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Comparative study between free and immobilized *Penicillium chrysogenum* mannanase: a local fungal isolate

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ABSTRACT

A search for fungal isolate with higher mannanase activity indicated that the local isolate *Penicillium chrysogenum* appeared as the most active one. *Penicillium chrysogenum* mannanase was eluted as one band near 30 kb by means SDS-PAGE at one step purification. Immobilization of the mannanase by entrapping this enzyme preparation in calcium alginate beads was carried out. The optimum temperature for both free and immobilized mannanase form unchanged (50 ºC). The optimum pH of the free enzyme was 6 while the immobilized form achieved it at pHs range 6-6.5. At 70 ° C the immobilized form could retain 41% of its activity after 180 min, while the free enzyme lost 88% of its original activity at the same time. The immobilization reduced the activating energy from 21.36 kcal mol^{-1} to 17.79 kcal mol^{-1}. Also, it was prolonged the half-lives and D values remarkably in compared to the free enzyme. Shelf stability study indicated that the immobilized form was stable at 4 °C retaining 90% of the activity after 60 days. Bioconversion of locust bean gum (LBG) and yeast mannan by partially purified mannanase (PPM) was similar (41%) for free PPM and 23% for the immobilized form. The prebiotic activity of mannooligosaccharides (MOS) are comprising bio-converted samples of LBG and yeast mannan towards the probiotics *Lactobacillus casei*, *Lactobacillus helveticus* and *Lactobacillus reuteri*. Noteworthy is the highest prebiotic activity (prebiotic index 247) was recorded for MOS (of LBG) towards *L. helveticus.*

INTRODUCTION

Polysaccharides, like mannans, are found as linear homopolysaccharides, heteropolysaccharides or branched form. Mannans are one of the major components of hemicelluloses and in the yeast cell wall. Mannanases are enzymes which have a great impact in the application of industrial processes such as biobleaching of pulp, detergent industry, food and pharmaceutical productions e.g. mannooligosaccharides (MOS) (Mc Cleary *et al.,* 1988), also in various other industries. The composition of mannan is mainly influenced by the action of more than one enzyme. So that degradation of heteromannan, like locust bean gum, can be achieved by fungi and bacteria using different microbial enzymes. Furthermore, MOS prepared by enzyme degradations are used as prebiotics namely as, a non-digestible food ingredient that have been affected the host by selective stimulation of the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health (Lee and Salminen, 2009).

It is generally known that microorganisms are the best source of enzymes. There are three kinds of mannanases which produced by filamentous fungi such as *Penicillium* strains. A restriction is that the organisms, employed to produce a product, should be a virulent and the resulting product, is non-toxic (Whistler and BeMiller, 1993). Actually, these restrictions become more eased by immobilization of enzymes. This technique is characterized by many advantages including stability and reusability of the immobilized enzyme. Enhancement of activities of immobilized enzymes was achieved using various processes, which are classified as chemical, somewhere the enzyme consists covalent and physical bonds, where the enzyme weakly interacted with support.

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According to Blibech *et al.*, (2011), locust bean gum (LBG) was mainly hydrolyzed into MOS i.e., mannotriose and mannotetraose. LBG is a galactomannan while yeast polysaccharide is a mannan. As a trail, the resulting MOS were examined for their benefit effect for growing of lactobacteria as a specific nutrient. The aim of this study is investigating the immobilization of mannanase and to evaluate its biological activity as prebiotic agent for hydrolysate MOS productions (Tomotari and Indus 1990; Titapoka *et al.*, 2008; Quigley, 2010; Gibson *et al.*, 2004; Yopi *et al*., 2006; Ariandi *et al.*, 2015) and efficiency of hydrolysis.

Currently, consumer is more aware of using of food that decreases the potential risk of diseases and enhancement of well-being better health. In this view, there is plenty of interest of non-digestible oligosaccharides as specific kinds of dietary carbohydrates regarded as prebiotics. For many years, modern societies have been consumed various products that promoting the level of health in the general population considered as prebiotic and probiotic properties and advocated for their benefits on health and gastrointestinal well-being. Recently, medical science has been a lot of attention paid in the population of micro-organisms, the intestinal gut microbiota which inhabit the human gut, and the scope of the tasks to be undertaken in the area of health. As a result, the list of disorders and diseases that will result from disruption of the normal microbiota and/or its interaction with the host is growing. A scientific reason for the utilization of probiotics and prebiotics is, therefore, beginning to widespread.

