Production and Immobilization of Halophilic Invertase Produced from Honey Isolate *Aspergillus niger* EM77 (KF774181)

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Abstract: Honey isolate *Aspergillus niger* EM77 was a good halophilic invertase producer in the presence of wheat bran as a complete medium (114.55 U/g), using solid state fermentation technique. Different parameters influence the enzyme productivity such as different pH values, temperature, incubation period, nitrogen and carbon sources were investigated. The optimum pH, temperature and incubation period for enzyme production were 5.5, 30°C and 72 hrs, respectively. Sucrose at 2 % was more suitable carbon source for invertase production (144.39 U/g) and $(NH_4)_2SO_4$ at 0.15 % was the ideal nitrogen source. Among different metals ions $MnSO_4$ enhanced the enzyme productivity than other tested ions to 194.71 U/g. The partially purified enzyme was successfully entrapped in polyvinyl alcohol sponge shielded with agar starch layer (PVAsp Gs) and achieved 71% immobilization yield. The optimum conditions for immobilization were: pH 5.2, an incubation time of 15 min and a protein concentration of 250 mg/ml. Immobilized enzyme was reused 12 times with 29% activity loss. The free enzyme lost its activity completely at 70°C after 45 minute and the immobilized form retained 80% of its activity at the same condition.The free and immobilized form reported extreme halophilic property since the highest enzyme activity was obtained between 3.5- 5 M.

Keywords: Honey isolate, invertase, *Aspergillus awamori*, immobilization, entrapment.

1. INTRODUCTION

Extreme environments are too harsh for normal life to exist, but a variety of bacteria and fungi could survive. These organisms have evolved to exist in these extreme environments and fall into a number of different categories, including halotolerant, moderately, borderline and extremely halophilic [1]. In the last few years, honey isolates take attention as new source for enzymes with unique feature [2, 3]. Since the halophilic enzymes possess salt-enriched solvation shells [4], they are constructed to retain catalytic activity in environments with low water activity, such as in the presence of high levels of organic solvents. Such properties could be of interest in a variety of applications [5].

Invertase is a commercially important enzyme known as a (β-D-fructofuranoside fructohydrolase, β-fructofuranosidase, sucrase, invertin, saccharase; EC 3.2.1.26) catalyses the irreversible hydrolysis of non-reducing fructofuranosidic terminal. The hydrolysis of sucrose yields an equimolar mixture of glucose and fructose, known as invert syrup, is widely used in food and beverage industries, residues of sucrose and related glycosides [6, 7]. Hence, one of the important applications of invertase lies in the production of non-crystallizable sugar syrup from sucrose. Due to its hygroscopic nature, invert syrup is used as a humectant in the manufacture of soft candies and fondants. Invertase is also used whenever sucrose containing substrates are subjected to fermentation viz. production of alcoholic beverages, lactic acid, glycerol etc. Due to the associated inulinase activity, it is also used for the hydrolysis of inulin (polyfructose) to fructose. Other uses of the enzyme include, manufacture of artificial honey, plasticizing agents used in cosmetics, drug and paper industries and as enzyme electrodes for the detection of sucrose. Invertase is also able to catalyze the hydrolysis of raffinose and stachyose [8, 9]. In addition it may be used to obtain fructooligosaccharides (FOS) used as prebiotic substance [10].

The production of invertase by filamentous fungi under submerged (SbmF) or solid-sate fermentation (SSF) has been reported [11, 12]. SSF is characterized by development of microorganism in a low aqueous content on a non-soluble material that can act as physical support and in some times also as nutrient sources [13]. Generally, the enzymatic production in SSF has advantages over SbmF, as higher productivity

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fermentation, absence of contaminant organisms, concentrated product formation and use of agro-industrial residues as substrates [14].

Enzyme immobilization is undertaken either for the purpose of basic research or for use in technical processes of commercial interests [15, 16, 17]. Immobilized enzymes offer advantages over the use of conventional chemical catalysts as they exhibit higher catalytic activity, higher degree of specificity could be produced in large amounts and are economically viable [18]. The use of safety and low cost matrices allows the immobilization method to be achieved successfully in industrial food field without additional production cost. Immobilization by entrapment is based on the localization of an enzyme within the lattice of a polymer matrix or membrane. The feasibility of using synthetic sponge as a carrier for immobilization was reported by many authors [19]. The lattice structure of polyvinyl alcohol (PVA) sponge characterized by very dense porosity and specific pore volume are very high. These properties recommended PVA sponge strongly to be used for the enzyme entrapment [20].

In this study, the honey isolate *Aspergillus niger* EM77 was a good invertase producer in the presence of wheat bran as the sole nitrogen and carbon source. The parameters influencing the enzyme productivity such as incubation time, temperature, pH, carbon and nitrogen sources, in addition to metals ions were studied. The enzyme was partially purified and successfully immobilized in (PVAsp Gs), the properties of the free and immobilized enzyme was studied. The results indicated to the novelty of *Aspergillus niger* EM77 (KF774181) invertase as thermo-philic and extreme-halophilic enzyme. This features recommended it strongly to be applied in industrial field.

