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Optimization, Characterization of Newly Immobilized Mannanase on Activated Chitosan Beads and Its Applications

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Abstract

Mannanase produced by mutant *Penicillium citrinium* Egy5 LC368457 isolated from Pharaoh Mummies of Ancient Egyptian Museum, Cairo, Egypt**,** was covalently immobilized onto activated chitosan beads by Polyethyleneimine, glutaraldehyde and dopamine. Loading time, enzyme's units, pH and temperature were optimized. The immobilized enzyme can keep its maximum activity at a broader range of pH and make it more stable at higher temperatures, including 60 °C, which is preferred to reduce microbial contamination of the enzyme. The reusability test indicated the high stability of immobilized enzymes that were still active at 75.82% of their activity at cycle number 9 and in the production of mannooligosaccharides, which showed antioxidant and prebiotic activity.

Keywords: *Penicillium citrinium;* reusability; β-Mannanases; immobilization technique; mannooligosaccharides

1. Introduction

According to the most recent Enzyme market analysis, the sum spent for enzymes in the global market reached up to $7,652.0 million, and the expanded catalyzed interest for enzyme are for the most part for nourishment and beverage industries.

The purpose of the immobilization technique is to improve an enzyme's operational presentation for industrial applications. The immobilized enzymes are more increasingly impervious to natural conditions. They can be effectively removed at the end of the reaction; the product is not contaminating with the enzyme and even more the immobilized enzymes can be reused as often as possible. In order to optimize the performance of immobilized enzymes, many matrices have been described in the literature. (Nadaroglu and Sonmez, 2016; and Mohapatra 2021).

In the constantly increasing biotechnology industry, hemicellulases have emerged as essential enzymes (Ismail *et al.*, 2022 and Nour *et al.*, 2022). Because of their diverse qualities, they gain a wide array of industrial applications. β-mannanases are an important group within hemicellulases that attack the mannan backbone's glycosidic linkages to release -1,4-manno-oligosaccharides (Chauhan *et al.*, 2012). Mannanase with biological origins has a broad range of applications in a variety of industries, such as production of manno-oligosaccharides which have prebiotic activity, pharmaceutical preparation, and pulp biobleaching, and as pre-treatment of plant biomass for second biofuel generation. The determination method of immobilization β-mannanase is necessary to prevent enzyme activity loss by avoiding changes in the chemical structure and reactive groups in the enzyme's binding site.

Adsorption, covalent coupling, entrapment, and cross-linking are the most well-known ways of enzyme immobilization (Hashem *et al*., 2016). Immobilized enzymes are more tight and resistant to environmental changes than unbound enzymes, which increases their thermal operational stability and recoverability. (Zhao *et al*., 2011).

The biocatalyst is more resistant to harsh industrial processes when organic (alginate, chitosan, collagen, and carrageenan) and manufactured (polyacrylamide, amberlite, and polyvinyl alcohol) components are adsorbed on the surface of different polymers (Sirisha *et al.*, 2016).

Because of its abundance, biocompatibility, biodegradability, and nontoxicity, chitosan nanoparticles have become a popular immobilized matrix in recent years (Mohapatra 2021). The high surface-to-area ratio of immobilization on nano-sized particles aids in increased enzyme loading, faster reaction rates, and resistance to fouling and diffusion. (Ansari *et al.,* 2016 and Verma *et al.,* 2016). The surface covalent attachment of an enzyme onto chitosan nanoparticles is achieved by using a crosslinker on the nanoparticles (e.g. glutaraldehyde). As a

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result, the biocatalytic –CHO groups in glutaraldehyde interact with the –Amino group molecules in glucosamine Nadaroglu and Sonmez (2016). The enzymatic activity showed that mannanase enzyme obtained from fungal Clitocybegeotropa was immobilized on magnetite (Fe_3O_4) nanoparticles using adsorption method for the clarification of some fruit juices (Verma *et al.*, 2016).

The properties of free and activated chitosan-immobilized *β*-mannanase from the gamma mutant *Penicillium citrinium* Egy5 LC368457 are described in this paper, in addition to the the biological activities of manno-oligosaccharides produced from the immobilized *β*-mannanase.

2. Materials and methods

2.1. Materials

Locust bean gum was obtained from sigma (St. Louis, Mo., USA). Chitosan, dopamine was purchased from Fluka. Polyethyleneimine (PEI) (50%, w/v) (MW: 423), Cat # 468533, was purchased from Aldrich. Glutaraldehyde, Dowex-IXD particle size 0.0730-0.15 mesh, acrylamide, bisacrylamide, TEMED, Potato dextrose agar and TLC cards coated with silica gel, layer thickness 0.2mm were obtained from Merck KGaA 64271 Darmstadt Germany. Coffee beans were bought from local market, Giza, Egypt. The rest of the reagents were of analytical quality.

2.2. Microorganism and its maintenance medium

Penicillium citrinium Egy5 LC368457 was isolated from Pharaoh Mummies of Ancient Egyptian Museum, Cairo, Egypt**.** The fungus was grown on potato dextrose agar (PDA) and grown for 7 days at 30°C until being maintained at 4°C and subculturing on a monthly basis. The probiotic beneficial bacteria *Pediococus lactis 1,2* , *Lactobacillus lactis, Lactobacillus acidophilus* and *Lactobacillus planterum* compared to that of pathogenic *Escherichia coli* cultured on MRS-medium (containing the sample as sole carbon source). The five probiotics were cultivated on MRS mixture for 24 hours, whereas *E. coli* was grown on nutritive broth medium for 24hrs with in laboratory.

2.3. Cultivation conditions and enzyme preparation

2.3.1. Mutagenesis with gamma rays treatment

Mutagenesis was carried out using gamma rays produced from Cobalt-60 (Co60) as a source of gamma radiation (by Egyptian Atomic Energy Authority) (Abosereh *et al.*, 2019).

2.3.2. Production of β- mannanase

The mutant *Penicillium citrinium* Egy5 LC368457 was used for the production of β- mannanase as previously described by (Khattab *et al.*, 2020 and Foda *et al.*, 2022). The medium used contain (g/L): KH_2PO_4, 8.5; Nitrogen complex (peptone + ammonium sulfate+ Urea), 1.8 and Coffee waste (10g/flask). The pH of the medium was adjusted at 5 before autoclaving. Each Erlenmyer flask contain 50ml inoculated with 8 percent spore suspension (on potato dextrose agar) cultured for 12 days from a 7-day-old age fungus shaking at 120rpm at 30°C. The crude extract was partially purified by fractional precipitation using ammonium sulfate. All the fractions were dialyzed.