MATERIALS AND METHODS

Materials

Fungal isolate

Penicillium chrysogenum was donated by Prof. Dr. A.F. Sehab, Department of plant protection, NRC. It was isolated from old deteriorated valuable manuscript (A) sultan, Malak, library No.1405 present in the stores of general Egyptian Book organization (G.E.B.O), Cairo Governorate, Egypt.

Bacterial Strains

Three bacterial strains were used for the study and investigation of the prebiotic activity. These included *L. casei, L. helveticus* and *L. reuteri* which are Gram-positive bacteria. They were provided by Chr. Hansen's Lab. Inc., Denmark. Pathogenic strain, *Escherichia coli* (Gram-negative), was obtained from the clinical lab of the El Demerdash Hospital in Cairo Governorate.

Culture Media

Potato Dextrose Agar (PDA): was used to maintain the fungal isolate, Modified Liquid Mandels medium was used for the growth of *Penicillum* strain which cultivated for mannanase production (Chaabouni *et al.*, 1995), De Man- Rogosa- Sharp-medium (MRS medium): was used for growing and maintenance of the probiotics, *L. casie, L. reuteri* and *L. helveticus* , M.R.S. broth medium was used to determined the growth intensity of the investigated probiotics by substitutable of carbon source by the produced MOS. The prepared MRS broth tubes were inoculated, then incubated anaerobically at 37°C for 24 hours and sub-cultured every month.

Methods

Screening of fungal isolates (microorganism) for their ability to produce mannanase

Many fungal isolates belonging to the genera *Penicillium, Trichoderma* and *Aspergillus* were examined for mannanase activity. So, the studied fungal isolates were grown on a modified medium known as Liquid Mandels. This medium comprised 2% KH_2PO_4; 5% $NaNO_3$; 0.3 % $MgSO_4.7H_2O$; 0.3 % $CaCl_2$; 1 % yeast extract; 10% guar gum, 1% tween 80. The same medium was also used for the preparation of crude mannanase.

Production of crude mannanase by P. chrysogenum

The fungal isolate *P. chrysogenum* was cultivated in the modified "Mandels" medium after sterilization medium at 121° C for 30 min, cooled and inoculated with 2% inoculum. The production of crude mannanase was done under aerobic conditions (shaking at 180 rpm) using guar gum as C source, at 30°C and pH 5.5 for 120 hr. The culture filtrate (CF) was used as the crude enzyme for assay.

Determination of mannanase activity and protein

Mannanase product (0.5 mL) was added to 0.5 mL LBG (0.5 % w/v) dissolved in 50 m M sodium acetate buffer (pH 5.5). The reaction mixture was incubated at 50°C for 30 min. Reduced sugars released from LBG were quantified by the method reported by Neish (1952) and based on those described by Somogi (1952). The assay was expressed as one unit of mannanase activity produce 1µmol of mannose per min. Protein concentration was determined as described by Lowry *et al.* (1951).

Fractional precipitation with acetone

Acetone was used by different concentrations (20–40–60–80%). It was added gradually to the ice-cold enzyme solution until reach the demanded concentration. The resulted supernatant were centrifuged at 4000 rpm for 10 min to remove the precipitated fraction, the process was repeated until the final acetone concentration was reached (80%). Different acetone fractions were dried over anhydrous calcium chloride, under reduced pressure at room temperature, weighed and their activity and protein were calculated.

Disc-electrophoresis for the purified mannanase

Polyacrylamide gel disc electrophoresis was carried out according to Laemmli (1970).

Enzyme immobilization by cross linking in calcium alginate beads

Two mL sodium alginate (4%) and 0.5 mL of the PPM (0.02 gm) in the ratio (2:0.5) were dissolved in 0.1 M acetate

buffer and the resulted viscous mixture was dropped (with suitable dropper) into cold 20 mL calcium chloride solution (0.2M). The formed calcium alginate beads (comprising the encapsulated mannanase) were stored in a refrigerator for 24 hr., and then the unbounded enzyme was removed by washing with distilled water

Immobilization yield was defined as follow

Yield % = **(**Total enzyme activity - unbound enzyme activity / Total enzyme activity**)** x 100

