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Production of Purified Free and Immobilized Exo-Inulinase from *Aspergillus terreus AUMC* 11628 by Solid State Fermentation for Degradation of Dahlia Tubers and Chicory Roots Inulin Mixture and Ethanol Production

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Abstract

The mixture of both Dahlia (Dahlia pinnata) tubers and Chicory (Cichorium intubus) roots has the potential to be an effective and cost-efficient substrate for Exo-inulinase production more than each alone. Five Aspergillus species were also examined for maximum production of Exo-inulinase activities when grown on aforementioned mixture. Aspergillus terreus was the potent on a ratio of (5:1 w/w Dalia: Chicory, respectively) producing the maximum levels of inulinase at 45 ºC, pH 7.0, seven days of incubation, 50 % moisture and peptone as the best nitrogen source. With 70.23 purification fold, inulinase was purified to homogeneity. Studying of inulinase thermal stability for 2 hours at 60 °C revealed a significant reduction in the free enzyme activity when compared to the immobilized one. With a 75.65 % immobilization yield, inulinase was successfully immobilized. The sodium alginate beads passed the reusability test after 10 cycles and 20 days of storage at 4°C. These findings imply that inulinase in its immobilized form could be a potential candidate for pure fructose syrups production. It was also found that ethanol production by both free and immobilized inulinase was achieved using two processes; direct conversion and Sequential hydrolysis and fermentation of inulin into ethanol using Saccharomyces cerevisiae for fermentation sugars into ethanol. To the best of our knowledge, there is the first report about immobilized inulinase production by Aspergillus terreus using Dalia tubers and Chicory roots mixture and its usage in ethanol production.

Keywords: Exo-Inulinase; Aspergillus terreus; Immobilization; Saccharomyces cerevisiae; ethanol production

1. Introduction

Inulin (a fructan-type plant polysaccharide) accumulates widely in plant sources include, Agave, Coffee, Chicory, Asparagus, Dandelion, Globe Artichoke, Dahlia, Jerusalem artichoke, Onion, Jicama, Garlic, Yukon and Wild yam, and etc [1] where it is stored as a carbon and energy source. Agro-industrial wastes (Dahlia tubers and chicory root) are widely regarded as a source of pollution at the environment. As a result, bio-conversion of these agro-industrial residues into valuable products used for various industrial applications such as the production of fermentable sugars [2] can assist in reducing their pollutant effect on the environment.

Inulin possesses various biological importance, such as regulation of blood sugar and blood lipids, a prebiotic improves the intestinal microbe environment, anticancer, antioxidant and immune regulating factor. Currently, inulin is widely applied in the food industries because of its functional and nutritional properties as fat replacer, thickener, water retaining agent and sweetener. It also can be used in the pharmaceutics as drug carrier, stabilizer and auxiliary therapeutic agent for some diseases such as diabetes and constipation [3]. It also considered as natural sources of potential intestinal antioxidants as well as prebiotics, which will be valuable in further studies and new applications of inulin-containing health products [4].

The inulin concentration enhances product texture and may significantly affect the sensory attributes of various food products. The physico-chemical significance of inulin is associated with its degree of polymerization that the short chain fraction of it possesses more solubility and contains more sweetness than the long chain oligosaccharides. Additionally, it can improve the mouth feel because of its properties related with those of other sugars [5]. Inulin, is fully proven to have a prebiotic function

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with the advantage of could be added to a wide range of commonly consumed foods such as yogurts, desserts, nutrition bars, cereals, ice cream, beverages, and others. Among its health benefits for the host is the reduction effect of lipids and cholesterol then a reduction in the hypertension risk. It has also been studied that the absorption of lactate in the colon, enhances the absorption of ions such as Mg, Fe, Ca and Zinc and favours both the digestive health and immune system [6]. In gums production, starch can be substituted with inulin as a gelling coagulant with dietary fibres. Unlike starch, inulin gel simplifies the manufacturing process because it can be formed at hot temperatures without prior term food preparation giving a slightly more elastic, softer, and sticky texture, enhanced the sour and sweet flavors without any effect on the colour. Therefore, inulin acted as a neutral flavouring and non-degradable ingredient that could be used as a coagulant to prepare resin jellies enriched with dietary fibres with a prebiotic effect [1].

Inulin is made up of many fructose units that are linked together by -2, 1-glycosidic bonds [7]. Fructose is a safe substitute for sucrose, which is related to a variety of diseases, such as diabetes and carcinogenesis [8]. Inulinases catalyzing the hydrolysis of plant inulin to produce inulo-oligosaccharides and fructose. Many microorganisms can produce Inulinases [9] particularly filamentous fungi, that have Inulinases have the ability of optimizing inulin hydrolysis in various food industries and alcohol, acetone and butanol production [10].