2.3.3. Enzyme activity and Protein content

The Nelson–Somogyi method was used to determine the enzyme's activity (Smogi *et al*., 1952). The partial pure enzyme was added to 1ml of locust bean gum (1% (w/v) for 10 minutes at 50°C in sodium citrate buffer (50mM) at pH 5.5 (Hashem *et al.*, 2001). Under the assay conditions, the quantity of enzyme that releases 1 mol of mannose per minute is defined as one unit of mannanase (U). Lowery-Folin technique was used to determine the protein content (lowery *et al.*, 1951).

2.4. Screening for the best carrier

48 carriers were screened individually for immobilization of β-mannanase according to Hashem *et al*. (2016). These carriers were made by dissolving their components in distilled water, then using an encapsulator, dropping the polymer solutions into 3 percent CaCl2, 3 percent KCl, or 0.05 M FeCl3 to form homogenous gel beads. To complete the hardening process, the gel beads were placed in the same solution for three hours. The gel pellets were submerged in a 4 percent (v/v) polyethylenimine (PEI) liquid at pH 9.5 for three hours to set up them for immobilization via covalent bonds of the enzyme, and then steeped in glutaraldehyde (GA) 2.5 percent for three hours before rinsing with dist. After soaking in H_2O, the pellets were available for enzyme immobilization.

2.5. Immobilization of enzyme

1g of the previously manufactured gel beads carrier was combined with 1 milliliter of partly purified mannanase. The mixture was allowed at room temperature overnight to immobilize the beads, which were then rinsed with buffer solution and stored at 4°C for following studies, the Yield of Immobilization was computed using the equation as follows:

$$\text{The Yield of Immobilization (\%)} = \frac{\text{Immobilized enzyme activity (U/g carrier)}}{\text{Enzyme added (U/g carrier)} - \text{Unbound enzyme (U/g carrier)}} \text{ (\%).}$$

2.6. Optimum conditions for enzyme immobilization process

2.6.1. Effect of different chitosan with different concentrations enzyme

Different activated gel beads with different aldehyde groups were used to determine the enzyme concentration required to react with all of the aldehyde groups identified on the gel beads:

A. Chitosan+dopamine

chitosan + glutraldhyde (2.5%)+ polyethelene+ dopamine were soaked in different concentrations of enzyme (9.16, 20.02 and 29.65 IU). Enzyme activity was determined as mentioned before. Unbounded enzyme was determined.

2.6.2. Effect of different concentration of glutraldhyde

The effect of different glutraldhyde concentrations (1, 2.5, 3.5, 5 and 7%) on the catalytic activity of the immobilized enzyme was determined by soaking aminated chitosan beads in different concentrations of glutraldhyde for 3hrs at room temperature on a shaker (150 rpm).

2.6.3. Loading time for immobilization of β-mannanase

The effect of time loading of partially purified β-mannanase on chitosan beads treated with 3.5% glutraldhyde at various time periods (2, 4, 6, 8, 20, 24 and 26 h) at room temperature on a rotary shaker (150 rpm) was studied.

2.6.4. Different pHs of enzyme solution

The effect of pH of loading partially purified β-mannanase on chitosan beads treated with glutraldhyde at different pHs of enzyme (4, 4.5, 5, 5.5, 6 and 6.5) at room temperature on a shaker (150 rpm) after 6hrs was determined.

2.6.5. Effect of different temperature of enzyme solution

The effect of different temperatures of loading partially purified β-mannanase on chitosan beads ranged from 20 to 37°C with 3.5% glutraldhyde at pH 5.5 on a shaker (150 rpm) after 6hrs was studied.

2.6.6. Operational stability

This was done by incubating 0.5 g of immobilized β-mannanase with locust bean gum at 60°C for 10 minutes, comprising roughly 20.02 IU of the enzyme. The pellet was removed by filtration at the conclusion of the reaction period, washed with buffer, to start a new run; the pellets were re-suspended in freshly prepared substrate. The activity of β-mannanase was measured in the supernatant.

2.7. Characterization of the gel beads

2.7.1. Infrared Fourier Transform (FT-IR)

Fourier transform infrared spectroscopy was used to record the infrared spectra of all formulations (chitosan), (chitosan + PEI), (chitosan +PEI + dopamine), (chitosan + PEI + dopamine + GA), and (chitosan + PEI + dopamine + GA + Enzyme) (FTIR-8300, Shimadzu, Japan). At room temperature, FT-IR spectra were collected in the wavelength range of 4000 to 400 cm^{-1}.

2.7.2. Scanning Electron Microscope (SEM)

The various gel compositions' surfaces (chitosan), (chitosan + PEI), (chitosan +PEI + dopamine), (chitosan + PEI + dopamine + GA) and (chitosan + PEI + dopamine + GA + enzyme) Scanning electron microscopy was used to investigate the samples. (SEM, S-590, HITACHI)

2.8. Stability and optimization of free and immobilized β-mannanase

The following studies were carried out to verify the effectiveness of the novel carriers for mannanase immobilization in industry.

2.8.1. The pH Effect

Identical reaction mixes of free and immobilized enzymes were incubated in sodium citrate buffer (0.05M) in this experiment at different pHs ranging from 4 – 6.5 at 50^{o}C for 10 min. The enzyme assay was done in normal circumstances.

2.8.2. Effect of temperature

The kinetics on the activity of free and immobilized mannanase was evaluated with executing the reaction for 20 minutes at various temperatures varying between 40 and 70°C., in agreement to Nagar *et al.* (2012). The activation energy (Ea) including both free and immobilized mannanase was estimated utilizing the Arrhenius plot, as shown in equation (1):

$$\text{Slope} = -\text{Ea}/R \quad (1)$$

Where R denotes the gas constant

Both free and the immobilized enzyme was investigated in the absence of the substrate at temperature range from 40-70°C up to 60min to determine the thermal stability. The residual activity of the free and the immobilized enzyme was determined at the optimum condition previously calculated. Without pre-incubation, the enzyme's activity was assumed to be 100 percent.

2.8.3. Thermodynamic research

A regression plot of log relative activity (percent) versus time was used to get the kd (min). For both free and immobilized mannanase, the t1/2 (the time it takes for activity to fall to half of its original level) and D-value (the time required to reduce 90 percent of enzyme activity) were calculated from the Eq. (2, 3).

$$t_{1/2} = \ln 2/kd \quad (2)$$

$$D-\text{value} = \ln 10/kd \quad (3)$$

A plot of log denaturation rate constants (lnkd) vs reciprocal of absolute temperature was used to determine the activation energy (Ed) for both free and immobilized mannanase denaturation (K) using the Eq. (4)

$$\text{Slope} = -E_d/R \quad (4)$$

2.8.4. pH stability

The free and immobilized enzymes were treated to varied pH values ranging from 4 to 7 for different time intervals in the absence of the substrate (15, 30, 45, 60, 90 and 120 min.). The residual activities were then tested under the same conditions as before.

