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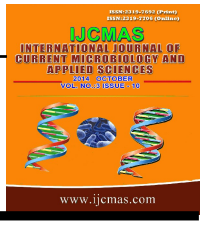

Original Research Article

Optimization of medium composition for alkali-thermostable mannanase production by *Bacillus nealsonii* PN-11 in submerged fermentation

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Keywords

Alkali-stable mannanase, Thermo-stable mannanase *Bacillus nealsonii*,

A B S T R A C T

Alkali-thermostable mannanase producing microorganism *Bacillus nealsonii* PN-11, isolated from forest soil, having potential application in pulp and food industry was used in this study. To enhance the production of mannanase by this strain, various physical and nutritional factors were optimized by one factor at a time method. Locust bean gum was the most influencing factor followed by bactopeptone and inoculum age. The mannanase yield increased from 6 U ml^{-1} at the start to 55 Uml^{-1} at the end of optimization experiments with approximately 9.25 fold increase. To the best of our knowledge yield of mannanase achieved in this study was better than most of the reports of mannanase production in OVAT method.

Introduction

Hemicellulose is the second most abundant heteropolymer present in nature, usually associated with cellulose and lignin in plant cell walls. Hemicelluloses are estimated to account for one third of total components available in the plants. They make upto 25-30% of total wood dry weight. The two most important and representative hemicelluloses are hetero-1,4-β-D-xylans and the hetero-1,4-β-D-mannans (Chauhan *et al.*,, 2012; Chauhan *et al.*, 2014a). Mannan is the predominant hemicellulosic polysaccharide in softwoods from hemicellulose in hardwood from angiosperms.

Major enzymes involved in the hydrolysis of linear mannans and glucomannans are 1,4-β-D mannan mannohydrolases (called mannanases, EC 3.2.1.78), 1,4-β-D-mannopyranoside hydrolases (called β-mannosidases, EC 3.2.1.25) and 1,4-β-D glucoside glucohydrolases (called β-glucosidases, EC 3.2.1.21). The mannanases are endo-acting hydrolases, attacking the internal glycosidic bonds of the mannan backbone chain, releasing short β-1,4-manno-oligosaccharides (Dhawan and kaur, 2007; Chauhan *et al.*, 2012; Chauhan *et al.*, 2014b).

The β-mannosidases are exoacting hydrolases that release mannose from the oligosaccharides by attacking the terminal linkage at the non-reducing end as well as cleaving mannobiose into mannose units (Chauhan et al 2012). The β-mannanases have been grouped into two families, glycosyl hydrolase 5 (GH5) and glycosyl hydrolase 26 (GH26). The protein folding, catalytic mechanism, and mechanism of glycosidic bond cleavage are conserved in both enzyme families (Henrissat B, 1991).

Various mannanases from fungi, yeasts and bacteria as well as from germinating seeds of terrestrial plants have been produced (Lin *et al.,* 2007; Kote *et al.,* 2009; Meenakshi *et al.,* 2010; Blibech *et al.,* 2010; Chauhan *et al.,* 2014b, Chauhan *et al.,* 2014c; Chauhan *et al.,* 2014d). Production of mannanase by microorganisms is more promising due to its low cost, high production rate and readily controlled condition (George *et al.,* 2014a). Microbial mannanases are mainly extracellular and can act in wide range of pH and temperature because of which they have found applications in pharmaceutical, food and feed technology, coffee extraction, bioethanol production, oil and textile industries, slime control agents. Owing to the increasing biotechnological importance of thermo-alkali stable mannanases in pulp and paper industry and other applications, the present study was undertaken.

Studies on medium optimization for mannanase production are the worthwhile technique for multifactor experiments because it is less time consuming and capable of detecting the true optimum conenctration of the factor. In addition, medium composition greatly influence the microbial production of extracellular mannanase and their interaction play an important role in the synthesis of this enzyme. On the other hand, medium optimization is very important not only to maximize the yield productivity, but also to minimize the product cost (Lin *et al.,* 2007; Aziz *et al.,* 2008; Sondhi *et al.,* 2014; George *et al.,* 2014b; George 2014c). Studies on the medium optimization for mannanase production using classical one parameter at a time method and statistical approach have been done (Mohamad *et al.,*, 2011). Previously we isolated, purified and characterized an extracellular alkali-thermostable β-mannanase from the *Bacillus* nealsonii PN-11 having temperature and pH optima are 65°C and 8.8 and also showed its role in pulp biobleaching, reduction in viscosity of coffee extract as well as its prebiotic potential (Chauhan *et al.,* 2014a, Chauhan *et al.,* 2014b, Chauhan *et al.,* 2014c, Chauhan *et al.,* 2014d). The objective of the present work was to characterize the medium and growth conditions of *Bacillus nealsonii* PN-11 for maximum mannanase production in submerged fermentation, using classical one factor at a time method.