Simultaneous Saccharification-Fermentation (SSF) and Sequential Hydrolysis and Fermentation (SHF) are two methods for bioethanol production from inulin. SSF is a single-step process that accomplishes both hydrolysis as well as fermentation at the same time, while, the SHF process on the other hand, has two steps; first, enzymes are used to decompose raw plant residues into simple sugars then the fermentation of sugars to ethanol [11], these two processes could be accomplished without any pre-treatment in the case of *Saccharomyces cerevisiae* usage for ethanol production by inulin-rich raw materials such as cassava pulp [12].

To the best of our knowledge, it is the first study on inulinase production using *Aspergillus terreus* growing on (Dalia tubers and Chicory roots) mixture and its industrial application for bioethanol production.

1. Materials and methods

Materials

Chemicals

All chemicals such as bovine serum albumin, inulin, Sodium alginate and 3, 5-dinitrosalicylic acid were purchased from Sigma Company.

Microorganisms

The microbial isolates screened for inulinase production in this investigation were five *Aspergillus* species; *A. flavus* AUMC11629, *A. terreus* AUMC11628, *A. niger* AUMC11615, *A. ochraceous* AUMC8966 and *A. conjunctus* AUMC11636, while, *Saccharomyces cerevisiae* was used for production of ethanol.

Media

Two types of media were used; enhancement medium (g/l) which consists of (glucose, 10; Yeast extract, 1; Peptone, 5; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.5 and distilled H_2O) and fermentative medium (g/l) which contains ($NaNO_3$, 10; Yeast extract, 10; $MgSO_4.7H_2O$, 1; KH_2PO_4, 5 and distilled H_2O).

Methods

2.1. Identification of fungal isolates and Inoculum preparation

Fungal isolates were identified by AUMC (Assuit University Mycological Centre) then maintained on (PDA) medium. *Aspergillus terreus*was the potent species that achieved the high inulinase production so, chosen for further studies. For all experiments, 1 ml of the suspension of 2.0 x 10^6 spore/ml was firstly inoculated in the enhancement medium then shaking incubated for 48h at 30°C, then 5 ml of pellets were inoculated on the fermentative medium.

2.2. Screening the most inulinase producing Aspergillus species

The studied species were inoculated on inulin agar medium composed of (g/L): 10, inulin; 1.5, $NaNO_3$; 1, KH_2PO_4; 2, $(NH_4)2SO_4$; 0.1, $FeSO_4.7H_2O$; 0.5, $MgSO_4.7H_2O$; and 20, agar. After inoculation and incubation for three days at 27°C, the plates were flooded by 1% iodine for 10 min to allow absorbance of iodine [13] then, the formation of the clear zones around the microbial colonies indicating the presence of inulinase activity that hydrolyze inulin of the medium.

2.3. Screening of the agro-industrial residues for the highest inulinase synthesis by Solid state fermentation

10 g of dried Dalia tubers, Chicory roots and a mixture of them (Mixture, DC) in various ratios were used for Fermentation. After washing and blending, these residues were used as sole carbon sources. Fermentative medium with pH 6.0 was and inoculated by fungal pellets then incubated at 30°C for 7 days [14]. The contents of each flask were gathered and filtered after ending the fermentation process. The produced filtrate was used for the determination of inulinase activity in order to determine the best plant residue and fungus species for further studies.

2.4 Analytical methods

In the enzyme preparations, the protein content, inulinase activity and reducing sugars were estimated according to [15,16 and 17], respectively. According to Rastogi and Shrivastava (2017) [12], one unit of inulinase (U) was defined as the amount of enzyme which produced 1 µmol/min of fructose. The reducing sugar in the reactant mixture was determined as a percentage of fructose content, while, inulin hydrolysis was estimated according to the following equation; (Reducing sugar (%) / total sugar) x 100. Total sugar in hydrolyzed products was determined according to Miller (1957) [18].

The concentration of ethanol (g/L) was determined according to the redox back titration method described as follows; into a conical flask, a mixture of 20 ml supernatant 1:25 & distilled water, 20 ml of potassium dichromate (0.04 N) and 10 ml H_2SO_4 (40%) was added, then the mixture was heated at 45 ºC in water bath for 10 minutes after covering with stopper and cooling the flask, then 2g of potassium iodide were added and titrated with sodium thiosulfate solution (0.01 M) the appearance of pale yellow colour and then added 1 ml of (0.1%) starch solution and continue titrating until reaching the endpoint by disappearing the blue colour with turning the reaction to clear green. The sodium thiosulfate volume (end point) was recorded for calculating the ethanol concentration.

2.5. Optimization of inulinase production conditions

A mixture of our agro-industrial wastes consists of Dalia tubers and chicory roots was added to the fermentative medium in various ratios (w/w) to determine the best ratio which next was supplemented with 1% (w/w) different inorganic and organic nitrogen sources. Physical parameters such as pH, incubation period, temperature and moisture level were optimized for the highest enzyme production. 5 ml pellets of the studied fungus were used then incubated as previously stated, finally, enzyme activities were estimated.

