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Production of Thermostable β-Mannanase from *Aspergillus niger* using Submerged Fermentation

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A B S T R A C T

Mannanases are one of the important lignocellulose degrading enzymes having potential biotechnological applications in a wide range of industries, including detergent industry, food and feed technology, coffee extraction, bioethanol production, pharmaceutical field, pulp and paper industry. The present study deals with the β-mannanase producing fungi. Fungal Strain *Aspergillus niger* was isolated from compost soil samples collected from Indore region and it was found to produce a significant amount of β-mannanase on solid agar plate and liquid media. Optimization was carried out for various physico-chemical parameters for maximum production of mannanase. After standardization we found that *Aspergillus niger* gave maximum production of mannanase (21U/ml) on the 10th day of incubation. Among different carbon and nitrogen sources 1% LBG and 0.5% yeast extract shows maximum enzyme production i.e. 21 U/ml and 39.89 U/ml respectively. The best temperature and pH for maximum yield of mannanase were 37°C (21.34 U/ml) and pH 6.0 (26.46 U/ml). The optimum inoculum size was found to be 10^7 spores/ml, giving maximum mannanase yield up to 22.63 U/ml. Mannanase was partially purified using ammonium sulfate fractionation and desalting using G25 column. The partially purified mannanase had optimum pH and temperature for activity at 3.8 and 75°C respectively and was found stable at 3-7 pH and 40-70°C temperature range.

Introduction

Mannanases are pivotal enzymes that break the polysaccharide mannans to monosaccharide mannose, which have tremendous biotechnological applications including coffee extraction, food and feed, prebiotics, paper and pulp industry, detergent industry, pharmaceutical industry and bioethanol production. Thermophilic and thermostable β-mannanase have great advantages such as reducing the risk of contamination, increasing the substrate solubility and improving the mass transfer rate, thus attracting considerable research interest in recent years (Maijala *et al.,* 2012). Many industrial applications involve high temperatures, thermophilic mannanases appear to offer a lot of promise for overcoming these thermal obstacles (Sadaqat *et al.,* 2024).

Since enzymatic hydrolysis of mannans happens under mild conditions and does not produce any toxic or damaging by-products, it is a sustainable and

environmentally beneficial (Zhou *et al.,* 2018; Liu *et al.,* 2020). Mannans are a common type of sugar present in hemicellulose. They are hydrogen bonded to cellulose fiber in softwood, hardwood and the seeds of legumes and beans, where galactomannan is the main storage polysaccharide (20% dry weight). Mannans have a great deal of potential for the synthesis of useful derivatives, including prebiotic mannooligosaccharides (MOS) (Dhawan and Kaur, 2007; Do *et al.,* 2009; Chauhan *et al.,* 2012).

Materials and Methods

Locust bean gum (LBG), was obtained from Sigma St. Louis, MO, USA. Mannose, dinitrosalicylic acid, yeast extract, peptone, beef extract, guar gum, Luria broth, dextrose, PDA (Potato Dextrose Agar Media) and pH strips (pH 1-14) were obtained from Himedia laboratories, India. Ammonium sulfate, tween 80, magnesium sulfate, sodium dihydrogen phosphate, di-sodium hydrogen phosphate, citric acid, sodium citrate, sodium nitrite, sodium carbonate, sodium bicarbonate, sodium hydroxide, sodium sulphite, methanol, ethanol, acetone, acetic acid, potassium iodide and manganese chloride were obtained from Merck India Ltd.

Microorganism

Fungal Strain *Aspergillus niger* was isolated from compost soil samples collected from Indore M.P. region. It was identified on the basis of morphological characteristics and molecular basis at NFCCI (National Fungal Culture Collection of India, Pune). The strain was maintained on PDA agar media slants at 4^{o}C.

Submerged Fermentation

Inoculum Preparation

Aspergillus niger was grown on PDA media at 37^{o}C for 5 days and spores were harvested using 5 ml sterile 0.01% tween 80 with the help of sterile glass rod. Spores were counted using a counting chamber. After autoclaving 10^7 spores/ml of spore suspension was inoculated in a 250 ml Erlenmeyer flask containing 50 ml liquid media.

Optimization of Culture Condition for Mannanase Production

Optimization of physico-chemical parameters for maximum production of mannanase were studied by using time scale, different carbon source, nitrogen sources, pH, temperature and inoculum size. Modified submerged fermentation mediums of Kote *et al.,* (2009) were used for optimization of culture conditions. SmF media containing 1% LBG, 0.1% K_2HPO_4 and 0.05% $MgSO_4.7H_2O$. 50 ml of liquid media was taken in each 250 ml Erlenmeyer flask and pH of the medium was adjusted to 5.5.

After autoclaving the media, 10^7 spores were inoculated from 5th day old fungal cultures. Flasks were incubated at 37^{o}C temperature and 140 rpm shaking condition for 5 days. After incubation supernatants were collected by centrifugation at 12,000 rpm for 10 minutes as the crude enzyme source.

Effect of Time scale on Mannanase Production

Effect of fermentation period on mannanase production using submerged fermentation was done on 250 ml Erlenmeyer flask containing 50 ml media. After autoclaving 10^7 Spores/ ml were inoculated in it and incubated at 37^{o}C and 140 rpm shaking condition for 0-12 days. 0.5 ml of sample was withdrawn from the flask each day after 24 hours interval and performed mannanase assay under standard assay condition.