Materials and Methods

Strain

The bacterial strain *Bacillus nealsonii* PN-11 was isolated from landfill site, Chandigarh, India (GenBank Accession No. JN624311) and deposited in the Microbial Type Culture Collection, Instiute of Microbial Technology, Chandigarh, India (MTCC No. 11401).

Optimization of mannanase production by one variable at a time method (OVAT)

Optimization of different nutrient and physical parameters for mannanase production were studied by maintaining all factors constant except the one being studied.

Effect of time

Viable cell count and mannanase activity in the cell free supernatant was measured for *Bacillus nealsonii* PN-11 for a period of 0-168 hours. For this, 350 ml of Minimal medium broth was inoculated with 1% inoculum of 20-24 hour old culture of *Bacillus nealsonii* PN-11.

The inoculated medium was distributed equally (20 ml each) in 15 pre sterilised 100ml flasks .These were incubated at 37°C, 150 rpm. Flasks were taken out at regular intervals (0h,1h, 2h, 4h, 8h, 12h, 18h, 24h, 36h, 48h,72h, 96h, 120h, 144h, 168h) and with these growth curve was plotted by measuring culture O.D at 600 nm for each time interval. Enzyme activity was also measured in the cell free supernatant for each time interval.

Effect of pH

The effect of pH was studied by preparing the media in the buffer of different pH viz. pH 4 (Acetate buffer), pH 5 (Acetate buffer), pH 6 (Phosphate buffer), pH 7 (Phosphate buffer), pH 8 (Tris buffer) and pH 9 (Glycine-NaOH buffer) and then inoculating these media with 0.1% inoculum of 24h old culture of the *Bacillus nealsonii* PN-11.The inoculated flasks were incubated at 37°C under shaking conditions (150 rpm) and the enzyme from the respective flask was harvested after 96h of incubation for the mannanase assay.

Effect of temperature

The effect of temperature was studied by incubating the inoculated Minimal medium flasks (pH 7) at various temperature at 27°C, 32°C, 37°C, 42°C and 47°C for 96 h. Enzyme activity was estimated in the cell free supernatant after 96 h of incubation.

Effect of inoculums %

The medium was inoculated with different inoculums sizes (0.1%, 0.2%, 0.3%, 0.4%, 0.5%) of 24h old culture and their effect on the mannanase production was studied after 96h of incubation.

Effect of inoculum age

The medium was inoculated with 0.3% inoculum of different ages *viz.*12h, 18h, 24h, and 36h and enzyme activity was estimated after 96h of incubation.

Effect of different carbon source

Various C-sources like pectin, wheat bran, copra meal, defatted copra meal, LBG, GG and potato peel were added in the minimal medium at a concentration of 0.8% and their effect on mannanase production was studied.

Effect of LBG concentration

Different concentrations of LBG *viz*. 0.1%, 0.2%, 0.4%, 0.6%, 0.8% and 1.0% were added in the Minimal medium to determine the effect of concentration of LBG on the production of mannanase.

Effect of different N-sources

For studying the effect of different N-sources ammonium nitrate was replaced with various N sources like ammonium chloride, ammonium sulphate, urea, sodium nitrate, potassium nitrate, tryptone, casein, bacto-peptone, meat extract, yeast exytract, sodium nitrate + meat extract, sodium nitrate + yeast extract).

Effect of bactopeptone at various concentrations

To study the effect of concentration of

bacto-peptone on the enzyme production, it was added in the minimal medium at different concentrations (01%-0.5%).

Effect of agitation rate

Mannanase production from *Bacillus nealsonii* PN-11 was studied at different agitation rates of 100 rpm, 150 rpm and 200 rpm. The medium flasks were inoculated and incubated rotary shakers set at different agitation rates. The mannanase assay was done after 96h of incubation.

Results and Discussion

Optimization of physicochemical factors for mannanase production from *Bacillus nealsonii* PN-11

To increase the production of mannanase from *B. nealsonii* PN 11, the physico-chemical factors effecting its production were optimized by one variable at a time in submerged fermentation.

Effect of time

Time course for mannanase production and growth for *B. nealsonii* PN-11 was studied. The organism showed a typical sigmoid growth curve with a short lag phase of 2-3 h and reached the stationary phase by 36 h. No extracellular mannanolytic activity was observed during the early exponential growth.