Effect of different parameters on free and immobilized form

In this experiment, equal reaction mixture of the free and immobilized enzyme was incubated at different temperature (40-70˚C) for 30 min and pH 5.5. Effect of pHs was done at the same procedure from pH (3, 3.5, 6, 6.5) and enzyme assay was achieved at the optimum conditions. Reaction time was also done at different time intervals (30-120 min) and same previous procedures**.**

Free and immobilized mannanase was expressed as activation energy (E_a) and determined from the slope of the Arrhenius plot [log V (logarithm of % residual activity) versus reciprocal of absolute temperature in Kelvin (1000/T)], which is given by the following Eq. (2)

$$Slope = -\frac{E_a}{R} \qquad (2)$$

Where *R* is the gas constant (8.314 $mol^{-1}k^{-1}$)

Thermal stability

The free and immobilized enzyme forms were pre-incubated at various temperatures in between 50 to 80 °C up to 3 h. Each sample was withdrawn after 30 min and its activity was determined at the optimized conditions. The residual activity was determined by considered the activity of enzyme at 0 minute as 100%. Previous results were also expressed as first order thermal deactivation rate constants (kd) (60-80°C). The t1/2 (half-lives) and D values for both was calculated from the equation 1, 2. Results were also expressed as first order thermal deactivation rate constants (*k*d) (60-80°C). The t1/2 (half-lives) and D values for free and immobilized amylase was evaluated according to the equation 1, 2.

$$t1/2 = ln2 /kd \quad (1)$$

$$\text{D-value} = \ln 10 \,(2)\, kd$$

The temperature rise necessary for reduction D-value by one logarithmic cycle (z value) was calculated from the slope of graph, log D versus T (◦C) using the equation:

$$\text{Slope} = \frac{-1}{z}$$

The activation energy (Ed) for mannanse denaturation was calculated by plotting log denaturation rate constants (lnkd) versus reciprocal of the absolute temperature (K) according to the Eq (2).

$$Slope = -\,Ed\,/R \quad (2)$$

Bioconversion of locust bean gum (LBG) and yeast mannan by the free and immobilized form

Bioconversion process was achieved applying a suitable enzyme: substrate (E/S) ratio: 37 U/2.5 mg substrate for free and immobilized PPM. Bioconversion conditions included the previously concluded optimal temp. (50°C) and pH value (pH6) after 90 min (in case of free form) or 120 min (in case of immobilized form).

Quantitative paper chromatography (PC) of* hydrolyzed *mannan

Hydrolysed mannan was separated by Chromatographic technique which carried out on Whatman chromatographic filter paper (No. 1) using the solvent: n-butanol – acetone – water in the ratio 4:5:1 v/v (Jayme and Knolle, 1956 and Percival, 1968). Quantitative determination of the separated sugars was done according to the method adopted by Wilson, (1959) as follows:-

Color reagent

O-phthalic acid **(**1.66 g**)** and 0.91 mL aniline were dissolved in a mixture of n-butanol, diethyl ether and water by ratio: 48: 48: 4.

Eluting agent

This consisted of 0.7 N HCl in 80% ethanol (v/v). The mixture consisted of 29 mL of 36% HCl to 420 mL of 95% ethanol and it was made up to 500 mL with distilled water.

Procedure

After the separation by chromatography, the air dried chromatograms were sprayed (40-50 mL) of the color reagent. The developing colors of spots were observed after the chromatograms were air dried and then heated in an oven at 105ºC for 10 min. The spots were divided into small strips and dropped into test tubes. Elution process occurred by adding 4 mL of eluting agent to each tube and shaken. The resulting solutions were determined by absorbance measurement in a BAUSCH and LOMB spectronic 2000 spectrophotometer at 390 nm for hexoses, and at 360 nm for pentoses. The sugars quantities were estimated by comparison to appropriate standard curves.

Prebiotic activity

Prebiotic activities of two samples of the enzymatically prepared mannooligosaccharides were evaluated. Experimentally, the three probiotics *L. Casei*, *L. reuteri* and *L. helveticus* were grown on the MRS medium, while *E. coli* was grown in nutrient broth medium, at 37°C for 24 h. Aliquots of 0.1 mL of each of the resulted bacterial culture were used as inoculum for 10 mL studied medium supplemented with 150 mg studied MOS samples as carbon source. After incubation at 37°C for 24 h, the resulted

bacterial growth was measured at 625 nm against a blank of un inoculated medium Hussein *et al.*, (2015). The prebiotic activity was calculated as "Prebiotic Index" (I):

Prebiotic index = (Optical density of probiotic culture at 600 nm/ Optical density of *E. coli* culture at 600 nm) x 10

Storage stability

Both the immobilized and free PPM was stored in distilled water at 4 ºC for 2 months. The activity was measured every 2 weeks using 5 mg of protein.