Effect of Carbon Sources on Mannanase Production

Effect of various carbon sources on mannanase production done by using different carbon sources namely LBG, konjac Powder, guar gum, gum arabic, copra meal, rose stem powder, wheat bran and rice bran. 1% of each carbon source was added on a 250 ml flask containing 50 ml liquid media and incubated at 37^{o}C and 140 rpm shaking condition for 10 days. Supernatants were collected by centrifugation at 12,000g for 10 minutes and used as crude enzyme sources for mannanase assay.

Effect of LBG Concentration on Mannanase Production

For enhanced production of mannanase concentration of LBG was optimized in the production medium using 0.2%, 0.5%, 0.8%, 1.0%, 1.5% and 2.0% of LBG. Flasks were incubated for 10 days at 37^{0}C and 140 rpm shaking. Supernatants were collected by centrifugation at 12,000 g for 10 minutes and used as crude enzyme sources for mannanase assay.

Effect of Nitrogen sources on Mannanase Production

The 50 ml Production medium was supplemented with various organic (0.5% w/v) and inorganic (0.5% w/v) nitrogen sources. Flasks were incubated for 10 days at 37°C and 140 rpm shaking condition, thereafter mannanase activity was determined.

Effect of Temperature and pH on Mannanase Production

The effect of different temperatures ranging from 25°C to 60°C and pH ranging from 3.5 to 8.0 on mannanase production were studied. 10 days culture supernatant was used as crude enzyme for mannanase assay.

Effect of Inoculum Size on Mannanase Production

Optimum inoculum size for mannanase production was determined by inoculating different spore's concentration to the production medium ranging from 10^3 to 10^9 spores/ml.

Enzyme Assay

Preparation of Substrate

The substrate used routinely for the study of mannanase is galactomannan from locust bean gum (*Ceratonia siliqua*) with a mannose:galactose ratio of 4:1 (De Nicolas-Santiago *et al.,* 2006). 0.5% LBG as a substrate for mannanase assay was prepared by dissolving 0.5 gram LBG in 50mM citrate buffer pH 3.8. Solution was kept on a magnetic stirrer for 15 min at 60°C. After this solution was centrifuged at 10,000 rpm for 10 min. Clear solution obtained from centrifugation was used for enzyme assay.

Mannanase Assay

0.5 % (w/v) locust bean gum dissolved in a 50 mM citrate buffer at pH 3.8 was used as the substrate mixture. 0.9 ml of the substrate mixture was added to 0.1ml of the crude enzyme solution and incubated at 75°C for 10 minutes. Afterwards 1 ml of dinitrosalicylic acid (DNS) was added to each enzyme – substrate mixture and boiled for10 minutes. The absorbance of the mixture was thereafter measured at 540 nm in a spectrophotometer. The amount of mannose released was determined by the method of Miller (1959).

One unit of mannanase activity was defined as the amount of mannanase that released 1 micro mole of mannose per minute in one ml of the reaction mixture under the assay conditions. The mannanase activity was determined using the standard curve of mannose.

Partial Purification of Enzyme Mannanase

Ammonium sulfate Fractionation

300 ml of cell free crude enzyme was taken from the SmF culture flask on the 10th day of incubation. 198.6 grams of solid ammonium sulfate was added slowly to the crude enzyme with continuous stirring in cold conditions with the help of a glass rod to get 0-90% saturation. The pH of the suspension was kept constant with the help of dilute ammonia.

After adding solid ammonium sulfate enzyme suspension was incubated at 4°C overnight to precipitate proteins. After incubation broth was centrifuged at 12000 g for 30 min in cold condition to collect precipitated protein. The pellets after centrifugation were dissolved in citrate buffer, pH 3.6. Enzyme activity and protein concentration was taken under standard conditions.

De-salting using Sephadex G-25 column

20 grams of Sephadex G-25 beads were soaked in distilled water overnight. After this, the beads were washed with distilled water 2-3 times. Now a column was packed and 15 ml sample was loaded on the top of the filter paper in the G-25 column. 5 ml fraction was collected every time and enzyme activity was taken. The fractions showing mannanase activity were pooled and used for enzyme characterization.

Characterization of Partially Purified Mannanase

Effect of pH on enzyme activity

The optimal pH for enzyme activity was investigated between pH 3.0–9.0 using different buffers (citrate buffer for pH 3–6, Tris–HCl for pH 7–9). Mannanase activity determined by preparing the substrate in different buffer solutions and mannanase assay was performed at 50°C on 10 min assay.

Effect of pH on enzyme stability

In order to determine the pH stability, the mannanase enzyme was preincubated in different buffers ranging from pH 3-9 (citrate buffer for pH 3–6, Tris–HCl for pH 7–9) at 4°C for 1 h and then assayed for mannanase activity with LBG as substrate. The residual activity was determined at 50°C on 10 min assay and LBG substrate was prepared in citrate buffer pH 5.

Effect of Temperature on enzyme activity

The effect of incubation temperature was determined by incubating enzyme and substrate reaction mixture at temperature ranging from 40°C to 90°C with difference of 5°C and enzyme assay was performed on 10 min assay and LBG substrate was prepared in citrate buffer pH 5.

