temperature used in food industries to prevent microbial contamination and to permit the use of higher inulin substrate concentration due to increased solubility. The properties of free and immobilized enzyme were determined and compared and the immobilized enzyme gave satisfactory results with regard to pH stability, thermal stability and operational stability, where the enzyme could be used for up to 10 cycles with only partial loss of activity. The reusability of the immobilized enzyme up to ten times tremendously reduces the cost of the products. In brief, the simplicity and effectiveness of the newly developed, modern methods for covalent immobilization of enzymes, in addition to the realized promising results for operational stability are encouraging with regard to industrial application of the endoinulinase described here.

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References

Balayan, L.M., Pivazian, I.A., Khachaturian, R.N., Afrikian, I.G., and Abelian, V.A. 1996. Inulinases from *Penicillium palitans* and *Penicillium cyclopium*. *Biochem*. **61**, 645–650.

Bankar, S.B., Bule, M.V., Singhal, R.S., and Ananthanarayan, L. 2009. Optimization of *Aspergillus niger* fermentation for the production of glucose oxidase. *Food Bioprocess Technol*. **2**, 344–352.

Barta, J. 1993. Jerusalem artichoke as a multipurpose raw material for food products of high fructose or inulin content, pp. 323–339. *In* Fuchs, A. (ed.) Inulin and inulin-containing crops. Elsevier, Amsterdam.

Betancor, L., Luckarift, R., Seo, H., Brand, O., and Spain, C. 2008. Three dimensional immobilization of β-galactosidase on a silicon surface. *Biotechnol. Bioeng*. **99**, 261–267.

Bickerstaff, G. 1995. Impact of genetic technology on enzyme technology. *Biotechnol. Genet. Eng*. **15**, 13.

Chen, H.Q., Chen, X.M., Li, Y., Wang, J., Jin, Z.Y., Xu, X.M., Zhao, J.W., Chen, T.X., and Xie, Z.J. 2009. Purification and characterisation of exo- and endo-inulinase from *Aspergillus ficuum* JNSP5-06. *Food Chem*. **115**, 1206–1212.

Chi, Z.M., Zhang, T., Cao, T.S., Liu, X.Y., Cui, W., and Zhao, C.H. 2011. Biotechnological potential of inulin for bioprocesses. *Bioresour. Technol.* **102**, 4295–4303.

Cruz, V.D., Belote, J.G., Belline, M.Z., and Cruz, R. 1998. Production and action pattern of inulinase from *Aspergillus niger*. 245: hydrolysis of inulin from several sources. *Revista-de-Microbiol.* **29**, 301–306.

Ettalibi, M. and Baratti, J.C. 2001. Sucrose hydrolysis by thermostable immobilized inulinases from *Aspergillus ficuum*. *Enzyme Microb. Tech.* **28**, 596–601.

Fawzi, E.M. 2011. Comparative study of two purified inulinases from thermophile *Thielavia terrestris* NRRL 8126 and mesophile *Aspergillus foetidus* NRRL 337 grown on *Cichorium intybus*. *Braz. J. Microbiol.* **42**, 633–649.

Fleming, S.E. and Groot Wassink, J.W.D. 1979. Preparation of high fructose syrup from the tubers of the Jerusalem artichoke (*Helianthus tuberosus* L.). *CRC Crit. Rev. Food Sci. Nutr.* **12**, 1–28.

Gill, P.K., Sharma, A.D., Harchand, R.K., and Singh, P. 2003. Effect of media supplements and culture conditionon inulinase production by an Actinomycetes strain. *Bioresour. Technol.* **87**, 359–362.

Gill, P.K., Manhas, R.K., and Singh, P. 2006. Comparative analysis of thermostability of extracellular inulinase activity from *Aspergillus fumigatus* with commercially available (Novozyme) inulinase. *Bioresour. Technol.* **97**, 355–358.

Glantz, A.S. 1992. Primer of biostatistics, pp. 2–18. edited by McGraw Hill, Inc., USA.

Hamdy, H.S. 2002. Purification and some important characters of extracellular inulinase of *Alternaria alternata* (Fr.) Keissler. *Indian J. Exp. Biol.* **40**, 1393–1398.