2.6. Enzyme purification

Cell-free dialysate (CFD) Aliquots were separately treated with $(NH_4)_2SO_4$ in saturation range from 0.5 to 0.9, acetone, methanol, iso-propanol and ethanol in different ratios of (alcohol: CFD). The dialyze was chromatographed through Diethylaminoethyl Sepharose column according to Scott and Melvin (1953) [19]. Proteins were eluted with linear gradient of NaCl. Active fractions were lyophilized before subjecting to gel filtration using a Sephadex G-100 column. Inulinase activity and protein concentration of each fraction were estimated as previously stated, after that, active fractions were dialyzed, lyophilized and stored for further studies at 0ºC.

2.7. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out according to Laemmli (1970) [20].

2.8. Enzyme immobilization by encapsulation technique:

The natural polysaccharide organic carrier (Sodium alginate) was used for inulinase immobilization via encapsulation technique as follows: 2% sodium alginate solution was used for the preparation of the anionic 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. sodium alginate gelation is instantaneous via calcium chloride. The capsules were rinsed with 100 mM acetate buffer pH 4.8 and hardened in 2% calcium chloride for 2 h at 4 ºC. The capsules were rinsed again with acetate buffer and incubated in 1.25% glutaraldehyde for 30 min at 4 ºC to activation the alginate beads. The activated alginate beads were contacted with inulinase solution (1ml, 140.43 Units/gm carrier) and the residual inulinase activity in the solution was assayed. Immobilized enzymes were kept under refrigeration. Then, they were washed with distilled water and acetate buffer for the last wash, then immersed in calcium chloride solution (0.05 mol/L).

The immobilization yield was calculated by the following equation:

$$\text{Immobilization yield (\%)} = \frac{\text{Immobilized enzyme}}{\text{Enzyme added–Unbound enzyme}} \text{ x } 100$$

2.9. Free and immobilized inulinase Catalytic properties

The following catalytic properties should be investigated in order to estimate the efficiency of novel carriers for immobilisation to be used in industries.

2.9.1. pH and pH stability

The ideal pH of both free and immobilized inulinase activities should be estimated, the reaction mixture was incubated under the various pHs (2-8) in the presence of our agricultural mixture as a carbon source for 20 minutes at 50°C. pH stability (at 3-9) for both free and immobilized inulinase was investigated under conditions that previously stated, then the residual activity was measured after incubation of the reaction mixture at 50 °C for 1 hour.

2.9.2. Temperature and thermal stability

The optimum temperature of both free and immobilized inulinase was determined as follows, 0.1ml of inulinase were incubated with 0.9 ml of inulin 0.5% (w/v) at pH 5.0 for 20 minutes and temperatures degrees from 25 to 70 °C. The optimum temperature has been taken as hundred percentage activity and the relative activity is expressed as a percentage of the 100% activity at each temperature. To prove the immobilized inulinase thermal stability, inulinase was incubated for a period of two hours in the buffer solution at 50 and 60 °C and then enzyme activity was examined as previously mentioned.

2.9.3. Operational stability of the immobilized inulinase

The operational stability of the immobilized enzyme was determined using this procedure; overnight, the enzyme was immersed in a calcium chloride solution containing 0.05 mol/L. After 20 minutes of incubation at 50 ºC, 0.5 percent inulin was added to allow the reaction to begin, and the reactive mixture was analysed as previously described. Finally, the mixture was filtered and washed by distilled water, experiments were repeated with the conditions previously mentioned.

2.10. Applications of Aspergillus terreus inulinase

2.10.1. Hydrolysis of inulin

According to Palmer (1991) [21], inulin hydrolysis was measured by adding immobilized inulinase (4 percent w/v) inulin in the presence of sodium acetate buffer (pH 5.2) and incubating for 0 to 120 minutes at 45°C. Fructose concentration (percent) in the reaction mixture was assessed as a reducing sugar, and inulin hydrolysis was estimated using the following equation: (Reducing sugar (percent) / total sugar) x 100. Determining of total sugar was measured according to the method of Saber and El-Naggar (2009) [22].

2.10.2. Ethanol production

Production of ethanol in the current study was carried out using two processes. The first was the Direct Conversion (DC) of inulin into ethanol by immobilized inulinase produced by *Aspergillus terreus* that degraded inulin to fructose then fermented to ethanol, while, the second process was Separate Hydrolysis and Fermentation (SHF) which involved inoculating free and immobilized inulinase with *Saccharomyces cerevisiae* at 120 rpm for 48 hours. After the fermentation process, the fermented cultures were centrifuged for ten minutes at 4 °C and 8496 xg then in supernatant, the total and reducing sugars as well as ethanol concentration were determined as described previously. Finally, according to Dysseler et al. (1999) [23], ethanol productivity (g/L/d) and percent saccharification were calculated as the following equations:

Ethanol productivity (g/L/d) = Ethanol concentration (g/L) / Fermentation time (day).

% Saccharification = ((Reducing sugars (g/L) x 1.15)/ initial substrate concentration (g/L) x 100.