Effect of Temperature on enzyme stability

The thermal stability was investigated by incubating the partially purified mannanase enzyme alone at temperature between 40 to 90°C with an interval of 10°C for 1 h. At each temperature, residual activity was determined at 50°C on 10 min assay and LBG substrate was prepared in citrate buffer pH 5.

Results and Discussion

Optimization of submerged fermentation conditions for mannanase production from *Aspergillus niger*

To increase the production of mannanase from *Aspergillus niger*, various physicochemical parameters affecting its production were optimized by one variable at a time in submerged fermentation.

Effect of time scale

Effect of Time course for mannanase production from *Aspergillus niger* was studied. The production medium used for mannanase production was viscous due to LBG. Production medium inoculated with fungal spores of *A. niger* kept in orbital shaking incubator to provide shaking condition. After 1-day of incubation it was observed that fungal mycelium encapsulated in LBG and large beads were formed. This action divides the production medium into two parts: one was beads and the other was a clear solution having extracellular mannanase. There was no mannanase activity observed from 0 to 1 day. On 2-day production medium broth showed mannanase activity, which kept on increasing and reached its maximum on 10-day of incubation period, which was found to be 21U/ml. After 10-day there was a slight decrease in mannanase production but no further decline was observed in subsequent days (Fig 1).

Effect of different carbon source

The mannanase production was done with different carbon sources viz. LBG, konjac Powder, guar gum, gum arabic, copra meal, rose stem powder, wheat bran and rice bran at a concentration of 1.0%. Maximum enzyme production was observed with LBG (Fig 2).

Effect of LBG concentration

Different concentrations of LBG viz. 0.2%, 0.5%, 0.8%, 1.0%, 1.5% and 2.0% in 50 ml broth was used to determine the effect of concentration of LBG on the production of mannanase. The enzyme production increased with increase in LBG concentration. Although maximum mannanase production was observed with 2% LBG (24.54 U/ml) but for further study 1% LBG (21 U/ml) concentration was selected for mannanase production because LBG is costly substrate and mannanase production does not increase in the ratio of LBG concentration after 1% concentration (Fig 3).

Effect of different nitrogen source

Effect of different nitrogen sources on the production of mannanase with *A. niger* was analyzed. Different nitrogen sources viz. Yeast extract, beef extract, peptone, soybean meal, ammonium sulfate, ammonium nitrate and urea were added at a concentration of (0.5% w/v). Maximum mannanase activity was observed with yeast extract i.e. 39.89 U/ml (Fig 4).

Effect of temperature

The optimum temperature to produce mannanase from *A. niger* was investigated from 25°C-60°C. Since the organism is a mesophile, very less growth as well as low amount of mannanase production was observed at higher temperature. Maximum enzyme production was observed at 37°C (21.34 U/ml) (Fig 5).

Effect of pH

The effect of pH on the production of mannanase, the organism was grown at different pH values by preparing the production medium in the buffers of different pH values. The organism showed minimal enzyme production at lower pH values of 4 and 5 and at higher pH values of 7 and 8. It showed optimal enzyme production at pH 6 (Fig. 6).

Effect of Inoculum size

To ensure a high production of mannanase in the limited volume of medium, the fungal inoculum size should be controlled. Small inoculum size leads to higher surface area to volume ratio as well as improved distribution of dissolved oxygen resulting in increased enzyme production. However, if the inoculums size is too small, insufficient fungal mycelium would lead to reduced amounts of secreted mannanase. Conversely, higher inoculums size results in reduced dissolved oxygen and increased competition towards nutrients. 10^7 spores/ml inoculums size gave maximum enzyme production (22.63 U/ml) (Fig 7).

Characterization of Partially Purified Mannanase

Effect of temperature on enzyme activity and stability

Mannanase showed optimum activity at 75^{o}C (Fig 08) and stable at 40-70^{o}C temperature range (Fig 10).

Effect of pH on enzyme activity and stability

The partially purified mannanase showed optimum activity at pH 3.8. (Fig 9a and Fig 9b) and was found stable at 3-7 pH range (Fig 11).

The large-scale synthesis of industrially significant enzymes is a crucial issue for their commercial utilization. The production of microbial enzymes is affected by the chemical components of the production medium as well as physical factors like temperature, pH, agitation, etc. (Rashid *et al.,* 2012; Olaniyi *et al.,* 2013). Various strategies have been adopted for the optimization of physicochemical parameters for obtaining the highest yield of industrially important enzymes. The most used method is one factor at a time (OVAT) technique where one parameter is changed while keeping the other parameter constant (Liu *et al.,* 2008). This method is useful for choosing factors that have a big impact on the production of enzymes. In optimization studies, this technique is useful for the initial screening of significant factors among a large number of potential components (Dan *et al.,* 2012).

Seven parameters were optimized by varying one variable at a time. Locust bean gum was the most influencing factor followed by yeast extract and inoculum size. Since fermentation duration is crucial, it is also important to find out the optimum period for mannanase production. Time course study for the enzyme production revealed that mannanase yield increased in the exponential phase and it reached its maximum at 10th day of incubation in the stationary phase. It indicated that enzyme yield is directly proportional to the number of fungal spores or microbial cells. Similar results have been shown in other cases of *Penicillium ocittanis* (Blibech *et al.,* 2011).