2.8.5. Effect of substrate concentrations and kinetic constant determination

For the determination of Michaelis-Menten kinetic parameters, different initial locust bean gum concentrations were used ranging from (2.5- 17mg/ml). The partial pure enzyme's dynamical parameters were calculated using the Lineweaver-Burk plot (Lineweaver and Burk, 1934), which was plotted using the formula giving:

$$1/V = (1/V_{max}) + (K_m/V_{max})\,(1/S) \quad (8)$$

$$K_{cat} = V_{max}/e \quad (9)$$

where V is the partial pure enzyme's activity (U/mL), Vmax is the maximal activity, Km is the Michaelis-Menten constant, S is the locust bean gum concentration (mg/mL), Kcat is the turnover number, and e is the enzyme concentration.

2.8.6. Effect of different salts on β-mannanase activity

The effect of Cu^{2+}, Zn^{2+}, Ca^{2+}, Hg^{2+}, Mn^{2+}, SDS, EDTA and Mercapto ethanol in concentration (1mM) for 30 minutes at 30°C on β-mannanase activity was determined. Each was added separately to free and immobilized β-mannanase at 1mM final concentration.

2.8.7. The immobilized β -mannanase's operating stability

0.5 g of immobilized β-mannanase was carried out in the experiment (wet), which contained approximately

20.02 IU of the enzyme. The immobilized form was incubated for 10 minutes at 60°C with Locust bean gum (10mg). It was filtered out at the conclusion of the reaction period, rinsed to start a new run, and re-suspended in solution with prepared substrate and pure water. The activity of β-mannanase was measured in the supernatant.

2.8.8. Thin – layer chromatography

Carbohydrates were separated and primary detected by (TLC) techniques using silica gel plates. The samples were spotted on plates with different authentic for mono, di, tri, tetra sugars. Plates were developed at room temperature in a saturated chamber containing the mobile phase which is propanol: water (8.5:1.5 v/v). Sugars were recognized using a spraying procedure. The dried plates were then added to the phenol-sulphuric acid reagent (3 g of phenol and 5 ml of concentrated sulphuric acid in 95 ml of ethyl alcohol), followed by incubation at 100 C in an oven for 10-15 min. (Adachi, 1965).

2.9. The biological actions of the manno-oligosaccharides synthesized (MOS)

Antimicrobial and antioxidant activity were measured for MOS's according to the following methods:

2.9.1. Antioxidant activity

The antioxidant activity of the samples was examined according to the approach provided; the scavenging activity of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals was determined by Brand-Williams *et al.,* (1995). The reaction was carried out by adding 0.1mL of the sample solution to 3.9mL of methanol solution of DPPH radical (1.1×10^{-4}mol/L). The reaction allowed for 30 minutes of standing in the dark. Using Trolox as a standard, the decrease in absorbance was determined spectrophotometrically at 515 nm. The outcomes are given in μMTrolox Equivalents (TE)/μg sample.

2.9.2. Determination of prebiotic activities

The oligosaccharide DP_4 was used as carbon sources for growing the three probiotics and (in parallel) a pathogenic strain *E. coli.* A medium-containing blank made out of uninoculated mannooligosaccharides (Hussein *et al.,* 2010). *E. coli* was cultured on nutritional broth medium, while probiotics were produced on conventional MRS broth medium. The optical densities of the growths were obtained at 625nm after 24 hours of incubation at 37°C, and The following formula was used to computed the prebiotic factor:

$$\text{Prebiotic factor} = \frac{\text{O.D of Bifido genic growth of culture}}{\text{O.D of pathogenic growth of culture}}$$

The prebiotic activity is determined by comparing the densities of growths of the probiotic beneficial bacteria *Pediococus lactis* 1,2, *Lactobacillus lactis*, *Lactobacillus acidophilus*, and *Lactobacillus planterum* cultured on MRS-medium with those of the pathogenic *Escherichia coli* (containing the sample as sole carbon source). The five probiotics were cultivated on MRS medium for 24 hours, whereas E. coli was grown on nutritional broth medium for 24 hours. As an inoculation, stock solutions of 0.1 ml of each resulting bacterial suspension were used. For a 10-ml study medium, including the researched material as a supply of carbohydrates. A carbohydrate source of 150 mg per 10 ml MRS-base medium was used to make this medium.

The resultant bacterial growth was detected at 625 nm after incubation during 24hrs at 37°C, against a blank made up of uninoculated MOS's including medium table (3).

The "Prebiotic factor" was used to determine the prebiotic activity: Prebiotic factor = Bifidogenic bacterial growth O.D. versus *E. coli* growth O.D. All studies were carried out in three different ways, and the findings are given as means SD (n= 3).

3. Result and discussion

3.1. Enzyme production

β-mannanase can be produced by microbes and filamentous organisms (Adigazel *et al.,* 2015 and Ismail *et al.,* 2019). The mutant *P. citrinium* β-mannanase was reported in our previous work as a good producer of β-mannanase that produce351 IU/ml with specific activity 21.57 U/mg protein (Ismail *et al.,* 2019).

3.2. Partial purification

The fraction brought precipitation at 60-70% ammonium sulphate saturation represented 34.03% of the total recovered activity with 7.99 fold increase in the specific activity. Our previous work (Ismail *et al.,* 2019) reported that the fraction determined at 60-70% (w/v) ammonium sulphate provided the most specific activities (27.83 U/mg proteins). Nadaroglu and Snomez (2016) and Olaniyi & Adebowale (2017) reported partial purification of mannanase using ammonium sulphate.

3.3. Enzyme immobilization

An important parameter in determination the economic viability of the industrial use is the reusability which shows the significance of the generated enzyme's immobilization (Fernandes *et al.,* 2013).

Among 48 different tested carriers, the immobilization by covalent binding with Chitosan-NaOH+ dopamine+ Polyethylenimine was the best giving the highest immobilization yield (34.41 %), which agrees with Mohy Eldin *et al.* (2012) who mentioned that covalent binding is progressively ideal as it leads to stable enzyme derivatives.

The immobilization process was optimized with different factors. The optimum conditions were the loading of 20.02 IU/ml on activated chitosan with glutraldhyde, polyethylenimine and dopamine, sufficient for achieving maximal immobilization yield (58.86%), while it was only 19.41%, in case of activated chitosan directly. Fig. 1 (A, B) showed the change in the surface of the beads for different gel formulation.

The increase in the loading enzyme decreases the immobilization yield, and this may be attributed to the increase on the surface of beads hamper diffusion of the substrate to the enzyme's active site (De Medeiros *et al.,* 2020 and Fernandez *et al.,* 2017).