Hendry, G.A.F. and Wallace, R.K. 1993. The origin, distribution, and evolutionary significance of fructans, pp. 119–139. *In* Suzuki, M. and Chatterton, N.J. (eds), Science and Technology of Fructans. CRC Press, Boca Raton, FL, USA.

Hugerth, A., Caram-Lelham, N., and Sundeliir, L. 1997. The effect of charge density and conformation on the polyelectrolyte complex formation between carrageenan and chitosan. *Carbohydr. Polym.* **34**, 149.

Kango, N. 2008. Production of inulinase using tap roots of dandelion (*Taraxacum officinale*) by *Aspergillus niger*. *J. Food Eng.* **85**, 473–478.

Kochhar, A., Gupta, A.K., and Kaur, N. 1999. Purification and immobilization of inulinase from *Aspergillus candidus* for producing fructose. *J. Sci. Food Agr.* **79**, 549–554.

Kuehnle, H.F., Von Dahl, K., and Schmidt, F.H. 1992. Fully enzymic inulin determination in small volume samples without deproteinisation. *Nephron.* **62**, 104–107.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lowery, O.H., Resenbrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.

Mahesh, N., Balakumar, S., Gayathiri, A., Arunadevi, A., and Arunkumar, M. 2013. Studies on the optimization and characterization for the biosynthesis of inulinase under solid state fermentation. *Int. J. Chem. Technol. Res.* **5**, 376–384.

Mazutti, M.A., Corazza, M.L., Maugeri, F., Rodrigues, M.I., Oliveira, J.V., Treichel, H., and Corazza, F.C. 2010. Hybrid modeling of inulinase bioproduction process. *J. Chem. Tech. Biotech.* **85**, 512–519.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Ann. Chem.* **31**, 426–428.

Mughal, M.S., Ali, S., Ashiq, M., and Talish, A.S. 2009. Kinetics of an extracellular exo-inulinase production from a 5-flourocytosine resistant mutant of *Geotrichum candidum* using two-factorial design. *Bioresour. Technol.* **100**, 3657–3662.

Nakane, K., Ogihara, T., Ogata, N., and Kurokawa, Y. 2001. Entrap immobilization of invertase on composite gel fiber of cellulose acetate and zirconium alkoxide by solgel process. *J. Appl. Polym. Sci.* **81**, 2084.

Nyanhongo, G.S., Gomes, J., Gübitz, G., Zvauya, R., Read, J.S., and Steiner, W. 2002. Production of laccase by a newly isolated strain of *Trametes modesta*. *Bioresour. Technol.* **84**, 259–263.

Palmer, T. 1991. Extraction and purification of enzymes, pp. 301–317. Understanding enzymes. Ellis Horwood. Ltd., England.

Pandey, A., Soccol, C.R., Selvakumar, P., Soccol, V., and Krieger, N. 1999. Recent developments in microbial inulinases Its production, properties, and industrial applications. *Appl. Biochem. Biotechnol.* **81**, 35–52.

Pawan, G.L.S. 1973. Fructose, pp. 65–80. Molecular structure and function of food carbohydrates. *In* Birch, G.G. and Green, L.F. (eds.). Appl. Sci. Publishers, London, UK.

Pessoni, R.A.B., Figueiredo-Ribeiro, R.C.L., and Braga, M.R. 1999. Extracellular inulinases from *Penicillium janczewskii*, a fungus isolated from the rhizosphere of *Vernonia herbacea* (Asteraceae). *J. Appl. Microbiol.* **87**, 141–147.

Peterson, E.A. and Sober, H.A. 1962. Column chromatography of protein: Substituted cellulases, Vol. 5, pp. 3–27. Methods in enzymology. *In* Colowich, S. and Kapllan, N. (eds.). New York, N.Y., USA.

Roberfroid, M.B., Van Loo, J.A.E., and Gibson, G.R. 1998. The bifidogenic nature of chicory inulin and its hydrolysis products. *J. Nuture* **128**, 11–19.

Sanal, F.E., Ertan, F., and Aktac, T. 2005. Production of exo-inulinase from *Alternaria alternata* growth on Jerusalem Artichoke and some biochemical properties. *J. Biol. Sci.* **5**, 497–505.