Microorganisms are very sensitive to pH therefore initial pH of the medium influence's growth as well as overall metabolism of the organism (Poorna and Prema, 2006). The optimum pH for the growth and enzyme production was in the range of pH 5.5-6.5. The optimum pH for the growth of fungi has been reported to vary from one organism to another. pH 3.5 *for Trichoderma reesi* by Eneyskaya *et al.,* (2009), pH 5.0 was reported for *Penicillium oxalicum* by Chantorn *et al.,* (2013) and pH 6 was reported for *Penicillium italicum* by Olaniyi *et al.,* (2015).

Production of any enzyme highly depends on the type of carbon source in the medium (Olaniyi *et al.,* 2013; Srivastav and Kapoor, 2014). When different carbon sources were explored, the mannan rich carbon source such as Locust bean gum, Guar gum etc stimulated the mannanase production. It indicated that production of mannanase from *A. niger* might be inducible.

The induction of mannanase by mannan containing substrates has been reported in most of the organisms; *Bacillus licheniformis* TJ-101 (Liu *et al.,* 2008), *Aspergillus niger gr* (Kote *et al.,* 2009), *Bacillus nealsonii PN-11* (Chauhan *et al.,* 2014).

Figure.1 Effect of Time Scale of Mannanase Production

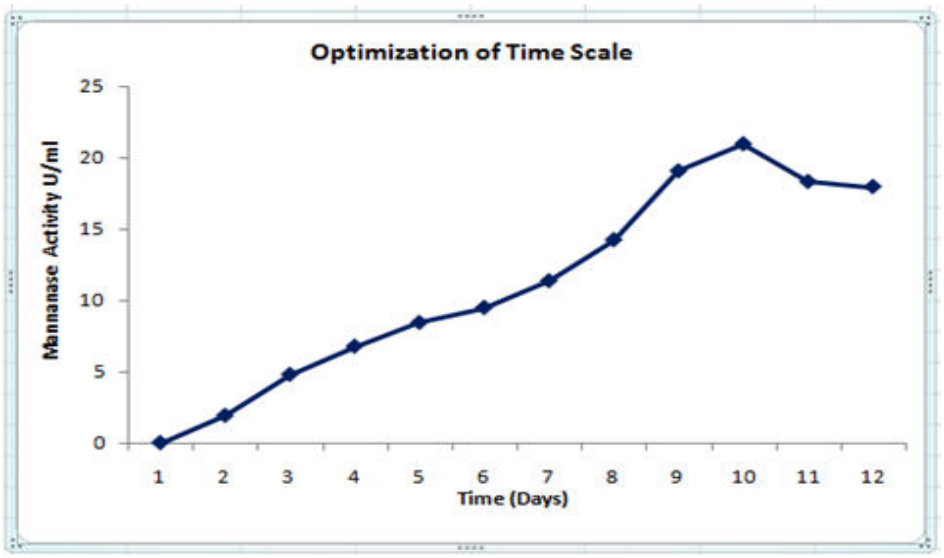


Figure.2 Effect of Various Carbon Sources on Mannanase Production

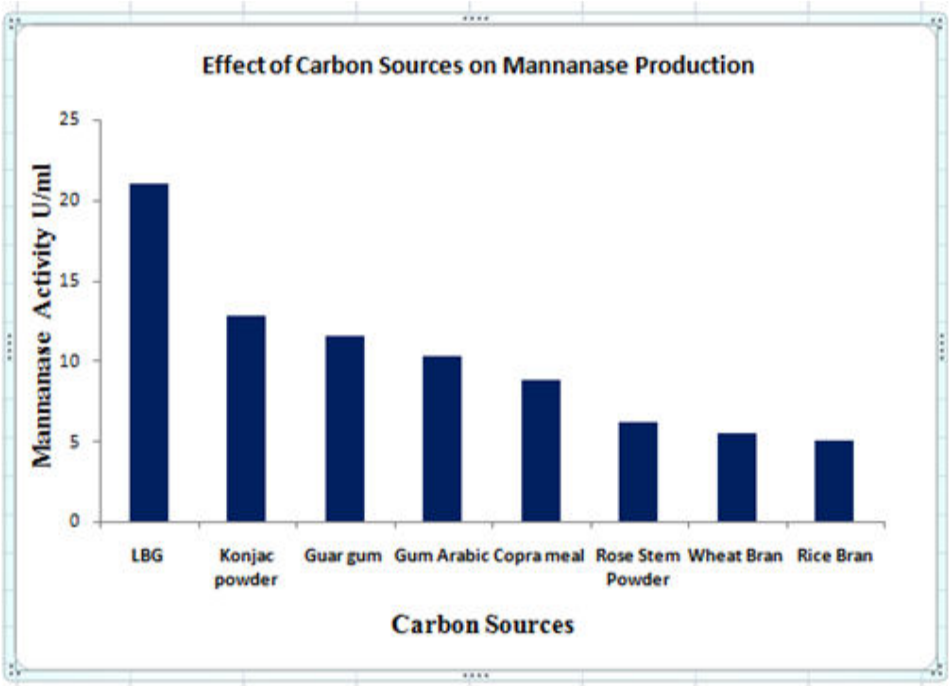


Figure.3 Effect of LBG Concentration for Mannanase Production

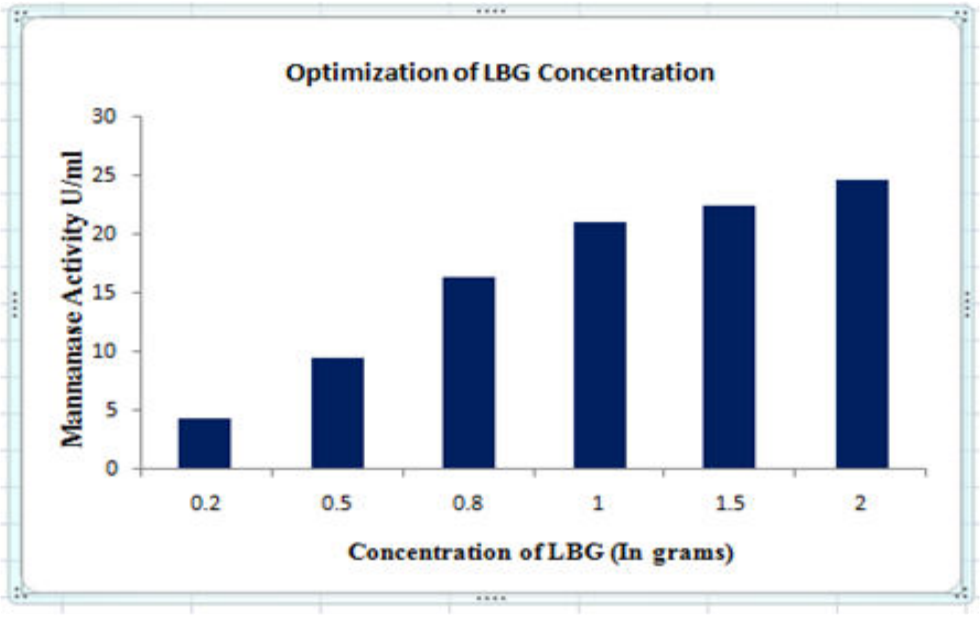


Figure.4 Effect of Various Nitrogen Sources on Mannanase Production

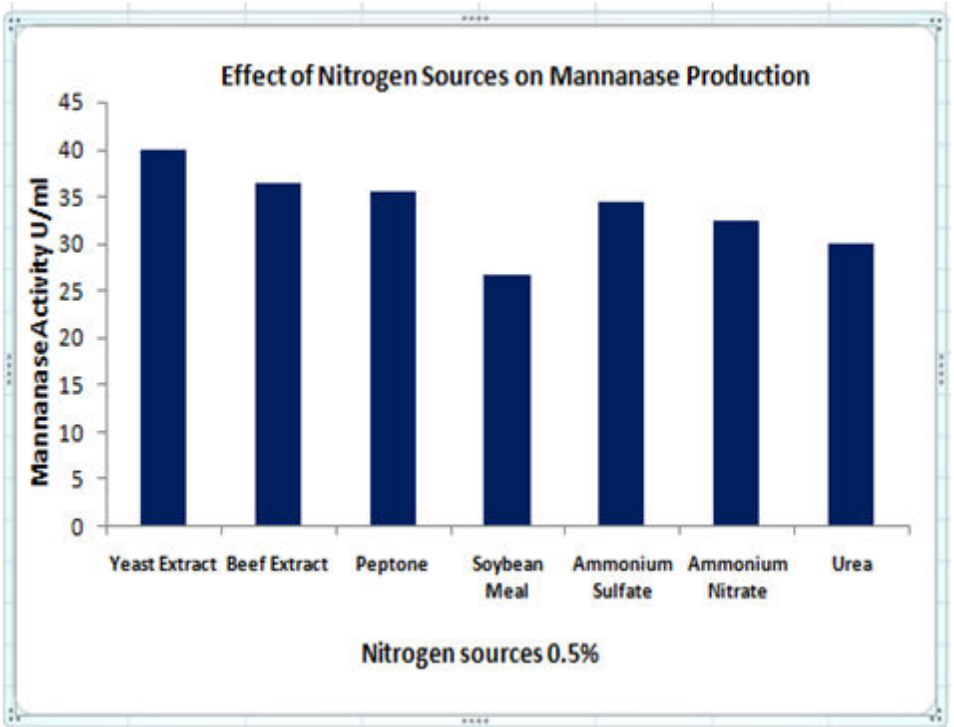


Figure.5 Effect of Temperature on Mannanase Production

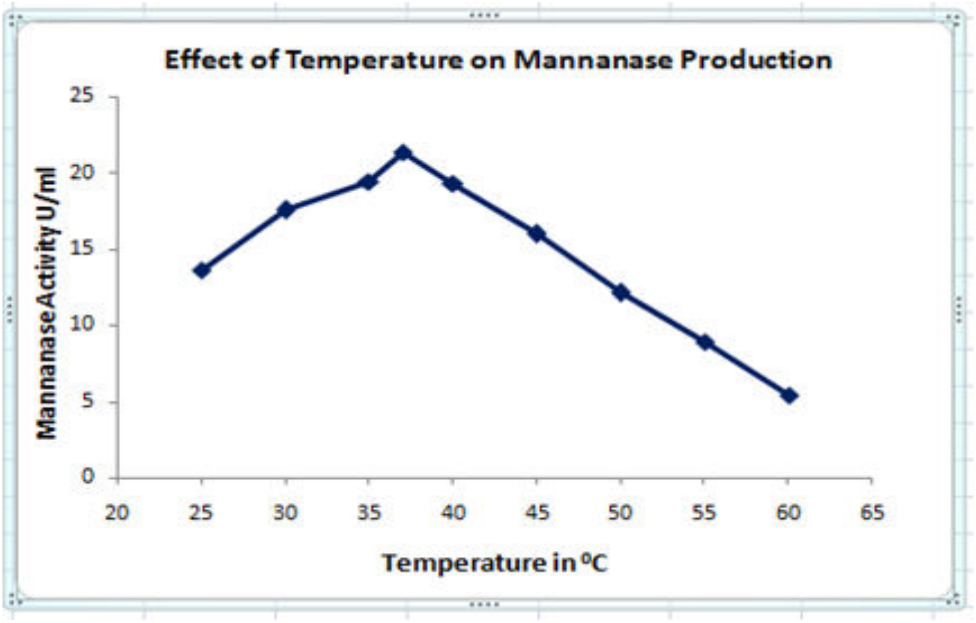


Figure.6 Effect of pH on Mannanase Production

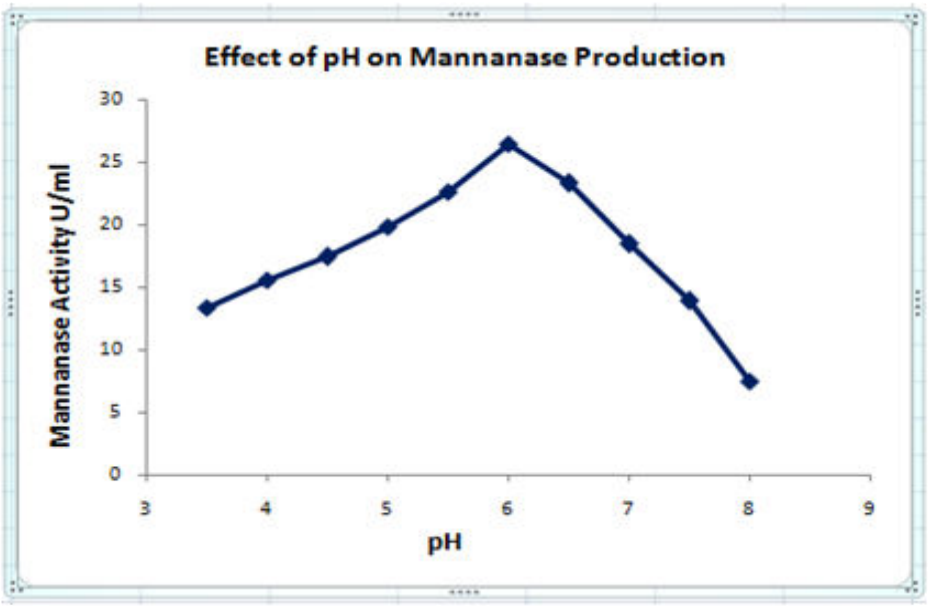


Figure.7 Effect of Inoculum Size on Mannanase Production

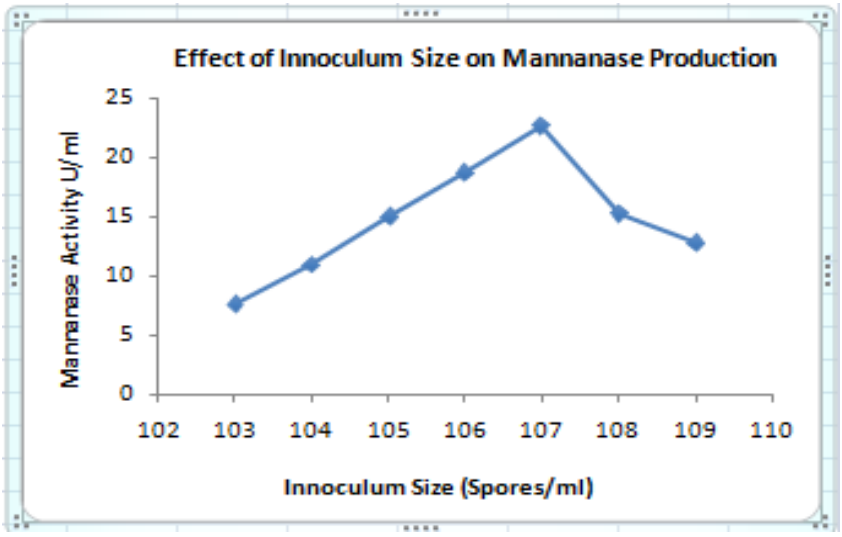


Figure.8 Effect of Temperature on Mannanase Activity

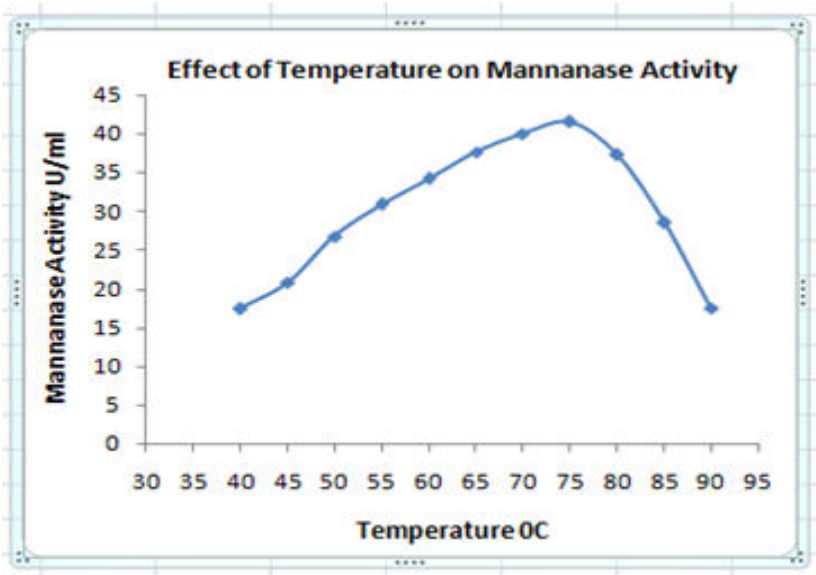


Figure.9(a) Effect of pH on Mannanase Activity