The yield of immobilization increased to 61.7% with 3.5% glutraldhyde (fig, 1C). Covalent binding through glutraldhyde most likely increased the carrier local surface area and, accordingly decreased the streric impediment that surrounds the active site (Siso *et al.,* 1990). Blibech *et al*., (2011) reported that 2.5% glutraldhyde was optimum for the immobilization yield.

In addition, the effect of different pH and time of loading enzyme was studied. The optimum yield was achieved (69.07%) at pH 6 after 6h (Fig, 1D) and (Fig, 1E). Also, the temperature affected the yield of immobilization. A yield of 77.2% was achieved at 20°C but decreased to 8.12% at 37°C (Fig, 1F). Similarly, Nadaroglu and Sonmez (2016) found that the immobilization of β-mannanse was significantly enhanced at the mild acidic pH 5 and at 20°C.

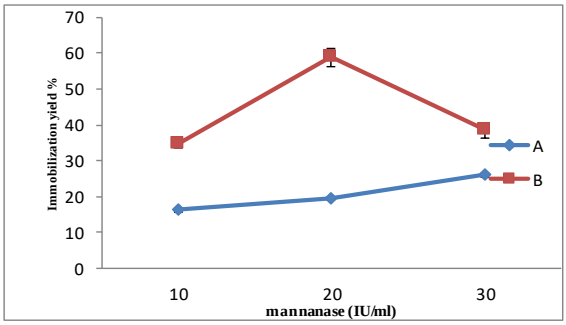

Figure (1A, B): Effect of different treatment of chitosan with different concentrations of enzyme
A-Chitosan+dopamine

chitosan + glutraldhyde(2.5%)+polyethelene+dopamine

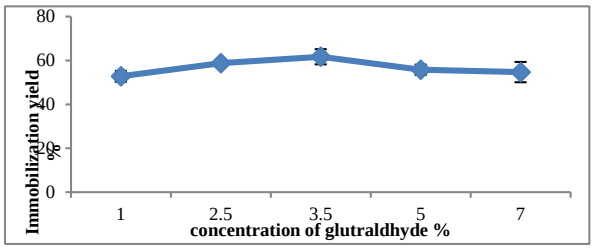

Figure (1C): Effect of different concentrations of glutraldhyde %

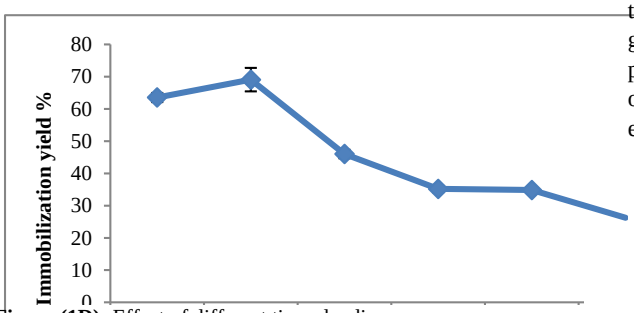

Figure (1D): Effect of different times loading

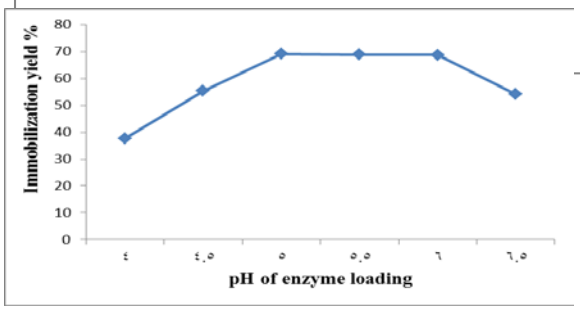

Figure (1E): Effect of different pHs of enzyme loading

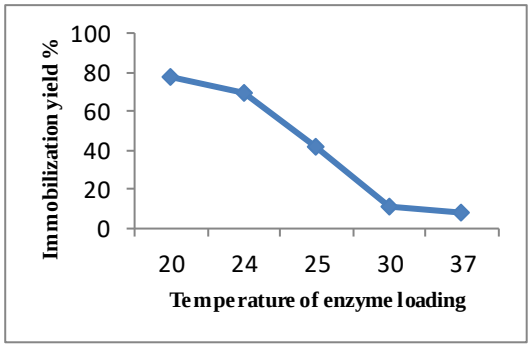

Figure (1F): Effect of different temperatures of enzyme loading

3.4. Characterization of the untreated and treated immobilized enzyme

3.4.1. Fourier Transform Infrared (FT-IR)

From 400 to 4000 cm-1, FT-IR spectroscopic investigation of chitosan with gel particles, triggered particles, and immobilized beads was done. (Fig,2). Chitosan gel beads had distinct peaks in their infrared spectra (curve A); in this figure the peak of amine group is clear at 3429cm^{-1} which is corresponding to NH_2 group. The spectra for aminated beads show that the peak at 3429 cm^{-1}became broader that more amine groups were found on the surface of the beads, indicating their existence (curve B). After treatment with dopamine, a distinguishable band appeared at 1109 cm^{-1}, corresponding to the C–O of the phenol group (curve C). Beads that have been activated with glutaraldehyde display two additional peaks. The first was at 1631 cm-1, referring to the (C=N-) group formed by the reaction of NH2 end groups with glutaraldehyde, and the second was at 1429 cm-1, referring to the (C=O) group formed by a free aldehyde end of glutaraldehyde (curve D). Finally, the immobilized beads produced a larger signal at 3433 cm^{-1}, reflecting a naturally occurring increase in the quantity of amino groups in the enzyme

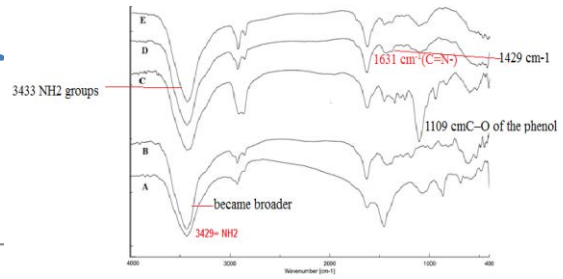

Figure 2. The examination of chitosan gel beads, activated beads, and immobilised beads using FT-IR spectroscopy

The FTIR spectra of the immobilized enzyme agree with novel *Streptomyces* β-mannanase Mohapatra (2021) and immobilized lipase from *Rhizomucor meihei* (Collins *et al.*, 2011) and β-galactosidase from *kluyveromyces lactis* (Klein *et al.*, 2012), and alginate lyase from Arthrobacter species AD-10 Mohapatra (2021).