2.11. Statistical validation of treatment effects

According to the mathematical principles described by Glantz (1992) [24], the mean and standard deviation of the three replicates of the investigated factors and the control were computed.

2. Results and Discussion

3.1. Screening of the agro-industrial wastes and fungal species

Inulinase can be made from a variety of substrates, but carbohydrate-rich feed stocks are preferable to pure substrates. Dahlia tubers, Chicory roots, and a mixture of both were employed in this work as inulin-rich substrates for fructofuranosidase synthesis. The carbon sources were chosen based on their potential to produce inulinase. Maximum activity of inulinase 410 U/ml was found by 5:1 (w/w) ratio of Dahlia tubers with Chicory roots (Table1&2). Without a doubt, the increasing popularity of microorganisms from numerous sources has the potential to develop industrially significant microbial products. Therefore, the development of novel strains capable of producing enzymes like inulinase could make a substantial contribution. After 7 days of incubation at 45 ºC, it was discovered that *Aspergillus terreus* AUMC 11628 was the most promising species from the five selected *Aspergillus* species for extracellular inulinase production (410 U/ml) when grown on the studied agricultural mixture under the previously described conditions.

2.2. Optimization of the highest inulinase production conditions

The selection of acceptable substrates in the most favourable concentrations and the adjustment of production conditions are critical when developing a fermentation process because of their impact on the process's economics and feasibility. Many studies have been undertaken with the goal of adopting low-cost optimization approaches to achieve high-yielding output. Traditional optimization methods were mostly used in the improvement tactics used in the manufacture of microbial enzymes. Traditional optimization procedures entail changing one component at a time, according to various sources [25].

2.2.1. Ratios of Dahlia tubers with Chicory roots in the agro-waste mixture

The optimal mixture component ratio for increasing inulinase activity in the fermentative medium was 5:1 (Dahlia tubers: Chicory roots w/w, respectively) (Table 2). Various optimum concentrations of plant extracts as carbon sources used for inulinase production were 5% for sunflower and 3% for Jerusalem artichoke as mentioned previously by Gill et al. (2003) [26] and these concentrations are primarily determined by the plant type [27].

2.2.2. Nitrogen sources

When the effects of organic and inorganic nitrogen sources on *A. terreus* inulinase production were investigated, it was discovered that peptone was the optimum nitrogen source at a concentration of 6.0 g/l. This was in contrast to Yu et al. (2009) [28] who stated that yeast extract was the optimum at 5g/L for the maximum inulinase production using *Aspergillus niger*.

2.2.3. Incubation periods

The most effective incubation period for inulinase production was seven days which corroborated the findings of Gill et al., (2003) [26]. After this time, the

enzyme activity continued to drop gradually because of nutritional deficiency (substrate constraint) or the accumulation autotoxic compounds in the media.

2.2.4. Initial pH

pH and temperature were established to be important parameters controlling the microbial synthesis of most substances. Recent studies have found that the optimum pH for inulinase synthesis by most fungal species ranges from 4.0 to 8.0 [29]. In the current work, the optimal pH of the highest inulinase activities was estimated as 4.0.

Table 1. Screening of the potentiality of some *Aspergillus* species for Exo-inulinase production using Dahlia tubers and Chicory roots as sole carbon sources by solid state fermentation.

Waste / Fungal isolate	Dahlia tubers			Chicory roots		
	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 niger	250±0.7 [a]	3.6±0.7 [b]	69.4. [a]	250±0.6 [b]	3.1±0.3 [a]	80.6 [c]
A. flavus	280±0.6 [b]	3.9±0.7 [c]	71.7 [b]	275±0.6 [c]	3.3±0.3 [b]	83.3 [c]
A. terreus	310±0.5 [c]	2.9±0.3 [a]	106.9 [d]	300±0.6 [d]	3.4±0.3 [b]	88.2 [d]
A. conjunctus	230±0.3 [a]	3.5±0.7 [b]	65.7 [a]	180±0.6 [a]	3.0±0.3 [a]	60.0 [a]
A. ochraceous	260±0.4 [b]	3.1±0.7 [a]	83.9 [c]	230±0.6 [b]	3.0±0.3 [a]	76.7 [b]

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

Table 2. Screening of the potentiality of *Aspergillus terreus* AUMC 11628 to produce Exo-inulinase in the culture medium containing Dalia tubers, Chicory roots and a mixture of both at different ratios.