2. MATERIALS AND METHODS

2.1. Microorganism (Isolation & Identification)

The fungal honey isolate was previously isolated from Yamane honey bee collecting nectar from mountain flower. Honeys samples are fresh non treated ripe honey (directly collected in beehives). It was identified based on morphological characterization and 18S rRNA sequence analysis (data not published yet). It was designed as *Aspergillus niger* EM77 and Gene Bank database was achieved in BLASTN searches at the National Center for Biotechnology Information (NCBI) site (http://www.ncbi.nlm.nih.gov) and take an Accs. NO (KF774181).

2.2. Inoculum Preparation

Four-days-old slant was gently scratched with sterilized needle, then one ml of *A. niger* EM77 spore suspension was injected in Czapek's Dox medium (CDM) contained (g/L): 20.0 sucrose; 2.0 $NaNO_3$; 1.0 K_2HPO_4; 0.5 $MgSO_4.7H_2O$ and 0.5 KCl, the pH was adjusted to be 6.0. The culture was incubated in a rotary shaker at 150 rpm for three days at 30˚C. The fungal growth was used for inculating the experimental flasks.

2.3. Solid State Fermentation

Five grams of wheat bran in 250 ml Erlenmeyer flasks was thoroughly mixed with eight ml of the following medium (BM) (g/l): 2.0 $NaNO_3$; 1.0 K_2HPO_4; 0.5 $MgSO_4.7H_2O$ and 0.5 KCl. at pH 6.0. The flasks were autoclaved at 121ºC for 30 min. They were, cooled to room temperature and each flask was inoculated with 2 ml (10^6 cells) (CDM). The flasks were incubated statically for three days at 30˚C.

2.4. Enzyme Extraction

One hundred ml of distilled water were added to each flask and the culture was shaked for 60 minutes on a rotary shaker at 150 rpm. Then, the suspension was filtered on whatman filter paper No 1. The obtained filtrate was centrifuged and the supernatant was used to assay the invertase activity.

2.5. Enzyme Assay

The invertase activity was determined according to the method described by Miller (1959) [21] using 2% sucrose as substrate in sodium acetate buffer, 0.1 mM, pH 5.2. The reaction mixture was composed by 500 µl of substrate and 500 µl of enzymatic sample. The reaction: one ml of dinitrosalicylic acid reagent DNS was added to each tube and boiled for 5 minutes in boiling water bath. The obtained color was measured at 540 nm according to Miller (1959) [21]. One unit of enzyme activity (U) was defined as amount of enzyme that releases 1 µmol of glucose per min under the assay conditions. The values of enzymatic activity were expressed as U/g of substrate for SSF.

2.6. Effect of Different Temperatures

Incubation was carried out at different temperatures (25, 30, 35, 40 & 45°C) to study their effect on the invertase activity. Cultivation was carried out in BM medium.

2.7. Effect of Different pH Values

The pH of culture medium was adjusted with diluted HCl or NaOH from 2 to 9 pH. Cultivation was carried out in BM medium at the optimum temperature obtained from the above treatment.

2.8. Effect of Different Carbon Sources

In order to measure the effect of different carbon sources on *A. niger* EM77 invertase activity, the sucrose in BM medium was replaced with 2% (w/v) of different carbon sources (fructose, galactose, mannose, lactose, casein, glucose, cellulose and pectin). The cultivation was done at the optimum conditions.

2.9. Effect of Different Nitrogen Sources

Addition effects of equi-molar amounts from different inorganic nitrogen (Na_2NO_3, NH_4NO_3, $(NH_4)_3PO_4$, $(NH_4)_2HPO_4$, $NH_4H_2PO_4$, NH_3Cl, and KNO_3) and organic nitrogen (yeast extract or peptone) sources on enzyme production of fungal isolate were also studied. The cultivation was applied at the above optimum conditions.

2.10. Fractional Precipitation with Ethanol

Ethanol (30, 50, 70 and 90%) was added slowly to the ice-cold enzyme solution and left to precipitate for 30 minutes. After removing the precipitated fraction by centrifugation in a cooling centrifuge at 4000 rpm for 10 min, further ethanol was added to the supernatant fluid and the process was repeated until the final concentration of ethanol was reached (90%). Enzyme fractions obtained at 30, 50, 70 and 90% ethanol concentrations were dried over anhydrous calcium chloride, under reduced pressure at room temperature, weighed and used for invertase activity and protein estimation.

2.11. Protein Determination

This was determined by the method of [22]. Bovine serum albumin was used as standard protein.

2.12. Immobilization Technique

Polyvinyl alcohol sponge (PVA sp) was washed several times by distilled water, squeezed to eject water and was cut into small pieces (4 mm × 4 mm × 4 mm) using scissors. Then, each piece of synthetic sponge was inserted into the invertase fraction (40-80% ethanol enzyme fraction) (173 U/g carrier) dissolved in 2.0 ml of 0.2 M acetate buffer, pH 5.2 at 4˚C for 24 h. For PVA sponge shielding agar starch (PVAsp Gs), the sponge pieces were immersed in (18% agar+ 2% starch) liquefied solution at 45˚C, then it was removed, and the carrier was used after solidification.