3.4.2. Scanning Electron Microscope (SEM)

The results shown in Fig (3) showed the changes in the different gel formulation after each step of the immobilization.

The surface of chitosan beads was smooth, and after activation with glutraldhyde molecule accumulated on the surface (figure, 3B) and finally Figure 3C shows the shape of beads homogenous, symmetrical and spherical beads, agree with (Zhao *et al*., 2016).

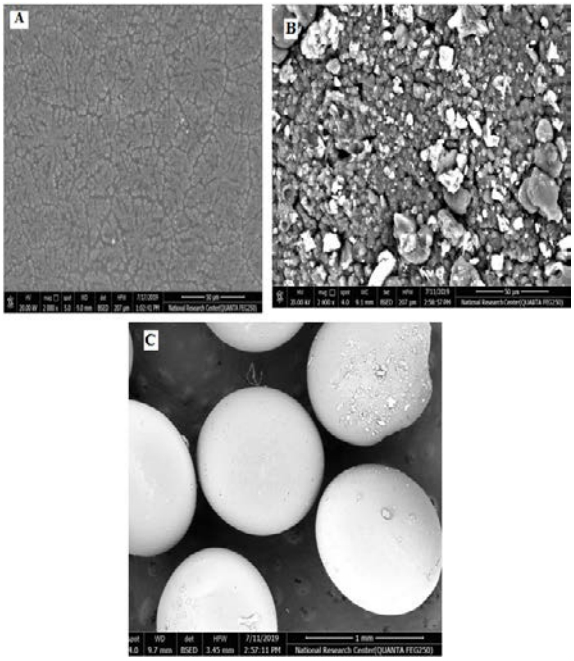

Figure 3. Scanning Electron Microscope (SEM) shows the shape of the beads

3.5. Effect of pH value of the reaction

The influence of pH on the activity of partly purified β-mannanase in a reaction combination and immobilized enzyme was studied in sodium citrate buffer (0.05M) at pH ranged from 4 – 6.5 at 50^{0}C. The results in Fig. 8 indicated that β-mannanase was optimally active at pH 5.5 (control) for both free and immobilized enzymes. The optimum pH of the free and immobilized enzyme was at pH 5.5 (Fig, 4A), and the immobilized enzyme still active (98.97%) at pH 5 and 98.67% at pH 6.0. These results indicated that the immobilized enzyme can keep its maximum activity at a broader range of pH.

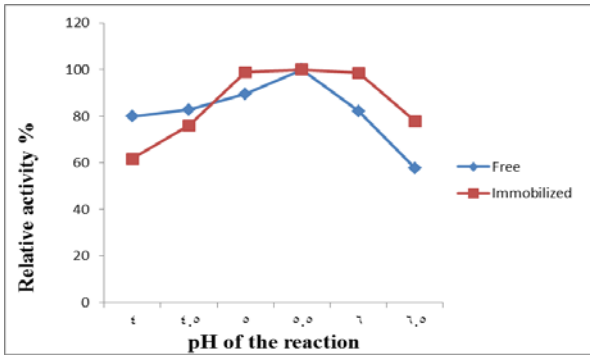

Figure 4A. Effects of varying pH levels on free and immobilized enzyme activity

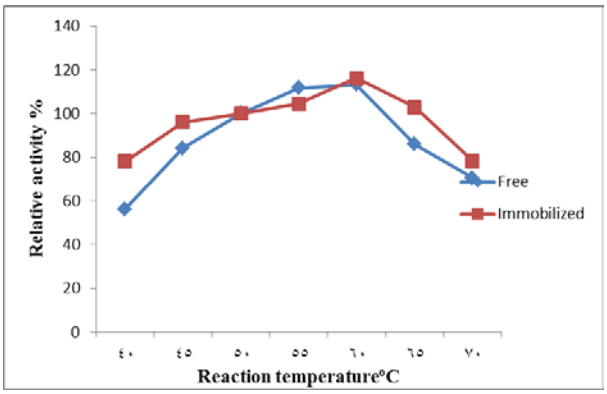

Figure 4B. Optimum temperature profile of free and immobilized mannanase.

Shalaby *et al.*, (2017) For *P. chrysogenum* mannanase, the optimal pH for the free and immobilized forms was found to be 6.0 and 6.5, respectively, respectively. Nadaroglu and Sonmez (2016) found that the immobilization of β-mannanase was determined at pH 5.0.

Most fungal β-mannanases have been found to be most active in the 4.0-5.0 acidic pH range (Lim *et al.*, 2012; Katrolia *et al.*, 2013), whereas β-mannanase is derived from *A. terreus* FBCC1369 was most active at pH 7.0. The optimal pH, according to Mohapatra (2021), is 7.5.

Additionally, the immobilized enzyme shows good pH stability that retained 100% of its activity at pH 5.5 and 6 for 2hr and retained more than 92% and 95% after 2hr at pH 6.5 and 5.0. Panwar *et al.* (2017) reported that between pH 6 and 10, the immobilized β-mannanase of *Bacillus sp.* CFR1601 was stable.

3.6. Effect of temperature

One of the most critical elements determining enzyme activity is temperature. With rising temperature, the activity of both free and immobilized β-mannanase enhanced up to 60°C. The free and immobilized enzyme showed activity of 113.28% and 116.26% of the control (50°C), respectively. Further increase of the reaction temperature up to 65°C leads to decrease of free β-mannanase activity (85.94%), while the immobilized one reaches 102.86%) (Fig. 4B). These findings revealed that the immobilized enzyme was more active than the free enzyme at higher temperatures. Covalent connections were formed between functional groups in enzyme molecules and the carrier surface, strengthening the flexibility of the enzyme's molecular structure and minimizing the effect of temperature on enzymes dissociation, may be referred to as shifting in the optimal temperature (Martin *et al.*, 2001 and Ferrarotti *et al.*, 2006). Covalent immobilization is one among the most extensively distributed utilized strategies for improving enzyme activity at high temperatures in enzyme engineering (Nwagu *et al.*, 2012 and Gill *et al.*, 2006). In general, high temperature is preferred and required for the majority of enzyme activity since it promotes conversion rates. Furthermore, high temperatures enhance the solubility of the substrate while also reducing microbial contamination (Ismail *et al.*, 2019).

The optimal temperature at previous studies were 40°C for Streptomyces galbus (Kansoh *et al.*, 2004), 50°C *Bacillus sp*. CFR1601 (Srivastava *et al.*, 2015), and 58°C *Streptomyces lividans* (Arcand *et al.*, 1993), and for the immobilized enzyme at 60°C for *Clitocybe geotropa*

(Nadaroglu *et al.*, 2016), and at 70°C for *Bacillus sp. CFR1601* (Panwar *et al.*, 2017) and *Penicillium occitanis* (Blibech *et al.*, 2011).