Plant residues	Inulinase activity Units/ml	Protein (mg)	Specific activity Units/mg protein
Dalia tubers	310±0.5 [a]	2.9±0.3 [b]	106.9 [b]
Chicory roots	300±0.6 [a]	3.4±0.3 [c]	88.2 [a]
Mixture of Dahlia tubers (D) and Chicory roots (C)			
Ratios of D:C (w:w)			
1:1	315±0.5 [a]	2.9±0.6 [b]	108.6 [b]
1:2	320±0.5 [a]	2.9±0.3 [b]	110.3 [b]
1:3	321±0.8 [a]	2.5±0.4 [b]	128.4 [b]
1:4	321±0.4 [a]	3.0±0.4 [c]	107.0 [b]
1:5	324±0.5 [a]	3.0±0.8 [c]	108.3 [b]
1:6	327±0.6 [a]	2.7±0.9 [b]	121.1 [b]
2:1	333±0.7 [b]	3.0±0.1 [c]	111.0 [b]
3:1	350±0.4 [b]	3.1±0.7 [c]	112.9 [b]
4:1	378±0.5 [c]	2.7±0.7 [b]	140.0 [c]
5:1	410±0.3 [d]	1.9±0.9 [a]	215.7 [d]
6:1	380±0.4 [c]	2.5±0.6 [b]	152.0 [c]

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

2.2.5. Temperature

A. terreus in this study was incubated at various temperatures, with a maximum inulinase activity of 410 U/ml obtained at 45°C (Table 3). It was established that 30°C and 28°C were the optimum temperatures causing maximum inulinase synthesis from *Kluyveromyces marxianus* and *Penicillium citrinum*, respectively which disagrees with that suggested by Selvakumar and Pandey (1999) [30] in which *K. marxianus* has the optimum temperature of (37°C).

3.2.6. Moisture level

Highest activity of inulinase 410 U/ml was recorded at 50% moisture. Similarly, Mahesh et al. (2013) [31] discovered that a moisture content of 40%

was ideal for *Aspergillus niger* inulinase synthesis when banana peel was used as a carbon source.

Table 3. Optimum cultural conditions for the best Exo- inulinase production.

Property	Parameters
Optimum ratios of Dahlia tubers and Chicory roots in the waste mixture	5:1 (w/w)
Optimum nitrogen source	Peptone
Optimum incubation period (days)	7
Optimum initial pH	4.0
Optimum temperature (ºC)	45
Optimum moisture level (%)	50

2.3. Enzyme purification

Protein precipitation from the cell-free dialysate is the first step in the enzyme purification. In terms of obtaining protein fractions with the highest overall inulinase activity, isopropanol (1:1 v/v) was shown to be preferred. With yields of 82.01 percent of the original activity, this resulted in a purification fold of 9.23 (Table 4).

Table 4 summarizes the inulinase purification steps. The precipitated inulinase was purified using a Sephadex G-100 gel filter. Inulinase was purified at this stage with yields of 34.92 percent and a specific activity of 3100 U/mg protein (Table 4). Purification was terminated by ion exchange using DEAE-Sepharose, with yields of roughly 28.86 percent, the specific activity of 10838.46 U/mg protein, with a purification fold of 45.5. (Table 4).

Isopropanol's superiority in getting the protein samples with the highest enzyme activities demonstrated that inulinase has a good structure that permits it to resist denaturation by organic solvents. Organic solvents have already been mentioned as being suitable in this regard in a number of articles.

Table 4. Purification steps of Exo-inulinase from *Aspergillus terreus* AUMC 11628 grown on a mixture of Dalia tubers and Chicory roots

Purification step	Total activity Units/ml	Total protein (mg)	Specific activity Units/mg protein	Yield %	Purification fold
Cell free filtrate	488.27	2.05	238.18	100.00	1.00
Cell free precipitate Isopropanol: filtrate (1:1 v/v)	400.43	0.182	2200.16	82.01	9.23
Gel filtration Sephadex G-100	170.5	0.055	3100.00	34.92	13.02
Ion exchange DEAE-Sepharose	140.9	0.013	10838.46	28.86	45.50

3.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The homogeneity of inulinase produced by *A. terreus* 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 80 kDa. This value is in accordance with Housseiny (2014) [15] who found that the relative molecular mass of inulinase from *Aspergillus niger* was 75 kDa.

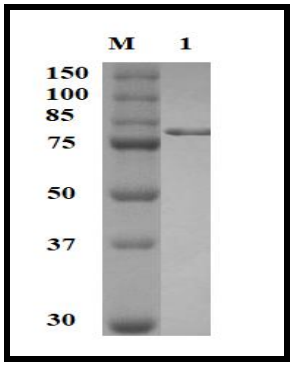

Fig. 1 SDS-PAGE analysis of the purified exo-inulinase. Lane 1, Inulinase sample. Lane M, molecular weight standards (Marker).

3.5. The general performance of inulinase by encapsulation

Tables 5 and 6 show that inulinase was immobilized successfully with immobilization yield of 75.65%, a best specific activity of 11430.76U/mg protein, and a retained activity of 93.38 % compared to the free inulinase specific activity of 10838.46U/mg protein. As a result, the immobilized enzyme had unique catalytic characteristics compared to the free one. The mentioned catalytic discrepancies may be attributed to the conformational changes and protein's mass diffusion coefficients as well as the substrate's interaction with the polymer matrix.