Immobilization Yield (U/g Carrier)

It was calculated from the equation:

Immobilized enzyme (U/g carrier) = Immobilized enzyme activity (U/g carrier)/ Enzyme added (U/g carrier) - Unbound enzyme (U/g carrier) (%) [23].

Activity and Protein Determination

The protein concentration of the immobilized enzyme was estimated by taking into consideration the protein concentration in the initial solution and of the unbound protein. The amount of protein immobilized on to and into the gel carrier Pg (mg/g) was calculated using the following equation:

$$Pg= C_oV_o-C_fV_f/W$$

Where C_o is the initial protein concentration (mg/ml), C_f the protein concentration of the filtrate (mg/mL), V_o the initial volume of the enzyme solution (ml), V_f the volume of filtrate (mL), and w is the weight of gel carrier used (g). For determination of activity of the immobilized enzyme, one piece carrier equivalent to (0.3g) were incubated into 2 mL of 2% sucrose (w/v) for 15 min at 40°C and invertase activity was assayed as described above [24].

2.13. Operation Stability

It was performed with immobilized invertase on (PVAsp Gs) contained about 135U of the enzyme. The tested sample was incubated with 0.5 ml buffered sucrose solution (0.2 M acetate buffer at pH 5.2) at 40˚C for 20 min. At the end of the reaction period, it was removed, washed with distilled water and re-suspended in 0.5 ml of freshly prepared substrate to start a new run. The supernatant was assayed for invertase activity.

2.14. Optimization of the Immobilization Yield (%)

2.14.1. Loading Time

For optimum loaded time determination, the free enzyme (173 U) was loading in (PVAsp Gs) carrier and incubated at 4˚C for different periods (2, 6, 14, 18, 24,

36, and 48 hours). After loading time was finished, the carrier was washed twice thoroughly for 30 min with 0.2 M acetate buffer at pH 5.2 to get rid of any unbound enzyme and the usual assay has been implicated. Then, the invertase activity was determined for each time interval and expressed as immobilization yield (%).

2.14.2. Enzyme Loading Capacity

Different enzyme protein concentrations (69.4, 104.7, 138.8, 173.5, 208.8 and 242.9 mg) incubated on (PVAsp Gs) at 4°C for 18 h. Then the invertase activity was determined and expressed as immobilization yield (%).

Properties of free and immobilized invertase enzyme by *A. niger* EM77 (KF774181)

2.14.3. Reaction Time

The reaction mixture consisted of 0.5 ml buffered sucrose solution (0.2 M acetate buffer at pH 5.2) 2% and 0.5 ml of the enzyme solution (free enzyme) or one piece of immobilized enzyme. Enzyme reaction was conducted at 30˚C at different time intervals (5 – 30 min).

2.14.4. Effect of Different Temperatures

Identical reaction mixtures were incubated at different temperatures (30 – 80˚C) for 15 min at pH 5.2. The enzyme assay was done as discussed previously.

2.14.5. Thermal Stability

The effect of temperature on enzyme stability was carried out by preheating the invertase enzyme at different temperatures (40 – 85˚C) for different time intervals (15, 30, 40 and 60 min). At the end of the incubation time, the usual enzyme assay has been carried out at the preferable conditions for each of the partially purified and immobilized enzyme. Control was also carried out using the free and immobilized enzyme without preheating. Its activity was taken as 100%.

2.14.6. Effect of pH Value

The effect of pHs on the enzyme activity was investigated in 0.1 M citrate phosphate buffer (pH 5.0 - 7.0), 0.1 M phosphate buffer (pH 7.0 - 8.0) and 0.1 M Tris - HCL buffer (pH 8.0 - 9.0), using the experimental assay conditions.