Arrhenius plots were used to compute the activation energy (Ea) of immobilized and free mannanase (Fig. 5). The energy needed to form enzyme- substrate complex was 2.5 higher than the immobilized enzyme. The lower activation energy needed in the immobilized enzyme makes it more suitable for the industrial application, and this would reduce the total cost of the industrial process (Naggar *et al.*, 2012).

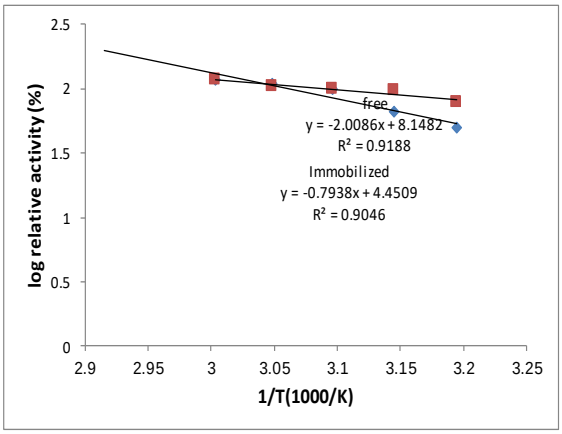

Figure 5. Calculation of activation energy (Ea) for free and immobilized enzymes using Arrhenius plots.

Many studies have suggested that the rise in the optimal temperature for immobilized mannanase is linked to a rise in the reaction's energy barrier as a result of the activation energy (Ea) (Guo *et al.*, 2017 and Arrhenius *et al.*, 1889).

Thermal stability profile (Fig, 6A, B) showed that the immobilized enzyme improved the stability of the enzyme at temperature from 50-60°C. After 2 hours at 50°C, the immobilized enzyme maintained 86.4 percent of its activity, compared to 56.27 percent for the free enzyme. After 2 hours at 60°C, the immobilized enzyme preserved 54.9 percent of its activity while the free enzyme was completely destroyed. This could be because of the covalent interaction between free enzyme and nanoparticles reduces protein unfolding and denaturation by enhancing structural rigidity and lowering thermal vibrations (Hanefeld *et al.*, 2009).

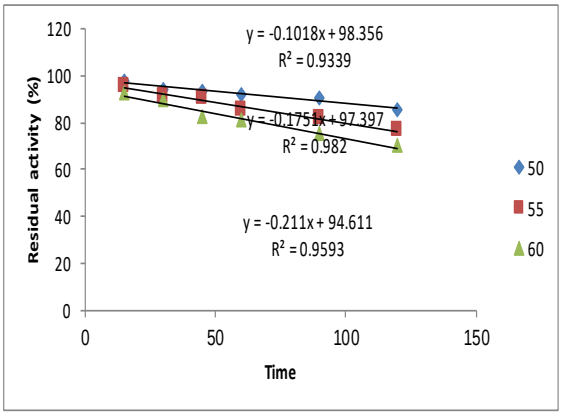

Figure 6A.Thermale-stability profile of immobilized mannanase.

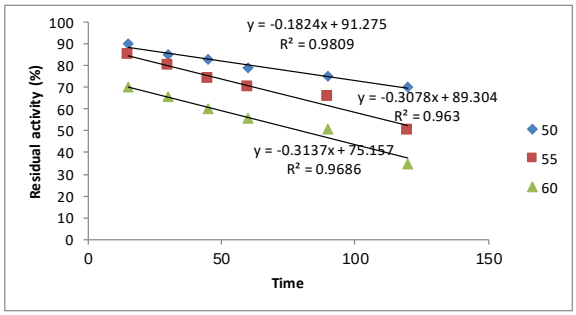

Figure 6B.Thermal-stability profile of free mannanase.

At temperatures of 50-60°C, the heat inactivation rate of free and immobilized mannanase were examined. Plotting the log of residual activity against time yielded a linear relationship, confirming a first-order kinetic response of the free and immobilized enzyme (Fig. 7A, B). The stability of enzymes was investigated as a function of temperature in this thermodynamic study. The thermal stability parameters of free and immobilized mannanase were shown in Table, 1. It was evident that as the temperature was raised, the $t_{1/2}$ fell, as did the (kd), the first order thermal deactivation rate constant (Fig, 8 A, B).

When the half-lives of free and covalently immobilized mannanase on chitosan beads were compared, the difference was striking, especially at higher temperatures. The results showed that the lower the kd the greater is the thermal stability of the enzyme which agrees with what was mentioned by Tayefi-Nasrabadi *et al.*, 2008. Furthermore, after covalent immobilization, D-values of mannanase increased, confirming the enhanced thermal stability of the immobilized mannanase. Previous studies also concluded that after immobilization, the enzyme's half-life increases (Mateo *et al.*, 2007).

Table 1. Thermodynamic parameters for thermal inactivation of free and immobilized mannanase.

	Free enzyme				Immobilized enzyme		
Temp ºC	50 ºC	55 ºC	60 ºC	Temp ºC	50 ºC	55 ºC	60 ºC
kd (min-1)	0.001	0.002	0.0027	*kd* (min-1)	0.0005	0.0009	0.0011
t1/2 (min)	693	346.5	256.67	*t1/2* (min)	1389.29	770.164	630.134
D-value (min)	2303	1151	852.96	*D*-value (min)	4605.17	2558.428	2302.585

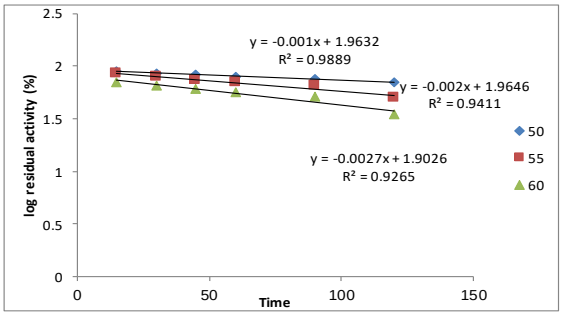

Figure 7A. Arrhenius plot for Kd calculation of free mannanase (free)

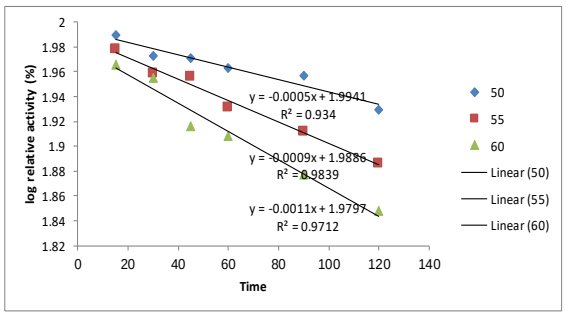

Figure 7B. Arrhenius plot for Kd calculation of immobilized mannanase (immobilized)