3.6. Properties of both free and immobilized inulinase on sodium alginate beads

3.6.1. pH and pH stability

Figure 2 indicates that free inulinase is most active at pH 4.0, whereas the ideal pH for immobilized inulinase has been decreased to alkaline range of (pH 6.0). This pH change is due in part to differences in the microenvironment, such as the surface charge of the carrier material. As demonstrated in Fig. 3, the immobilized enzyme was stable in pH ranges of (5 - 7.5) while the free enzyme was stable in pH ranges of (4.0 - 6.0).

Table 5: Immobilization of inulinase by encapsulation technique:

Immobilization technique	Carrier	Enzyme added (U/g carrier)	Unbound enzyme (U/g carrier)	Immobilized enzyme (U/g carrier)	Immobilization yield (%)
Encapsulation	Alginate beads	159.14	52.08	80.99	75.65

Table 6: The overall performance of the free and immobilized inulinase by encapsulation technique:

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	0.013	159.14	12241.53	-	0.70	0.015
	Immobilized inulinase	0.013/g carrier	148.60	11430.76	93.38	1.56	75.65

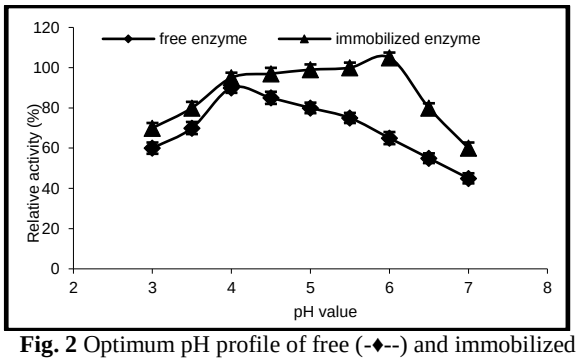

Fig. 2 Optimum pH profile of free (-♦--) and immobilized (▲-) inulinase.

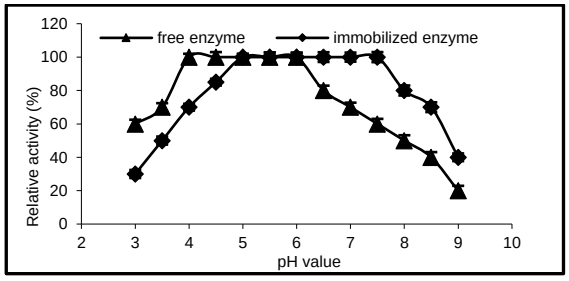

Fig. 3 pH stability profile of free (-▲-) and immobilized (-♦-) inulinase.

3.6.2. Temperature and thermal stability

Optimum temperature for the immobilized inulinase climbed to 60 °C in this investigation, compared to 45 °C for the free enzyme, as shown in Fig. 4. The creation of a molecular cage around the enzyme's molecules, which insulated the enzyme's molecules from the bulk temperature, could explain this shift in the enzyme's optimal temperature after immobilization. The temperature of 60 °C is high enough to be employed on an industrial scale to limit microbial contamination and to allow for higher sugar concentrations, which are optimal for inulinase to create fructooligosaccharides.

Figure 5 shows how immobilization improves the enzyme's heat stability. As a result, a thermostable inulinolytic enzyme is likely to play a key role in the fructose food industry. The results showed that the free enzyme activity dropped to 8% at 60 °C after two hours, while, the immobilized inulinase retained 90% of its activity (Fig. 4) which more suitable to be employed in most industrial applications [27].

3.7. Stability of stored inulinase under refrigeration (4 °C):

Immobilized inulinase activity after twenty days at 4 °C dropped from 160 to 145 units (Fig.6) however,

the free inulinase activity reduced considerably, from 140 to 95 units. These data revealed that the factory lacked ideal or convenient storage conditions for free inulinase, resulting in them failing to purchase all of the inulinase needed early in the season. This problem also highlights the importance of factory workers being able to monitor the enzyme activity all the season.

3.8. Operational stability of the immobilized inulinase

Enzyme stability is considered as a major consideration in the creation of repeatably stable biocatalysts and the fundamental primary advantages of enzyme immobilization are the facility of enzyme separation and reusability (operational stability) as well as the stabilization of the immobilized enzyme which can be tested using a shelf-life stability test. Enzyme stability is always a major factor during the creation of stable bio enzymes. The operational stability of the immobilized *A. terreus* inulinase was tested in a batch process as in (Fig. 7).

The operational stability of the immobilized *Aspergillus terreus* inulinase was investigated in a batch method (Fig. 7). According to the findings, following the fifth cycle, the concentration of reducing sugars produced decreased (15.2 %). This drop-in activity could have been caused by the release of untigthly bound enzyme from the carrier. Inulin hydrolysis decreased somewhat from the fourth to the fifth cycle (by just 1.3 percent compared to four cycles), which may be happened because of enzyme inactivation as a result of continuous use.