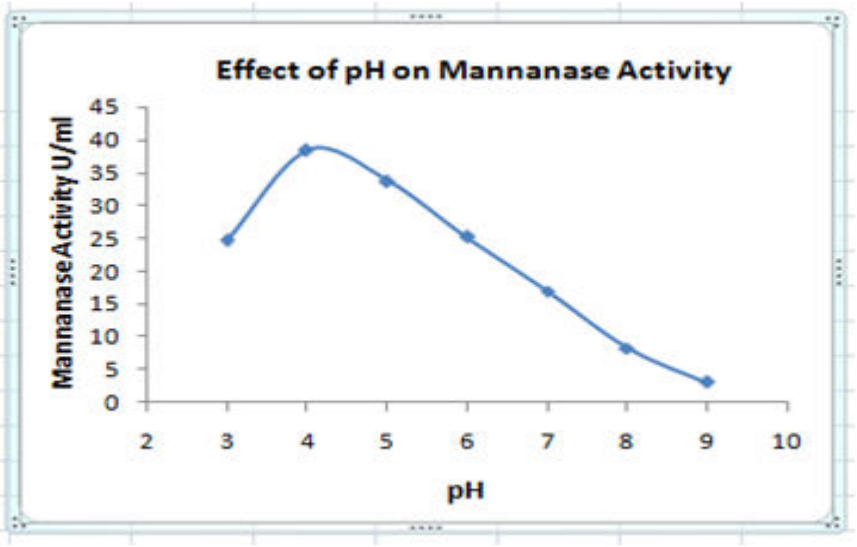


Figure.9(b) Effect of pH on Mannanase Activity

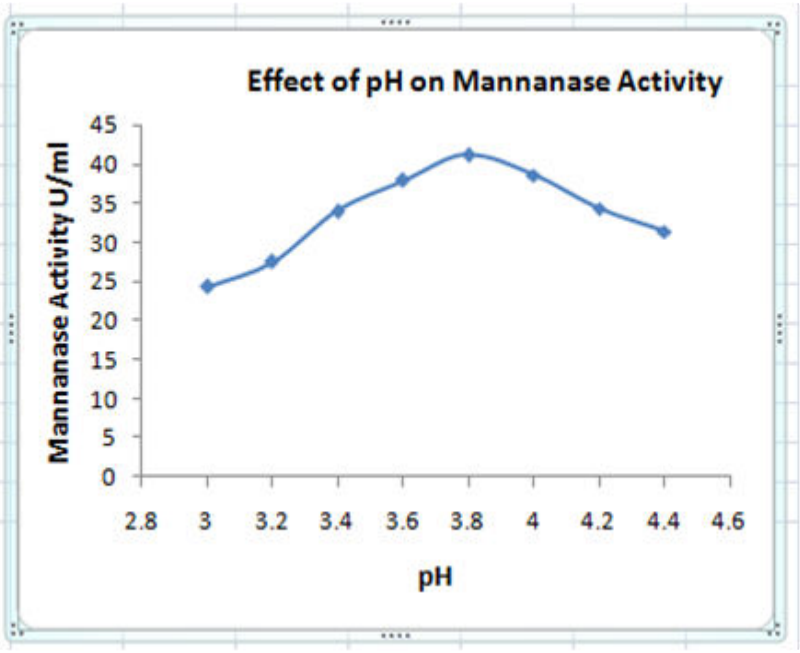


Figure.10 Effect of Temperature on Mannanase Stability

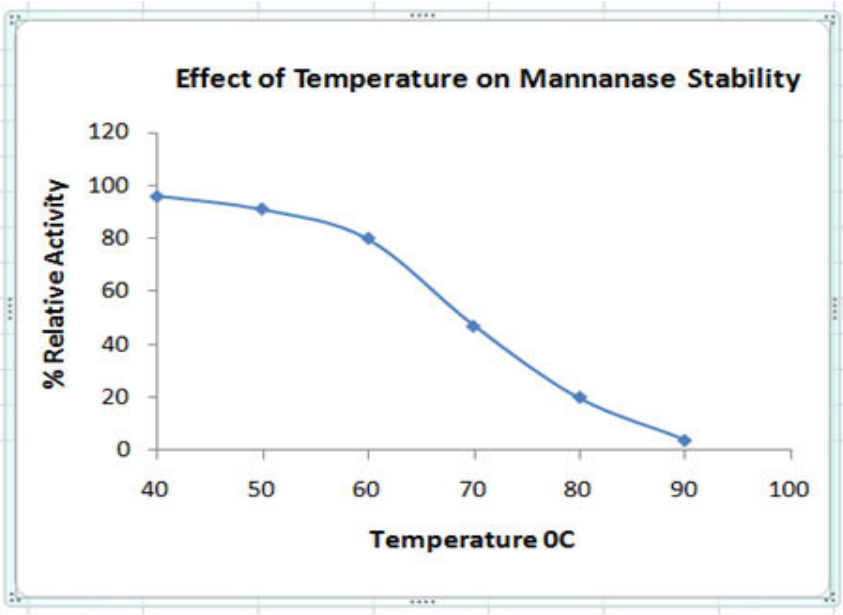
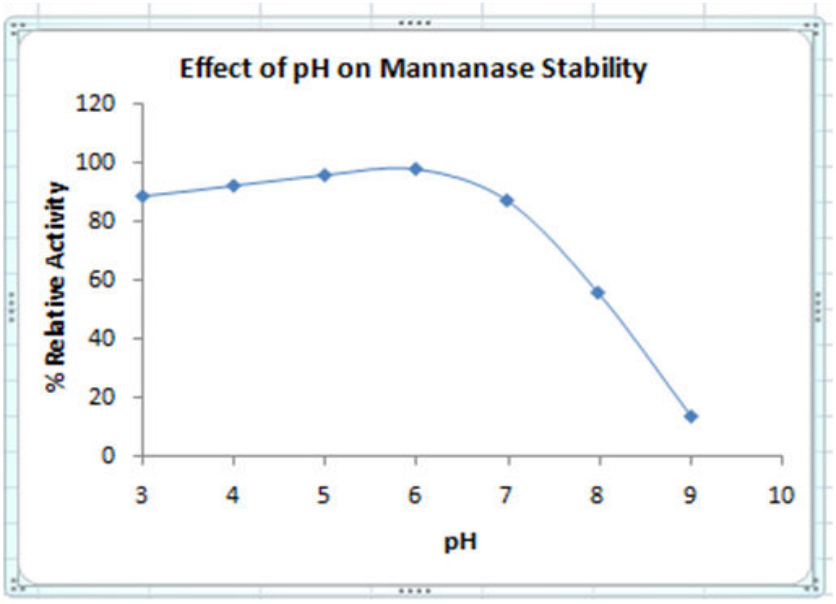


Figure.11 Effect of pH on Mannanase Stability



Further highest enzyme yield could be achieved with LBG. It can be because of the higher content of mannan in LBG than other mannan substrates. Locust bean gum has been reported to be the best carbon source for higher mannanase yield for another organism as well; *Klebsiella oxytoca* CW23 (Titapoka *et al.,* 2008), *Aspergillus niger* gr (Kote *et al.,* 2009). In this study *A. niger* mainly utilized Yeast extract, the organic nitrogen source for maximum mannanase production. For mannanase organic nitrogen sources have been reported to be better sources for higher enzyme yield (Rashid *et al.,* 2012; Chauhan *et al.,* 2014). This could be due to that inorganic nitrogen sources are