3. RESULTS AND DISCUSSION

Recent advances in industrial biotechnology process are exploited for an economic utilization of wastes for producing valuable products. Studies have been carried out by using synthetic medium for invertase preparation. A little attention has been paid on its production from un-conventional inexpensive sources [25; 26; 27; 28]. Within this context, the honey isolate *Aspergillus niger* EM77 (KF774181) showed good invertase productivity in the presence of wheat bran as a complete medium (114.0199 U/g) using solid state fermentation technique. In this finding, it was reported that filamentous fungus *Aspergillus caespitosus* is a good producer of intracellular and extracellular invertases under SSF condition and wheat bran as a substrate [29]. The optimum pH, temperature and incubation period for enzyme productivity were pH 5.5, 30˚C and 72 h (data not shown). Similarly, *A. flavus* produced high levels of invertase under optimized culture conditions [30]. The maximum productivity was on 4th incubation day, pH 5.0, and 30˚C using fruit peel waste as a substrate. Also, it was reported that the optimum pH, temperature and incubation period for *Cladosporium Cladosporioides* invertase production were 4, 30˚C and 4th day respectively [31]. The best carbon and nitrogen sources for invertase productivity were sucrose at 2% (144.39 U/g) and $(NH_4)_2SO_4$ at 0.15 % (158.21 U/g), respectively (Figures **1**, **2**). The results were supported by the findings of [32] who reported that invertase production in some other fungi was induced by sucrose. It was reported in enhancement of *A. flavus* invertase production by addition of sucrose and yeast extract as nutritional factors [30]. The evaluating of different metal ions effect on *Aspergillus niger* EM77 (KF774181) showed that the highest productivity was achieved in the presence of $MnSO_4$ (194.71 U/g). Enzyme secretion was strongly inhibited by the presence of Hg^{2+} in the medium and to some extent by Cu^{2+}, Ca^{2+}, Fe^{2+} and Mg^{2+}, Pb^{2+}. Also, significant increase in the enzyme production was observed when Mn^{2+} was added in the medium (Figure **3**). Alves *et al*., [33] reported that invertases from the *Aspergillus nidulans* and *Emericela nidulans* were activated by Mn^{2+}. The enzyme was partially purified by 40-80 % ethanol and achieved two fold purification (Table **1**). In immobilization studies, the matrix characteristics and the mode of attachment of the enzyme to the matrix play an important role in determining the properties of the bound enzyme [6]. PVA sponge has a continuous pore structure featuring high porosity. This will cause the enzyme to leak out from the matrix. In this study, to overcome this problem the PVA sponge pieces was shielded with a thin agar starch layer (PVAsp Gs), the agar starch shielding method improved the

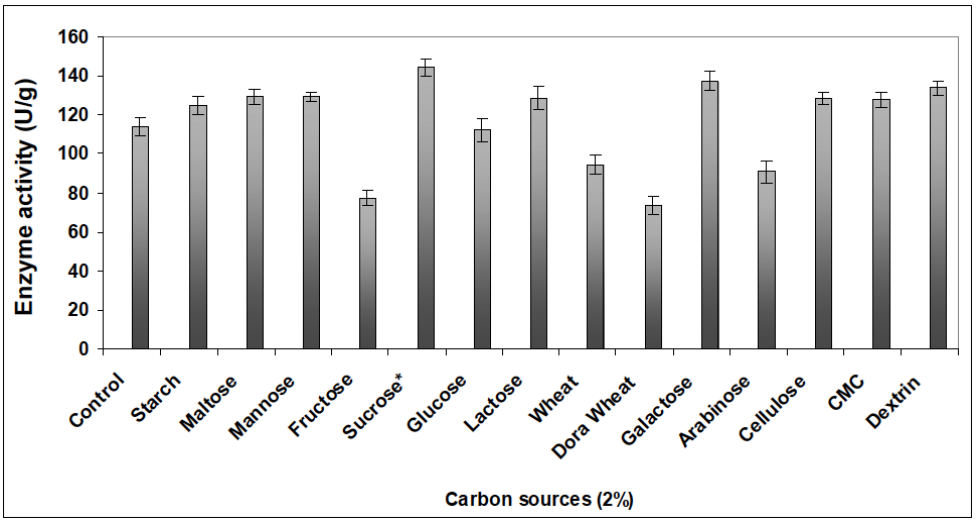


Figure 1: Effect of different carbon sources on *A. niger* EM77 (KF774181) invertase productivity.

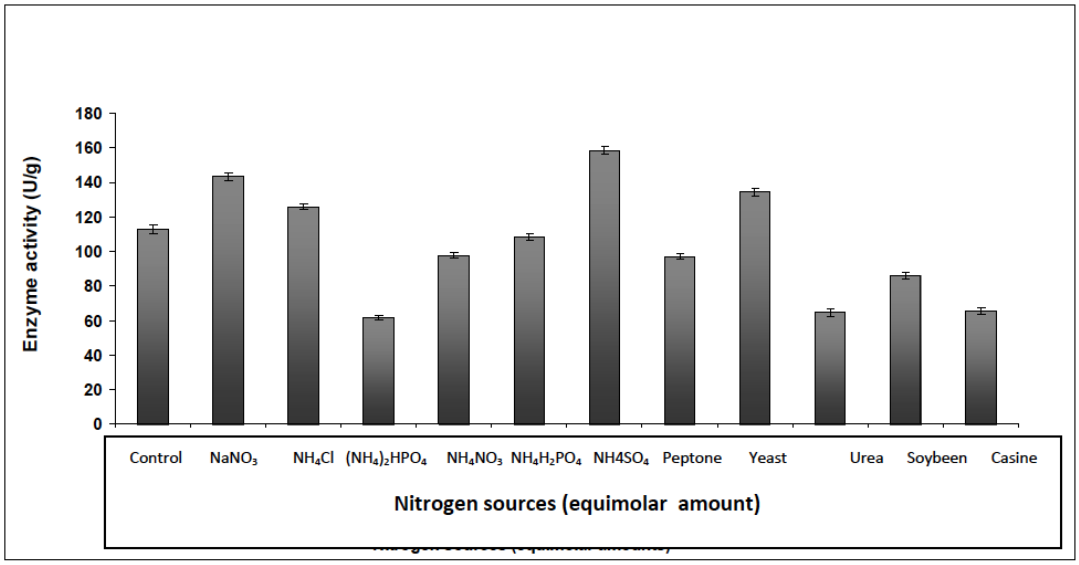


Figure 2: Effect of different nitrogen sources on *A. niger* EM77 (KF774181) invertase productivity.