The hydrolysis efficiency gradually fell after that, culminating at roughly 50.3 percent in the ninth cycle. The immobilized inulinase can maintain a best level of reducing sugar synthesis after the 9th cycle (50.3 percent). This revealed that, for nine cycles, we could use the bounded enzyme with reducing the sugar yield to 50.3 percent on average. These observations might be a result of ionic interactions formation between the enzyme and the gel polyelectrolyte complexes that increased the immobilized enzyme stability.

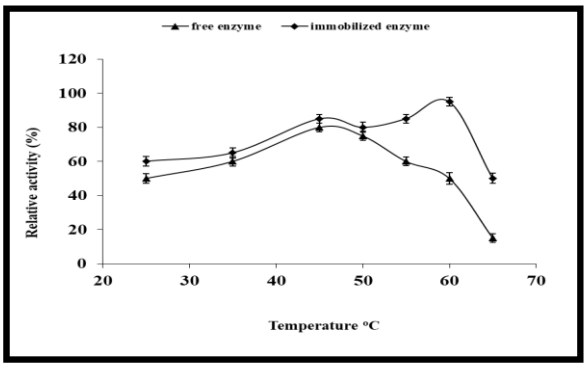

Fig. 4 Optimum temperature profile of free (-▲-) and immobilized (-♦-) inulinase

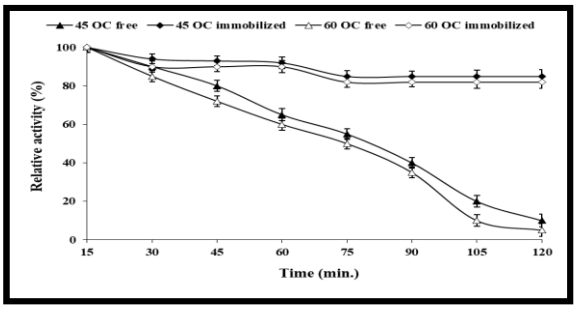

Fig .5 Thermal stability profile of free (-▲-) and immobilized (-♦-) inulinase

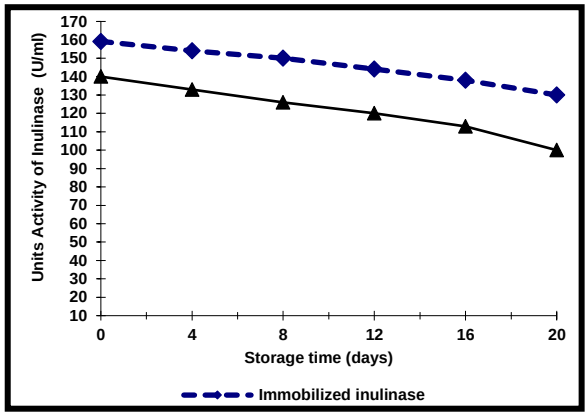

Fig. 6 Changes in activity of free and immobilized inulinase stored under refrigeration °C over 20 days.

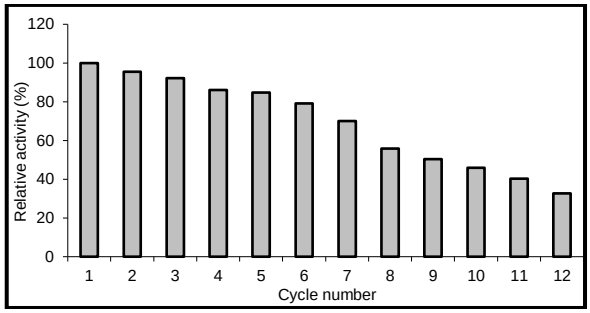

Fig. 7 Operational stability of immobilized *Aspergillus terreus* inulinase.

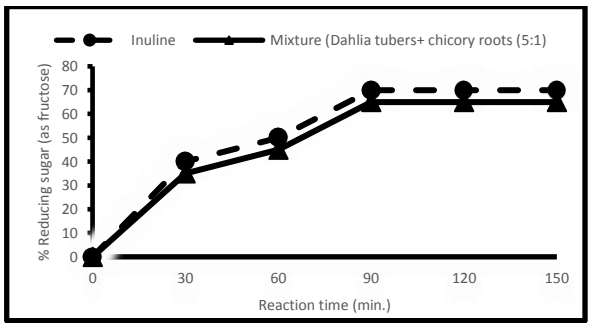

Fig. 8 % inulin hydrolysis by *Aspergillus terreus* crude inulinase during various reaction time.