RESULTS AND DISCUSSION

Searching for fungal isolate with higher mannanase activity

A Search for fungal strain characterized by its relatively higher mannanase activity was done. The search included many isolates belonging to the genera *Penicillium*, *Trichoderma* and *Aspergillus*. Qualitative examination (appears of reducing groups) of the hydrolytic action of these fungi (grown on guar), revealed that all isolates were negative except *P. chrysogenum* exhibited the promising mannanase activity (80 U/mL). In agreement with this observation, mannanas enzymes were found to be secreted by different microbes including fungi, yeasts and bacteria (Dhawan and Kaur, 2007; Blibech, *et al*., 2011; Dan *et al.*, 2012). The fungal strain was considered as mannanase producer. It produces three types of mannanase, one of them (mann III) can produce mannooligosaccharides (MOS) from LBG and ivory nut mannan (Blibech *et al.*, 2010). So, *P. chrysogenum* was used, in the next part of this work, for the preparation of considerable amount of crude and partially purified mannose products.

Production of crude mannanase by *P. chrysogenum*

The fungal isolate *P. chrysogenum* was grown on the modified "Mandels" medium under aerobic conditions (shaking at 180 rpm) using guar gum as C source, at 30°C and pH 5.5 for 120 hr. Similarly, it was reported in the production of *P. occitanis* mannanase using locust bean gum (Blibech *et al., 2011*). After centrifugation, the resulted culture filtrate was analyzed for its mannanase activity and protein content. Specific mannanase activity of the crude product (CF) as found: 24 U/mg protein. Blibech *et al.* (2011) reported in partial purified manno-oligosaccharides where the specific activity was 12 U/mg.

Preparation of a partially purified mannanase, produced by *P. chrysogenum*

Enzyme fractionation by acetone reported that all started mannanase activity was precipitated at 60% acetone (40000 U) and 1075 mg protein, while completely absent at 20%, 40% and 80%. Traces of protein were obtained at 20% and 40% acetone. SDS-PAGE results indicated that the enzyme was eluted in single band near 30 kb (Figure 1). Specific mannanase activity (of the prepared partially purified product) was found: 37 U/mg. These data indicated that the yield of the partially purified mannanase was 90% and 1.54 purification fold. In a similar study (Blibech *et al.*, 2011) the CF (crude enzyme) of *P. occitanis* was treated with 60 % $(NH_4)_2 SO_4$, the partially purified mannanase (precipitated) exhibited specific activity of 12 U/ mg protein with a yield of 99.4% and 2.33 purification fold.

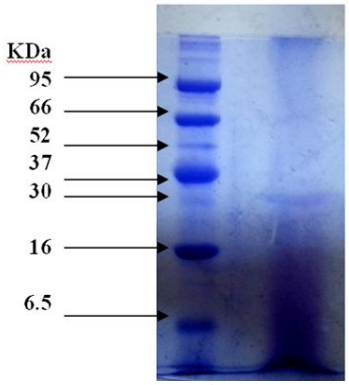

Fig. 1: SDS-PAGE analysis of mannanase enzyme.

Characterization of the partially purified mannanase product

This included studies on the effects of temperature, pH-values and reaction period on the enzyme activities of the partially purified mannanase product.

Immobilization of the partially purified mannanase

This was achieved by entrapping the partially purified enzyme, in calcium alginate beads. Approximately, the whole of enzyme was entrapped reported 100% immobilization yield.

Effect of temperature for free and immobilized PPM

The present investigation aimed to evaluate the effect of different temperature on free and immobilized form. Amongst the studied temperature (40, 45, 50, 55, 60, 65 and 70° C), the degree 50 °C was recorded as the optimal temp for both free and immobilized form (Figure 2a).