Santos, A. and Maugeri, F. 2002. Biotechnological production of bioflavors and functional sugars. Patent, PI. 02026023; Brazilian office. *Technology* **1**, 74.

Selvakumar, P. and Pandey, A. 1999. Solid state fermentation for the synthesis of inulinase from *Staphylococcus* sp. and *Kluyveromyces marxianus*. *Process Biochem.* **34**, 851–858.

Severian, D. and Esteban, C. 1998. Inclusion and release of proteins from polysaccharide-based polyion complexes. *Adv. Drug Deliv. Rev.* **31**, 223–246.

Shady, T.S.M., Hauka, F.I.A., and El-Sawah, M.M.A. 2000. Inulin hydrolysis by *Kluyveromyces marxianus* inulase produced on some agricultural wastes. *Egypt J. Microbiol.* **35**, 139–154.

Singh, R.S., Dhaliwal, R., and Puri, M. 2007. Partial purification and characterization of exo- inulinase from *Kluyveromyces marixianus* YS-1 for preparation of high-fructose syrup. *J. Microbiol. Biotechnol.* **17**, 733–738.

Skowronek, M. and Fiedurek, J. 2004. Optimization of inulinase production by *Aspergillus niger* using simplex and classical method. *J. Tech. Biotech.* **42**, 141–146.

Stanbury, P.F., Whitaker, A., and Hall, S. 1997. Principles of fermentation technology, 2nd ed., pp. 93–105. New Delhi, India. Aditya.

Tanriseven, A. and Dogan, S. 2001. Immobilization of invertase within calcium alginate gel capsules. *Process Biochem.* **36**, 1081.

Tor, R., Dror, Y., and Freeman, A. 1989. Enzyme stabilization by bilayer "encagement". *Enzyme Microb. Technol.* **11**, 306.

Uhm, T.B., Chung, M.S., Lee, S.H., Gourronc, F., Housen, I., Kim, J.H., Van Beeumen, J., Haye, B., and Vandenhaute, J. 1999. Purification and characterization of *Aspergillus ficuum* endoinulinase. *Biosci. Biotechnol. Biochem.* **63**, 146–151.

Vandamme, E.J. and Derycke, D.G. 1983. Microbial inulinases: Fermentation process, properties and applications. *Adv. Appl. Microbiol.* **29**, 139–176.

Vijayaraghavan, K., Yamini, D., Ambika, V., and Andsowdamini, N.S. 2009. Trends in inulinase production – a review. *Crit. Rev. Biotechnol.* **29**, 67–77.

Viswanathan, P. and Kulkarni, P.R. 1995. Properties and application of inulinase obtained by fermentation of Costus (*Saussurea*

lappa) root powder with *Aspergillus niger*. *Nahrung* **39**, 288–294.

Wang, Y., Xu, J., Luo, G., and Dai, Y. 2008. Immobilization of lipase by ultrafiltration and cross-linking onto the polysulfone membrane surface. *Bioresour. Technol.* **99**, 2299-2303.

Wenling, W., Wuguang, L.E., Huiying, W., and Shiyuan, W. 1999. Continuous preparation of fructose syrups from Jerusalem artichoke tuber using immobilized intracellular inulinase from *Kluyveromyces* sp. Y-85. *Process Biochem*. **34**, 643–646.

Yu, X., Guo, N., Chi, Z., Gong, F., Sheng, J., and Chi, Z. 2009. Inulinase overproduction by a mutant of the marine yeast *Pichia guilliermondii* using surface response methodology and inulin hydrolysis. *Biochem. Eng. J.* **43**, 266–271.

Yun, J.W., Kim, D.H., and Song, S.K. 1997. Production of inulo-oligosaccharides from inulin by immobilized endoinulinase from *A. niger. J. Fermentation Bioeng*. **84**, 371.

Yun, J.W., Park, J.P., Song, C.H., Lee, C.Y., Kim, J.H., and Song, S.K. 2000. Continuous production of inulo-oligosaccharides from chicory juice by immobilized endoinulinase. *Bioprocess Eng.* **22**, 189–194.