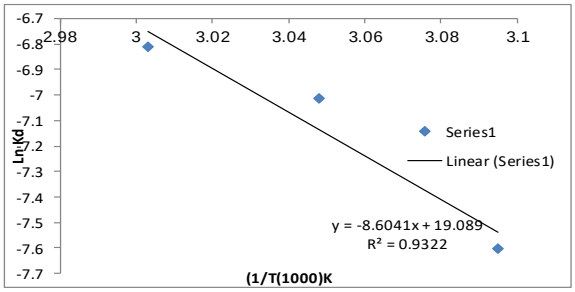

Figure 8A. Arrhenius plot to calculate activation energy (Ed) for denaturation (immobilized mannanase)

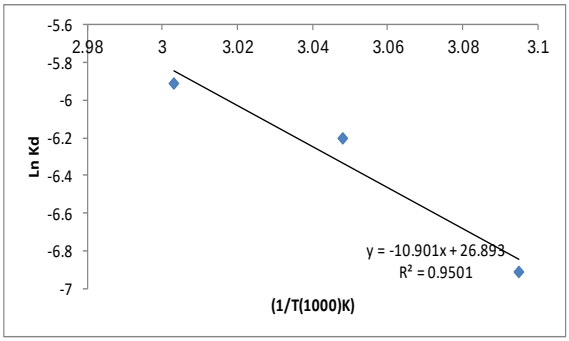

Figure 8B. Arrhenius plot to calculate activation energy (Ed) for denaturation (free mannanase)

coli with the highest activity of 24.9 and 15.65 times recorded for *Pediococus lactis 2 and Lactobacillus planterum*, respectively.

3.6.1. Antioxidant activity

By measuring the scavenging activity of the generated mannooligosaccharides mixture against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, the antioxidant activity of the mixture was determined. The results indicated that the produced oligosaccharides possessed antioxidant activity of about 166 µM Trolox Equivalents (TE)/mg. This result agrees with Amna *et al.* (2018) who indicated that the hydrolysate (rich in mannooligosaccharides) of konjac gum (linear mannan polymer) possessed significant antioxidant activity.

4. Conclusion

In our production, the Mannanase enzymeproduced by the mutant *Penicillium citrinium* Egy5 LC368457 was covalently immobilized onto activated chitosan beads by Polyethyleneimine, glutaraldehyde and dopamine. The immobilized enzyme possessed high stability and reusability as it retained 75.82% of its activity at cycle number 9. Furthermore, they produced mannooligosaccharides, which showed antioxidant and prebiotic activity. The influence of different substrate concentrations

In Lineweaver–Burk graph Fig. (9), the activities of free and immobilized enzymes for locust bean gum concentrations were plotted, and maximal activities (V_{max}) and Michaelis–Menten constants (K_m) values were computed. The results showed an increase in the K_m value of the immobilized enzyme. For free and immobilized enzyme, the K_m was 5 and 5.5 mg ml-1, respectively. In addition, the free and immobilized enzymes had V_{max} of 20 and 25 mg/min/ml, respectively. K_m value commonly increases after immobilization (Park *et al.*, 2005). Mohapatra (2021) reported 4.94mg/ml and 93.5U/mg protein as K_m and V_{max} value for the immobilized Streptomyces species ALg-S25 mannanase. Nadaroglu and Sonmez (2016) reported a K_m value of 0.122mg/ml for *Clitocybe geotropa*. Panwar *et al.*, (2017) reported a K_m value of 6.7mg/ml for *Bacillus sp. CFR1601*.K_m and V_{max} values are mostly determined by the enzyme's source, and hence represent the enzyme's sensitivity to the substrate.

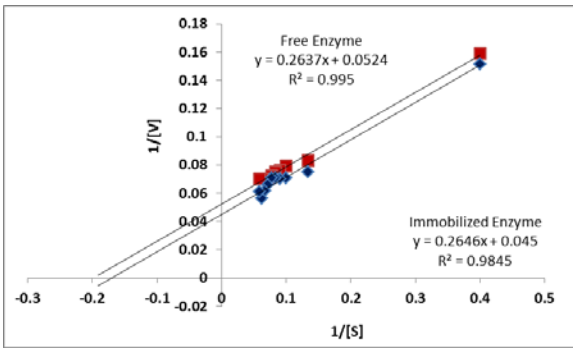

Figure 9. Effect of substrate (locust bean gum) concentrations on the activity of the free and immobilized *Penicillium citrinium* 150GY β-mannanase

4.1. The effect of metal ions on free and immobilized β-mannanase activity

The effects of different metal ions on the free and immobilized enzymes were investigated. The results indicated that EDTA stimulated both free and immobilized enzymes, resulting in increased activity by 14.3% and 15.6% respectively. Also, Ca^{+2} increased the activity by 12.9 and 8.2%, respectively. On the other hand, Hg^{2+} and Mn^{2+}decreased the activity of both free and immobilized enzyme (Fig. 10). Different concentration of EDTA ranged from 0.5 to 5M were examined, and the results showed that the activity reached its maximum at 3mM after 30 min

incubation reached 170.8% and 180.5% for free and immobilized enzyme, respectively (Table, 2). Mohapatra (2021) recorded that EDTA had strong inhibition on the free and immobilized enzyme, and different effect cation action of the enzyme may be due to adopt different geometrics in the same site in the absence of substrate.

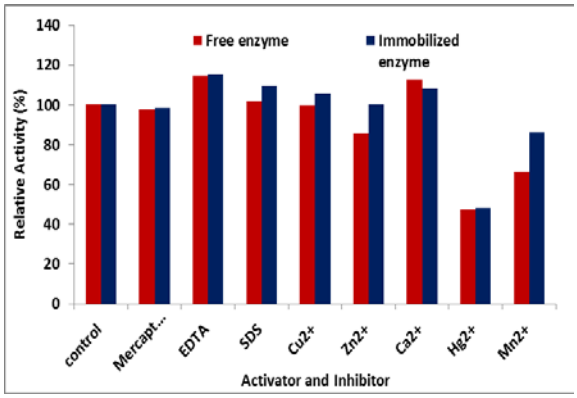

Figure 10. Effect of different activators and inhibitors on the activity of the free and immobilized enzyme

Table 2. Effect of different concentration of EDTA with different incubation time on the activity of the free and immobilized enzyme

conc	Time	Relative activity (%)	
		Free enzyme	Immobilized enzyme
0.5	30 min	129.34	128.92
	60 min	132.58	140.50
1	30 min	168.25	180.48
	60 min	148.35	163.94
3	30 min	170.78	180.48
	60 min	150.78	164.67
5	30 min	152.35	149.04
	60 min	143.28	135.82