Extracellular mannanase activity could be detected during the mid exponential phase and reached its maximum in the stationary phase at 96 h after which there was no further increase (Fig. 1). After studying the time course, various parameters affecting the mannanase production was optimized by OVAT method.

Effect of pH

To see the effect of pH on the production of enzyme, the organism was grown at different pH values by preparing the MM in the buffers of different pH values as explained in materials and methods. The organism showed minimal growth at lower pH values of 4 and 5 and at higher pH value of 10. It showed good growth in pH range of 6 to 9 and optimal enzyme production was seen at pH 8 (Fig. 2).

Effect of temperature

The optimum temperature for the production of mannanase from *Bacillus nealsonii* PN-11 was investigated from 27°C-47°C.The medium was inoculated and incubated at different temperature viz. 27°C, 32°C, 37°C, 42°C and 47°C. Since the organism is a mesophile, very less growth as well as low amount of mannanase production was observed at higher temperature of 42°C. Maximum enzyme production was observed at 37°C (Fig. 3).

Bacillus nealsonii PN-11 was grown for 24h and it was used as starting culture for inoculation in different.

Effect of Inoculum%

To ensure a high production of enzyme in the limited volume of medium, the bacterial 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 number of bacteria would lead to reduced amount of secreted mannanase. Conversely, higher inoculums size results in reduced dissolved oxygen and increased

competition towards nutrients. To investigate the effect of different inoculums sizes (0.1%, 0.2%, 0.3% 0.4% and 0.5%). 0.3% inoculums size gave maximum enzyme production (Fig. 4).

Effect of Inoculum age

Like inoculum size, the inoculum age (physiological state of cells) can also be an important parameter for the enzyme production. The enzyme production was done by inoculating the medium with 0.1% inoculums of different ages viz. 12h, 18h, 24h, 30h and 36h (the inoculum was prepared by inoculating MM with a loopful of culture from the plate and growing it for different time intervals). Maximum enzyme production was observed with 18h inoculum (Fig. 5).

Effect of different carbon source

The mannanase production was done with different carbon sources viz. pectin, wheat bran, copra meal, defatted copra meal, LBG, GG, and potato peels at a concentration of 0.8%. Maximum enzyme production was observed with LBG followed by Guargum (Fig. 6).

Effect of LBG concentration

Different concentrations of LBG viz. (0.1%), (0.2%), (0.4%), (0.6%) ,(0.8%) and (1%) in 20 ml MM broth (pH8) was used to determine the effect of concentration of LBG on the production of mannanase .Enzyme was harvested after 96 h of incubation. Maximum enzyme production was observed with 0.8% LBG in 20 ml MM broth (pH 8).The enzyme production increased with increase in LBG concentration i.e. from 0.1% to 0.8% and there was no further increase with increasing the LBG concentration (Fig. 7).

Effect of different nitrogen source

Effect of different nitrogen sources on the production of mannanase with *Bacillus nealsonii* PN-11 was analyzed. Different nitrogen sources viz. sodium nitrate, tryptone, potassium nitrate, meat extract, ammonium chloride, bacto-peptone, ammonium sulphate, ammonium nitrate, urea, casein and in combinations such as sodium nitrate+ meat extract, and sodium nitrate+ Yeast extract were added to the MM at a concentration of 0.03%. Maximum mannanase activity was observed with bact-peptone followed by ammonium nitrate, sodium nitrate+ meat extract, sodium nitrate+ Yeast extract, ammonium sulphate, casein, urea. Very less enzyme production was seen with ammonium chloride, tryptone, meat extract, potassium nitrate and sodium nitrate (Fig. 8).

Effect of Bacto-peptone concentration

Since bacto-peptone was the most favorable nitrogen source for the enzyme production, it was necessary to study the effect of its concentration on the enzyme production. The concentration of bacto-peptone was varied (0.01%-0.5%) and its effect was studied on enzyme production. Maximum enzyme production was observed with 0.05% bacto-peptone concentration (Fig. 9). The enzyme production increased with increase in bacto-peptone concentration i.e from 0.01% to 0.05% and then decreased with increase in bacto-peptone concentration i.e from 0.1 to 0.5%.

Effect of agitation rate

The mannanase production was done at different agitation rate viz. 100 rpm, 150 rpm, and 200 rpm. Results revealed that maximum enzyme production was seen at the agitation rate of 150 rpm. The enzyme

production increased from 100 rpm to 150 rpm and then decreased at 200 rpm (Fig. 10). At lower agitation rates, insufficient aeration and nutrient uptake perhaps causes inability of bacteria to grow efficiently and at optimum agitation, aeration of medium is increased which leads to sufficient supply of dissolved oxygen in the medium.