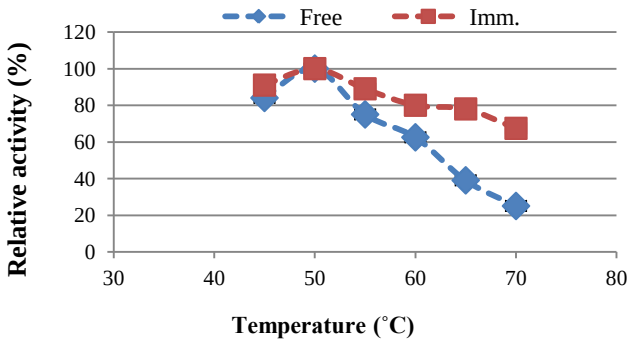

Fig. 2a: Effect of temp. on activity of free and immobilized PPM.

Also, the results indicated clearly to the effect of immobilization process in protect the enzyme activity to great extent in compared to the free form while, the free enzyme lost 75% of its original activity at 70˚C and the immobilized form kept 76.4 % of its activity at the same degree. On contrary,

Paenibacillus sp. DZ23 and *B.subtilis* NM-39 showed the optimum productivity of mannanase at 37°C (Chandra *et al.*, 2011; Mendoza *et al.*, 1994).

Also, the optimal temp. recorded for the mannanase produced by immobilized and free *P. occitanis* were 70°C (Blibech et al., 2011). This indicated that the *P. chrysogenum* mannanase is more susceptible to temp. higher than 50° C , as compared to that of *P. occitanis* (Blibech *et al.*, 2011). Accordingly, the result pointed to the role of immobilization process in PPM protection.

Activation energy of free and immobilized mannanase (E_a)

The immobilization process reduced the activating energy from 21.40 to 17.80 kcal mol−1 **(**Figure 2b). This means that the immobilization process save energy and consequently the immobilized form was economically most favorable. Similar observation was reported by Esawy *et al.* (2013) where the immobilization process reduced the activation energy from 5.1 to 2.7 kcal mol^{-1}.

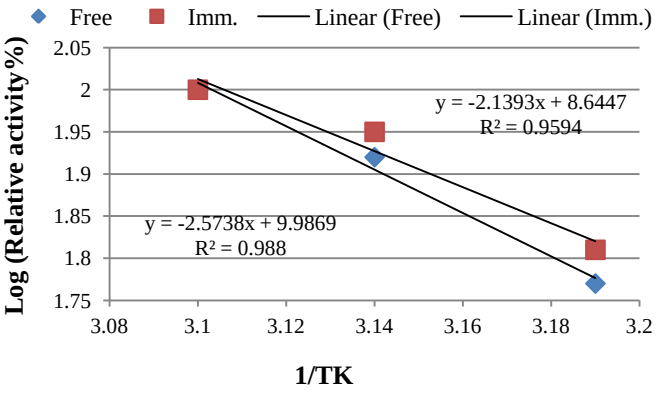

Fig. 2b: Arrhenius plot to calculate the activation energy of mannanase (E_a).

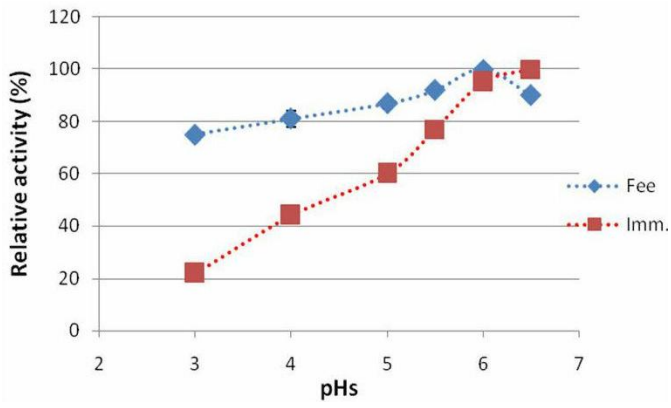

Fig. 3: Effect of pH-value on the activity of free and immobilized mannanase.

Effect of pH-value for the free and immobilized PPM

The goal of this experiment was to identify the optimal pH-value required for exhibition of the highest enzyme activity for the free and immobilized PPM at fixed temp. (50°C) and reaction time (30 min). The optimum pH was determined to be 6 and 6.5 for free and immobilized form respectively (Figure 3).