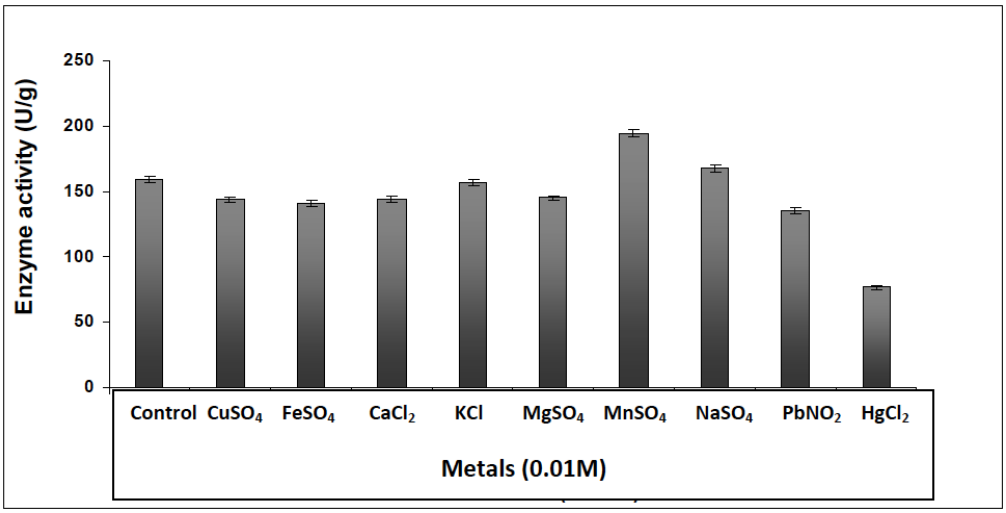


Figure 3: Effect of different metal ions on *A. niger* EM77 (KF774181) invertase productivity.

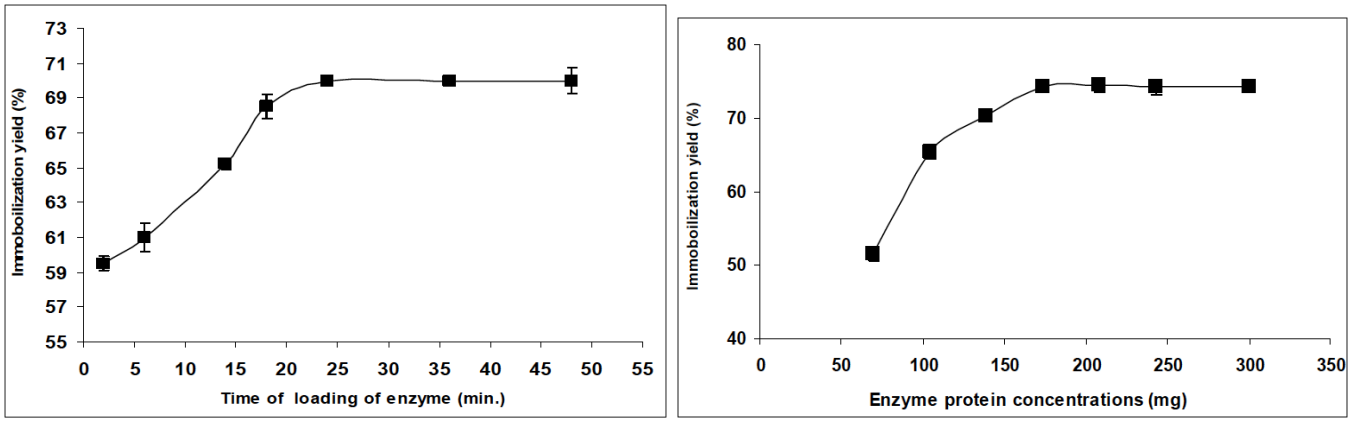
Table 1: Partial Purification of Invertase Enzyme by *Aspergillus niger* EM77 (KF774180)

Immobilization yield (%)	Purification fold	Specific activity U/mg	Total protein (mg)	Total unit (U)	Fraction precipitation with	
100	1	2.12	225	478	Culture filtrate	Ethanol
0.88	0.38	0.81	5.19	4.23	0-40	
25.76	2.73	5.80	21.21	123.18	40-60	
43.93	2.90	6.17	34.03	210	60-80	

Table 2: Immobilization of Invertase Produced by *Aspergillus niger* EM77 (KF774181) on (PVA) Sponge by Two Methods