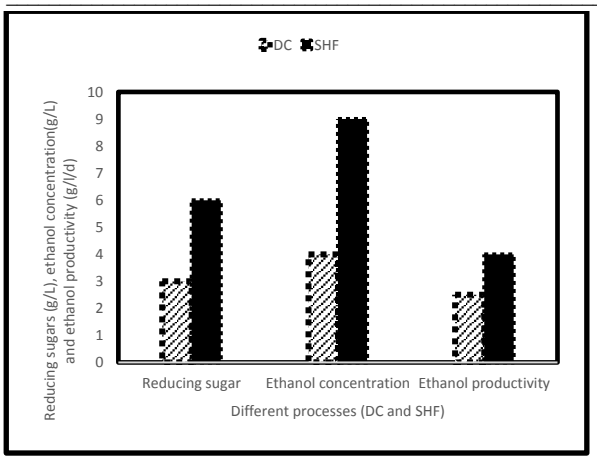

Fig. 9 Inulin saccharification and ethanol production from inulin by *Aspergillus terreus* inulinase using different processes.
DC= direct conversion of inulin into ethanol by *A. terreus*
SHF = Separate Hydrolysis and Fermentation

3.9. Applications of microbial inulinase in
3.9.1. Inulin hydrolysis

Inulinase of *Aspergillus terreus* has beneficial effect of environment that it can hydrolyse inulin in plant residues. The percent of inulin hydrolysis by immobilized inulinase produced from *Aspergillus terreus* cultivated on Dalia tubers & chicory roots mixture in a ratio of (5:1) achieving maximum values (50 and 45 percent of fructose) after one hour on pure inulin and inulin of our mixture in the presence of *Aspergillus terreus*, respectively (Fig. 8). Whereas the percent of fructose content was found to be more consistent as the reaction time was increased from 90 to 150 minutes. These results are consistent with Singh and Chauhan (2016) [32], which suggested that after 120 minutes of reaction time, % inulin hydrolysis using *A. tamarii* inulinase on Jerusalem artichoke, pure inulin, dahlia tubers and chicory roots was 71.64, 66.81, 67.55, and 55.11 percent, respectively. Sirisansaneeyakul et al. (2007) [33] also discovered that mixing inulinase from *C. guilliermondii* and *A. niger* resulted in 28 percent of Jerusalem artichoke tubers inulin hydrolysis after a reaction of 25-hour. Furthermore, it was claimed that *A. niger* inulinase after 30 minutes achieved 72 percent chicory inulin hydrolysis.

3.9.2. Ethanol production by Aspergillus terreus inulinase

In this procedure, ethanol could be produced via two processes; the first, direct conversion (DC) of inulin from our plant mixture (Dahlia tubers and Chicory roots) to ethanol by *Aspergillus terreus*, and the second, Separate Hydrolysis and Fermentation (SHF), where inulin hydrolysis was fermented to ethanol by *Saccharomyces cerevisiae*. As shown in Fig. 9, Inulin was fermented directly to ethanol by *Aspergillus terreus* by (DC process) yielding a low value of ethanol (4.0 g/L) and a productivity of 2.5 g/L/d due to the *Aspergillus terreus'* poor ability of inulin fermentation to ethanol, that was similar to *K. marxianus'* ability to directly produce ethanol after inulinase in fermented medium, by low amount when compared to *S. cerevisiae*, as stated by Chi et al. (2011) [34].

Whereas the SSF process produced much higher ethanol quantities (9.0 g/L) and productivity of (4.0 g/L/d) than the DC process. It's possible that the reducing sugar collected in the SHF process as a result of hydrolysis inhibits or reduces enzyme activity, as suggested by Margeot et al. (2009) [35]. It was discovered that using the SHF method for production of ethanol from Jerusalem artichoke tubers and pure inulin increased the yield with about 3 and 14 times, respectively, when compared to other methods, also, Suttikul et al. (2016) [36] found that the SHF method was the most effective for producing ethanol from wastes of sugarcane with a yield of 16.3 percent greater than the conventional method.

4. Conclusion

In summary, this work is divided into two sections: the first is concerned with inulinase production. When using a blend of Dalia tubers and Chicory roots, we discovered that solid state fermentation acted as more effective method for inulinase biosynthesis than liquid state fermentation, producing (410 U/ml) with 6.5g/l peptone at 50% moisture after a 7-day incubation period. This study is significant since it is the first to show that *Aspergillus terreus* can synthesize inulinase using SSF from our mixture. When compared to other inulin sources, this blend provides a high supply of inulin that is readily available and inexpensive. Furthermore, by facilitating the synthesis of inulinase, that has numerous industrial applications, this finding opens the door to commercialization and cost reduction.

The second step was to immobilize the enzymes, which raised their optimum temperature and thermal stability from 45 to 60^{o}C, which is the best temperature for preventing microbial contamination and allowing higher inulin substrate concentrations in the food industry because of increased solubility. Meanwhile, the free and immobilized inulinase activities were evaluated and compared, with good results for the immobilized enzyme in terms of thermal stability, pH stability, and operational stability, with recycling of immobilized inulinase for 10 cycles with no activity loss and consequently, the re-usability for tens of times that reduces the cost of enzymatic industries dramatically.

Inulinase from *Aspergillus terreus* was used to produce fructose via inulin saccharification, in addition to *S. cerevisiae* fermentation of inulin to ethanol via DC and SHF methods. When compared to DC, SHF produced excellent bioethanol yields.

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