Effect of reaction time in free and immobilized mannanase

The aim of this investigation was to define the longest reaction time at which, mannanase activity still appeared at its higher value. This was carried out at the optimum conditions for both free and immobilized form. The results indicated that the optimum time for the highest PPM was 90 and 120 min. for free and immobilized form respectively. This result could be explained that the immobilization process prolonged the diffusion time to contact with the substrate. In a similar work on P. occitanis mannanase (Blibech *et al.*, 2011), the longest reaction time at which the enzyme activity was still higher was found to be 30 min at 70 ℃ and pH 4.

This refers to *P. chrysogenum* mannanase as a more stable enzyme preparation (at its own optimal temp. and pH value) comparing to that of *P. occitanis*.

Thermal stability

Thermal study was done to evaluate the role of immobilization process in enzyme thermal stability improvement. The results (Figure 4 a, b) indicated that the immobilization process improved the enzyme stability remarkably. At 70 ºC the immobilized form could retained 41% of its activity after 180 min, while the free enzyme lost 88% of its original activity at the same time, while at 80 ºC the immobilized form retained 30% of its activity in compared to 10% for free form after the same time. The immobilized ß- mannanase showed more stability than the free enzyme, particularly if the temperature exceeded 50 ˚C. The heat inactivation rate was studied for both enzyme forms. Log (% remaining activity) plots vs. time were linear indicating the first order kinetics of immobilized enzyme (Figure 4c, d). The Ed of the immobilized form reported 26 kJ mol^{-1} in compared to 23 kJ mol^{-1} free enzyme (Figure 4e, f).

This means that more energy was required to denature the immobilized form (Driss *et al.*, 2014). Also, the halves lives and D values were prolonged clearly after the immobilization. Since the half lives for the immobilized form reported 383, 313 and 222 h at 60, 70 and 80 ˚C in compared to 202, 156 and 130 h for PPM at the same temperature. Also, D values reported 676, 522, 460 (min) and 1277, 1045, 741 (min) for free and immobilized PPM. The z value for the free and immobilized form was calculated to be 85, 119 ˚C respectively (Figure 4 g).

The high magnitude of z values pointed to more sensitivity to the duration of heat treatment and the low z value meant more sensitivity to the increase in temperature (Tayefi-Nasrabadi and Asadpour, 2008). All previous results suggested that the immobilization process acquired the PPM rigidity and stability.

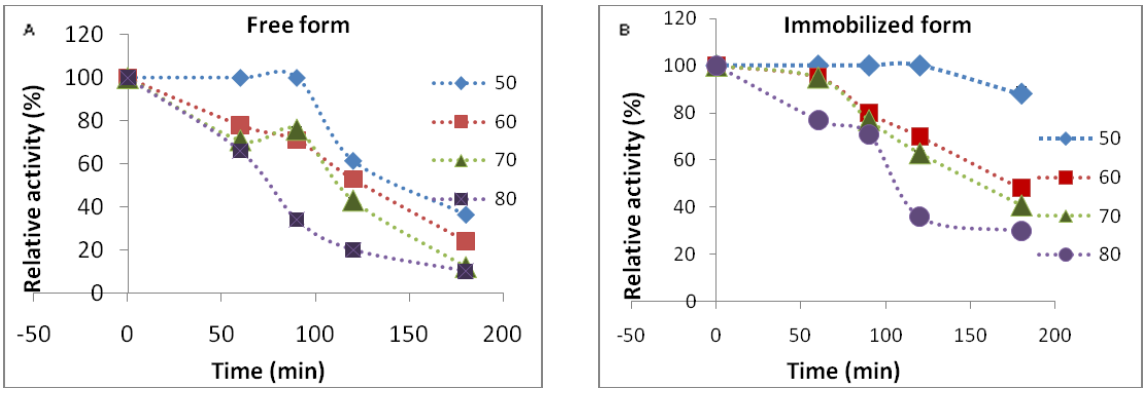

Fig. 4 a, b: Thermal stability studied on free and immobilized PPM.