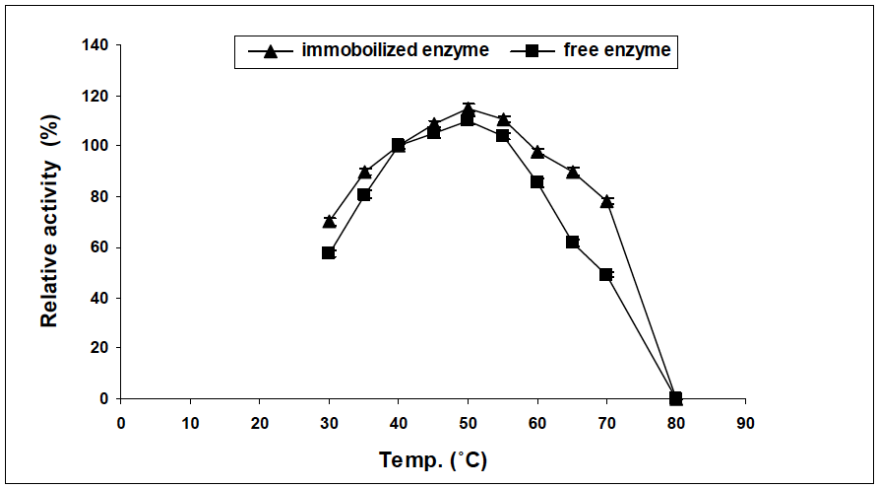
Carriers	Added enzyme (U) (A)	Protein of added Enzyme (mg) (C)	Bounded enzyme (U) (I)	Unbounded enzyme (U) (B)	Protein for bounded enzyme (D)	Protein for unbounded enzyme (C-D)	Immobilization yield (%) I/(A-B)%	
PVAsp	138.8	23.09	60.3	36.5	8.47	14.62	58.5	±1.83
PVAsp Gs	138.8	23.09	70.4	38.5	5.33	17.75	70.18	±1.27

immobilization yield from 58.9% in PVA sponge alone to 71% in (PVAsp Gs) (Table **2**). The specific activity of the immobilized enzyme was 13.12 U/ml compared to 6.03 free form. This result considered as partial purification step and could be attributed to sponge high porosity. It was reported in the sponge structure which helped in entrapment most of the pectinase letting the undesired protein abroad [20]. Similar result was obtained by Esawy and Combet-Blanc [34]. The highest loading capacity (71%) was reported at 18 h and remained constant till 48 h (Figure **4a**). The enzyme loading at 29.5 mg enzyme protein achieved the highest immobilization yield (75%) (Figure **4b**). The optimum pH and temperature of the free and immobilized enzymes remained unchanged at pH 5.2 and 50˚C (Figure **5a**, **b**). Similarly, it was reported that invertase immobilization in sawdust did not affect the optimum pH and temperature of the enzyme but it enhanced the pH and temperature stability [35]. As well as, It was reported that the immobilization did not affect the optimum pH but it increased the optimum temperature to 50°C compared to 45°C in case of the free enzyme [36]. Thermal stability was considered as a good enzyme criterion for industrial field. In this finding, thermal stability study was done at different temperatures (Figure **6a**, **b**). The result indicated that

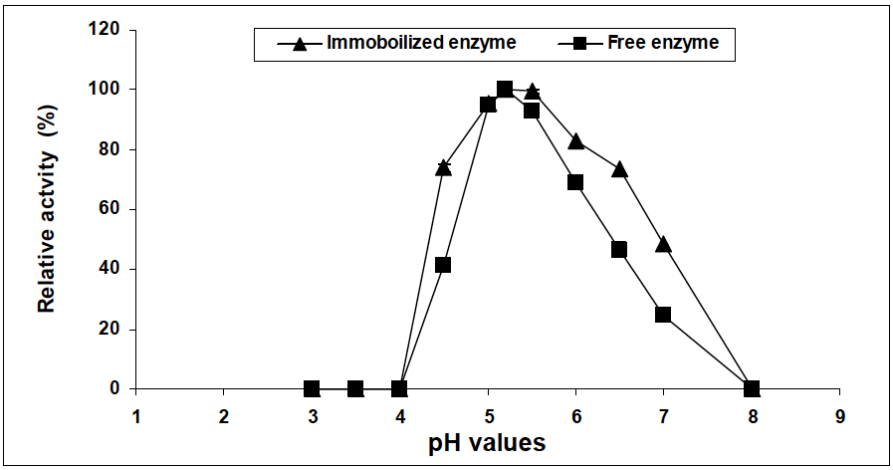


(**a**) (**b**)

Figure 4: Evaluation of different loading time (a) and enzyme protein concentrations on enzyme loading the result expressed as immobilization yield (%).



(**a**)



(**b**)

Figure 5: Effect of different temperatures (a) and pHs on the free and immobilized *A. niger* EM77 (KF774181) invertase.

the immobilization process improved the enzyme stability to great extent. Also, it showed that the free enzyme lost its activity completely at 70˚C after 45 minute. On the other hand, the immobilized enzyme kept 80% of its original activity at the same conditions. On the other side, the immobilized form could keep 18% of its activity at 80˚C after 15 min. Similar results was obtained by [36].