4.2. Production of manno-oligosaccharides

L.B.G was more suitable substrate for hydrolysis; therefore, different incubation periods (1hr, 2hr, 3hr, 4hr, 5hr, 6hr, 21hr and 24hr) for hydrolysis of gum locust bean (1%) were applied at 45°C and with 2.5 IU/ml for free and immobilized enzyme. The results showed that in each case for free and immobilized enzyme the hydrolysis increased by increasing the time of the reaction. The 1h hydrolysis reaction yielded 4.39 and 3.1mg/ml for free and immobilized enzyme, respectively, while the 24 h reaction yield was enhanced and reached 8.16 and 7.5 mg/ml of reducing sugars for free and immobilized enzyme, respectively. The TLC indicated that as the time increased the production of MOS (visualized by the spot intensity on TLC plate) increased and the production of monomer increased (Fig. ١١A).

The degree of polymerization of the produced mannooligosaccharide was detected by HPLC and indicated that the enzyme hydrolyzed the locust bean gum to DP4 which has several biological activities (Fig. 11B). The significant item freed from L.B.G was manotetraose (M4) with minor of di, tri oligosaccharides and mannose. Our outcomes concur with Hakamada *et al*. (2014).

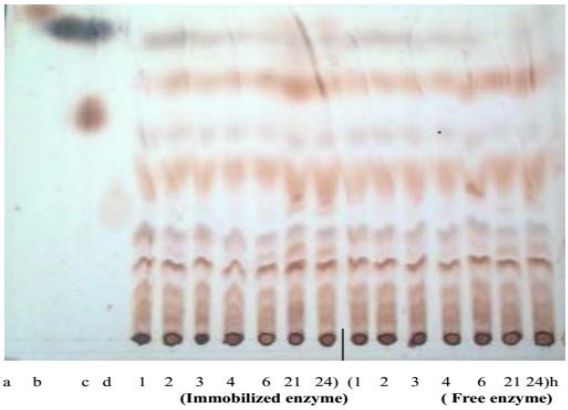

Figure 11A. TLC plate of hydrolysis product of LBG by (Immobilized enzyme and Free enzyme) of mutant of *P.citrinium*150GY β-mannanases at various periods 1, 2, 3, 4, 5, 6 and 7h.
Lan a: mannose, lanb: melibiose, lanc: raffinose, lan d :stachyose, lan 1: 1h, lan 2: 2h, lan 3: 3h, lan 4:4h, lan 5: 6h, lan 6:21h, lan 7: 24h

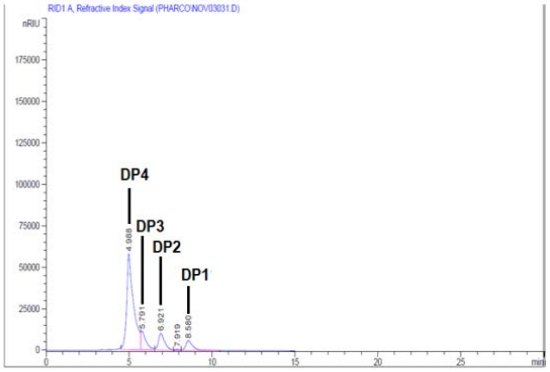

Figure 11B. HPLC for hydrolysis of locust bean gum

4.3. Operational stability

The immobilized enzyme was stable until 5th cycle as it retains 100% of its activity. The enzyme retained 75% of its activity at cycle number 9. The activity then dropped, which might be attributed to the released sugar during hydrolysis or the leaking of the enzyme from the carrier (Gouda *et al.*, 2002). The successive reuse of the immobilized enzyme is a major advantage of this technique due to enhancing the economic value (Fig. 12 A, B).

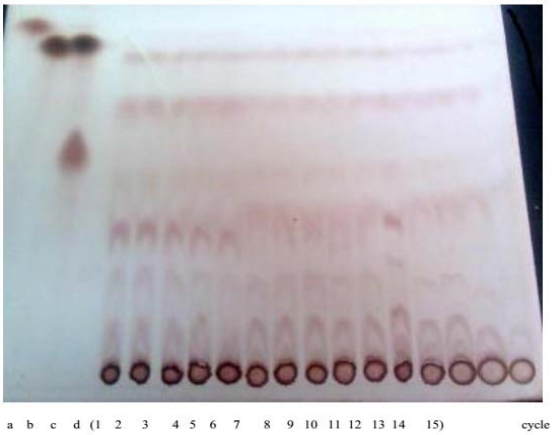

Figure 12A. TLC plate of hydrolysis product of LBG by immobilized enzyme and its reusability.

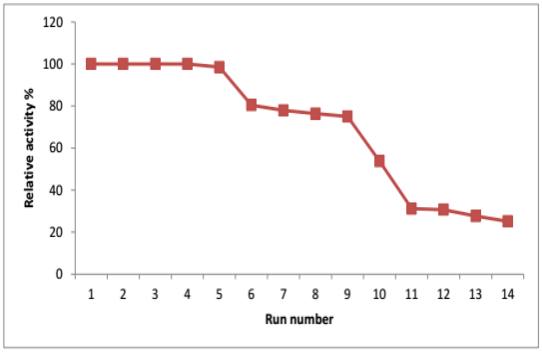

Figure 12B. Operational stability of immobilized from *Penicillium citrinium*150GY

Biological activities of galactomanno-oligosaccharide

4.3.1. Prebiotic activity

Mannooligosaccharides had been previously estimated as prebiotic compounds. They can increase the growth of good bacteria, such as *Lactobacillus* and *Bifidobacterium*, specifically. They can also prevent *Salmonella* and *E. coli* bacteria from attaching to each other in the digestive system (Chauhan *et al.*, 2014 and Dhawan *et al.*, 2015). In the current study, the prebiotic activity of the produced mannooligosaccharides mixture was evaluated by comparing its effect on the growth of various probiotic bacteria including *Pediococus lactis 1,2*, *Lactobacillus lactis*, *Lactobacillus acidophilus* and *Lactobacillus planterum* with that of the pathogenic *E. coli*. The results in table 3 indicated that the produced oligosaccharide mixture possessed a positive effect on the growth of all the tested probiotic strains as compared to the growth of *E.*

Table 3. Prebiotic activity of MOS

Probiotic microorganism	Ratio againt *E. coli*
Pediococus lactis 1	13.06
Pediococus lactis 2	24.9
Lactobacillus lactis	3.2
Lactobacillus acidophilus	4.7
Lactobacillus planterum	15.65

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