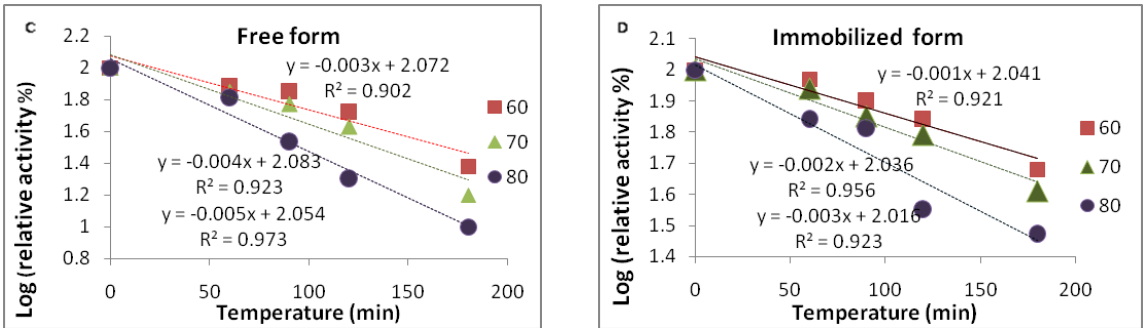

Fig. 4 c, d: First order of thermal deactivation of the free and immobilized form.

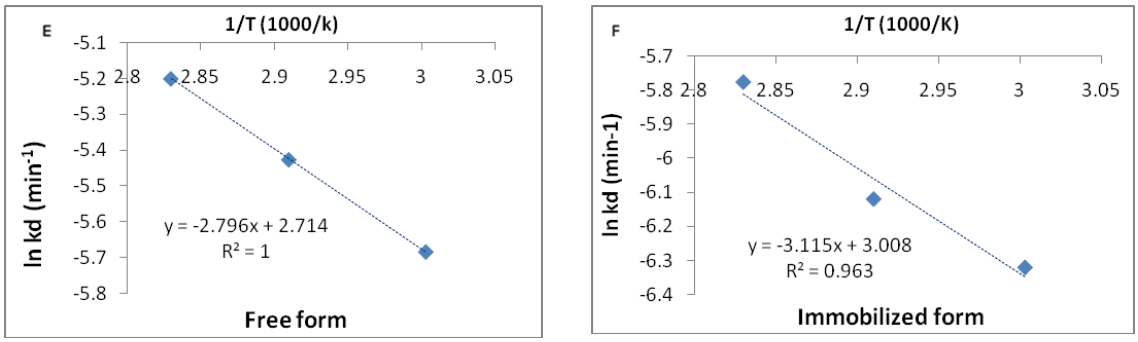

Fig. 4 e, f: Arrhenius plot to calculate activation energy for denaturation (Ed).

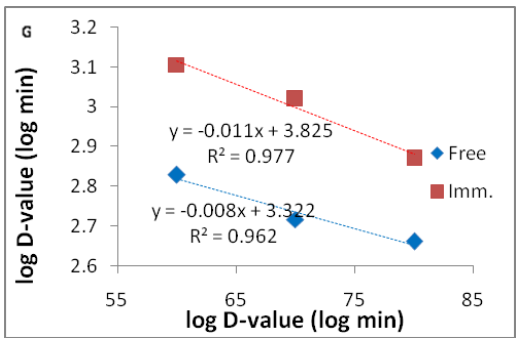

Fig. 4 g: Temperature dependence of the decimal reduction of free and immobilized PPM to calculate z- values.

Bioconversion of locust bean gum (LBG) and yeast mannan by free and mannanase form

Bioconversion of LBG and yeast mannan prepared according to Edwards (1965) means hydrolysis of this galactomannanase into oligosaccharides and probably monosaccharides. The bioconversion process was achieved at optimum conditions for free and immobilized enzymes. The bioconversion percentage of LBG and yeast mannans by free PPM was found: 41%. Also, Locust bean gum (LBG) and yeast mannan were subjected to a bioconversion process by using the immobilized mannanase preparation reported 23%. The resulted partially hydrolyzed product was examined (by PC) (Figure 5) and found a major component appeared as brown spot with R_{mann}: 0.46 for free and immobilized PPM.