not involved in the competition with active sites of the enzyme; moreover organic nitrogen sources are preferred as they are cheaper in cost (Karboune, 2009).

The partially purified mannanase was maximally active at 75°C compared with the optimal temperatures obtained for other β-mannanases (65°C for *Aspergillus niger gr;* 60°C for *Aspergillus flavus gr;* 40°C for *Penicillium occitanis* Pol6; 50°C for both *Bacillus circulans TN-31* and *B. subtilis B36*; 60°C for *Paenibacillus sp.DZ3*) (Maruyama and Nakajima, 2000; Kote *et al.,* 2009; Blibech *et al.,* 2010; Chandra *et al.,* 2011). The thermal stability was found in the range of 40-70°C temperature as compared to 60°C for *Aspergillus niger gr and Aspergillus flavus gr;* 50-60°C for *Bacillus sp. MG 33* (Kote *et al.,* 2009; Meenakshi *et al.,* 2010).

The partially purified mannanase showed optimum activity at pH 3.8. and was found stable at 3-7 pH range compared with *Aspergillus niger gr and Aspergillus flavus gr* has maximally active at pH 5.5 and 6 respectively and both enzyme has been stable at 4-8 pH range; *Paenibacillus sp.DZ3* mannanase has been active at pH 6 and stable at 5-7 pH range (Kote *et al.,* 2009; Chandra *et al.,* 2011).

In conclusion, Present study revealed the potential of *Aspergillus niger* isolated from the compost for the high level production of β-mannanase under submerged fermentation. Among the various carbon and nitrogen sources tested, LBG and yeast extract gave maximum mannanase production. The best temperature and pH for maximum yield of mannanase were 37°C and pH 6.0. The partially purified mannanase showed broad activity and stability at 40-70°C and 3-7 pH. This finding demonstrates the potential applications of mannanase and it could be used in different industries.

Author Contribution

Prashant Chourasia: Investigation, formal analysis, writing—original draft. S. Gaherwal: Validation, methodology, writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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