Halophiles represent valuable sources of various biomolecules which can offer potential applications for biocatalysis and biotransformation [37]. Within this context, the halo-tolerant property was studied for the free and immobilized form. The result indicated that both the free and immobilized form characterized by extreme halophilic property, since the highest enzyme activity was obtained between 3.5-5 M and the enzyme activity work with good efficiency till 6 M (Figure **7**). Halophilic enzymes, have identical enzymatic features like their non-halophilic counterpart, but they exhibit different properties mainly in structure. Among these, two main points could be mentioned, (i) a high content of acidic amino-acids located predominantly at the protein surface and (ii) requirement for high salt concentration for better biological functions Enache and Kamekura [1]. It was reported in yeast invertase isolated form saline soil that the highest invertase activity was observed in the medium containing 1.7 mM sodium chloride [38]. Mohmoud 2007 [35] reported that invertase kept 97% of its activity for a week even after washing with 6 M NaCl. Enzyme reuses is very important criteria to evaluate the immobilization process efficiency. The results showed that the immobilized enzyme was reused 12 times with 29% activity loss (Figure **8**). Awad *et al*., 2013 [24] reported that the reusability test proved the durability of the grafted alginate beads for 15 cycles with retention of 100% of the immobilized enzyme activity. All the previous properties recommended *Aspergillus niger* EM77 (KF774181) to be used in industrial field.

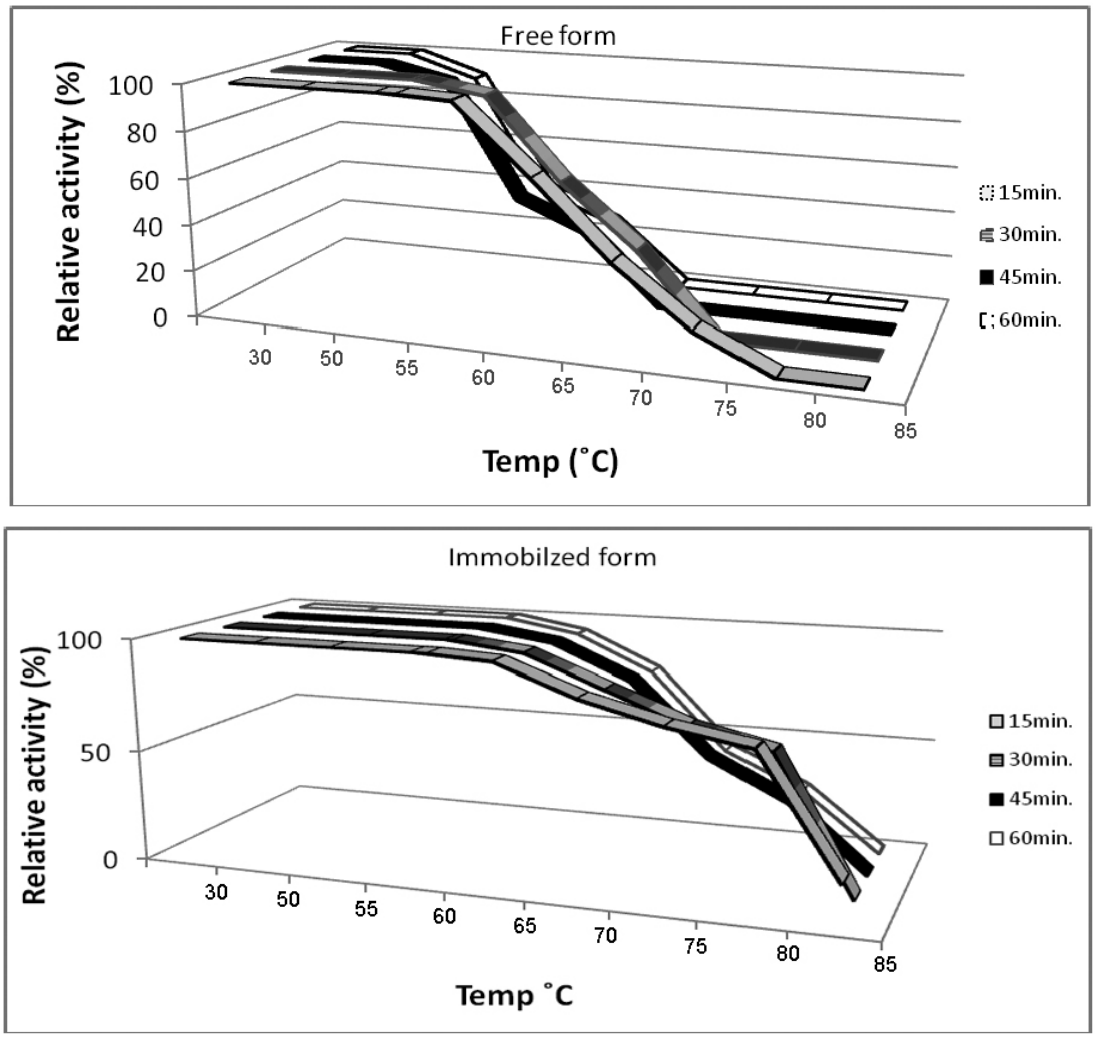


Figure 6*:* Thermal stability profile of free and immobilized *A. niger* EM77 (KF774181) invertase.