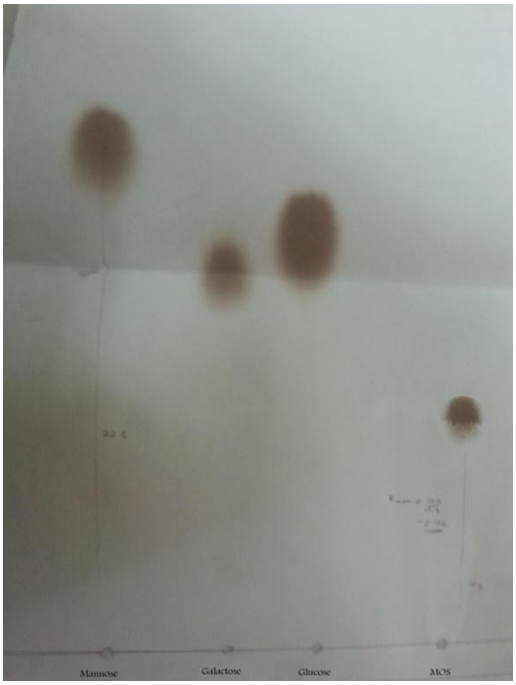

Fig. 5: Paper chromatography of MOS.

Prebiotic activities of mannnooligosaccharides (MOS) comprising bio-converted samples of LBG and yeast mannanase

Bioconverted samples (comprising MOS) of LBG and yeast mannan, were examined for their prebiotic activities towards the probiotics *L. casei, L. helveticus and L. reuteri*. The prebiotic indices were calculated on the proportion between the growth intensities of the probiotics (grown on MOS) and the growth intensity of *E.coli* (grown on MOS). The results (Figure 6) indicated that MOS originated from LBG characterized by their higher prebiotic activities toward *L. helveticus* and *L. casei* and slightly lower activity towards *L. reuteri*, comparing to the activities exhibited by MOS derived from yeast mannan. Noteworthy is that the highest prebiotic activity (**prebiotic** index 247) was recorded for MOS (of LBG) towards *L. helveticus*. On the other hand, the lowest prebiotic index (41.5) for *L. helveticus* grown on MOS obtained from bio-converted yeast mannan. This result was higher than the maltooligsaccharide effect obtained by Esawy *et al.* (2016) and galactooligosaccharide effect obtained by Hussein *et al*. (2009).

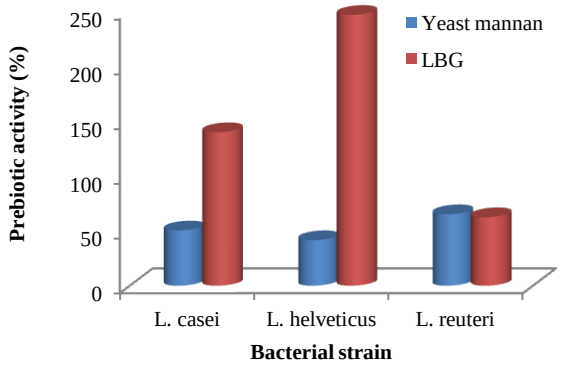

Fig. 6: Prebiotic activities of MOS.

Storage stability of the immobilized mannanase preparation

The stability of the immobilized mannanase preparation was studied; it was known that storage stability is an important parameter recommend the enzyme in industrial use. Immobilized enzyme was stored for a long time at (4°C) and periodically, the enzyme activity was measured during 60 days. The results clarified that the immobilized mannanase was stable for a long period at 4 ºC retaining 90% of the activity after 60 days. The immobilization appeared good storage stability which could be back to the holding of the enzyme in a same position. Also, the immobilization acquired the enzyme molecule rigidity and decreases the enzyme molecule interaction which manages it to overcome the deactivation and autolysis by proteolytic enzymes (Vu and Le 2008).

Finally, it is worthy to add that the results of the present work may offer a promising base for further studies on microbial mannanases. Thus, hope is holding out that further investigation will include other local fungal isolates to produce mannanase with higher activities and longer stability times.

CONCLUSION

A local isolate of *P. chrysogenum* was found as a higher producer of mannanase. A crude product (CF) of this enzyme was attained by growing the aforementioned fungal isolates on a guar-containing medium. Comparative studied was done between the free and immobilized PPM. The optimum temperature and pH, in addition to the thermal stability studied reported the superiority of the immobilized form in stability and rigidity in compared to the free enzyme. Also, both the free and immobilized form had the ability to convert LBG and yeast mannan to valuable MOS. The prebiotic indices indicated that MOS originated from LBG characterized by their higher prebiotic activities towards *L. helveticus* and *L. casei*. Noteworthy is the highest prebiotic activity (prebiotic index 247) was recorded for MOS (of LBG)

towards *L. helveticus.* All previous results recommended PPM to be used in different aspects such food supplementary.

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Conflict of interests: There are no conflicts of interest.

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