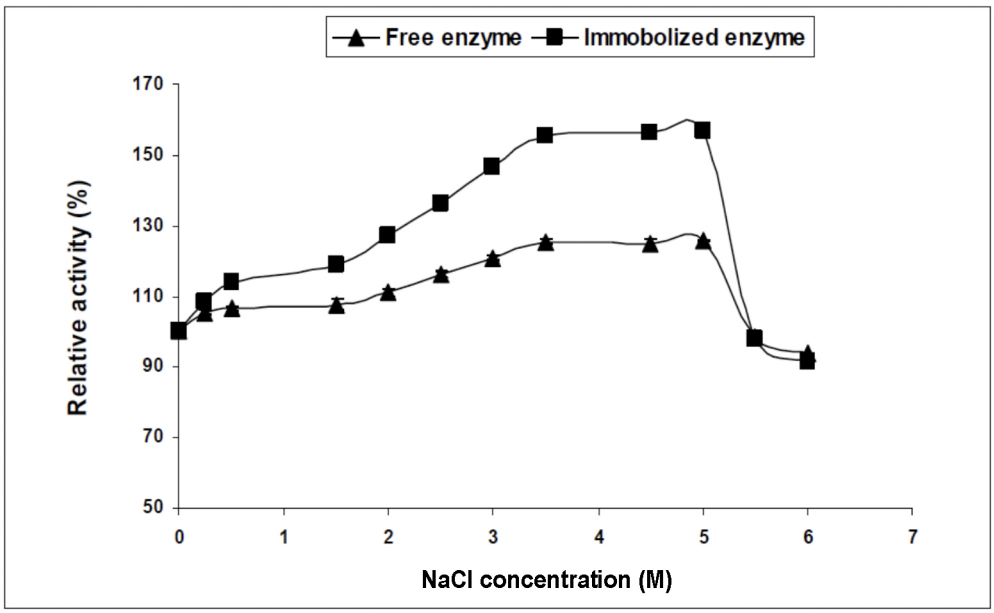


Figure 7: Effect of NaCl concentrations on free and immobilized *A. niger* EM77 (KF774181) invertase.

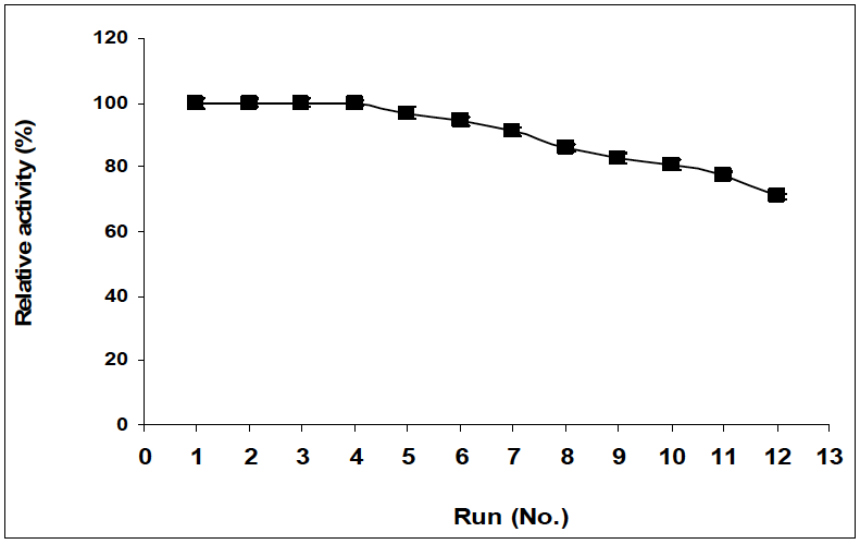


Figure 8: Reusability of enzyme immobilized.

4. CONCLUSION

Extreme environment is expected to have microorganisms have unique and valuable products. In this finding the honey isolate *Aspergillus niger* EM77 (NO.KF774181) was achieved a good invertase activity in the presence of waste material (wheat bran). Extensive investigations have been carried out aiming increasing the enzyme productivity including different incubation period, temp, pHs, carbon and nitrogen sources in addition to metal ions. Also, a simple novel and low cost method was achieved to immobilize *Aspergillus niger* EM77 (NO.KF774181) invertase in (PVAsp Gs). The properties of the free and immobilized invertase pointed out to its novelty. This conclusion comes from the extreme halophilic feature of the free and immobilized enzyme, also its thermophilic character. These properties recommended it to be applied in industrial field.

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