A total of 10 parameters were optimized by varying one variable at a time. The optimized conditions in preceding experiment were used in each subsequent experiment. Locust bean gum was the most influencing factor followed by bactopeptone and inoculum age. The mannanase yield increased from 6 U ml^{-1} at the start (unoptimized conditions) to 55 Uml^{-1} at the end of optimization experiments with approximately 9 fold increase.

For industrially important enzymes, its hyperproduction is an issue of central importance for commercial application. Microbial enzyme/s production is influenced by both physical factors such as pH, temperature, agitation etc. and chemical constituents of the medium (Rashid *et al.,* 2012; Olaniyi *et al.,* 2013).

Several strategies have been adopted for the optimization of physiochemical factors for increasing the yield of any enzyme, the most commonly being 'One factor at a time' (OVAT) technique where one parameter is changed while keeping the other constant (Liu *et al.,* 2008). Although this technique is helpful in selecting parameters significantly affecting the enzyme production. This system is beneficial in optimization studies for the preliminary screening of important factors amongst a large number of possible factors (Dan *et al.,* 2012).

In this regard parameters effecting the mannanase production from *Bacillus nealsonii* PN 11 was optimized by one variable at a time method (OVAT). Initially production of enzyme was optimized by one factor at a time. Time course study for the enzyme production and growth revealed that mannanase yield increased in the exponential phase and it reached its maximum at 96 h in the stationary phase. It indicated that enzyme yield is directly proportional to the number of cells. Similar results have been shown in other cases eg. maximum mannanase yields from *Bacillus* sp. MG-33 (Meenakshi *et al.,* 2010), *Bacillus subtilis* WY34 (Jiang *et al.,* 2006), *Aspergillus niger* LW-1 (Zhang *et al.,* 2008) have been reported in the stationary phase. Other factors which enhanced the mannanase yields were pH, LBG and bactopeptone.

Initial pH of the medium influences growth as well as overall metabolism of the organism (Poorna and Prema, 2006). The optimum pH for the growth of *Bacillus nealsonii* PN-11 was in the range of (7-8), higher enzyme production was also observed in this range being maximum at pH 8.0. Similarly in other cases it has been shown that maximum mannanase production is at the optimal pH of their growth for eg. *Bacillus* sp. N16-5 (Lin *et al.,* 2007), *Bacillus subtilis* BM-9602 (Cui *et al.,* 1999) and *Paenibacillus* sp. MSL-9 (Manjula *et al.,* 2010).

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 *viz,* Locust bean gum (LBG), Guar gum (GG) and potato peels stimulated the mannanase production where as non mannan containing carbon source could not do so. It indicated that production of mannanase from *Bacillus nealsonii* PN-11 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). Further highest enzyme yield could be achieved with LBG it can be because of high content of mannan in LBG than GG and potato peels. Moreover in LBG, the ratio of mannose to galactose is usually 4:1 (Prajapati *et al.,* 2013), while this is 2:1 for GG (Prajapati *et al.,* 2013) which indicate that LBG has less substituted structure than other manan containg carbon sources hence it is easy to metabolize. Locust bean gum has been reported to be the best carbon source for higher mannanase yield for other organism as well; *Klebsiella oxytoca* CW23 (Titapoka *et al.,* 2008), *Aspergillus niger gr* (Kote *et al.,* 2009).

The requirement of specific nitrogen supplement differs for organism to organism and for the enzyme/s to be produced. For mannanase organic nitrogen sources have been reported to be better sources for higher enzyme yield (Rashid *et al.,* 2012; Akinyele *et al.,* 2013). Similar results were obtained with *Bacillus nealsonii* PN-11 where organic nitrogen sources *viz,* tryptone, casein, bactopeptone, beef extract and yeast extract gave higher enzyme yield than the inorganic nitrogen sources. This could be due to that inorganic nitrogen sources are not involved in the competition with active site of the enzyme moreover organic nitrogen source are preferred as they are cheaper in cost (Karboune, 2009). Among different organic nitrogen sources bactopeptone enhanced the enzyme yield to the maximum, reason might be it has higher protein content than the others (Klompong *et al.,* 2009). Bactopeptone has been reported to be the best nitrogen source for mannanase production for number of other organism (Mohammad *et al.,* 2011; Chantorn *et al.,* 2013). Optimization of mannanase production with one variable at a time method increased yield from 6.0 to 55.5 Uml^{-1} leading to approximately 9.25 fold increase over unoptimized conditions.

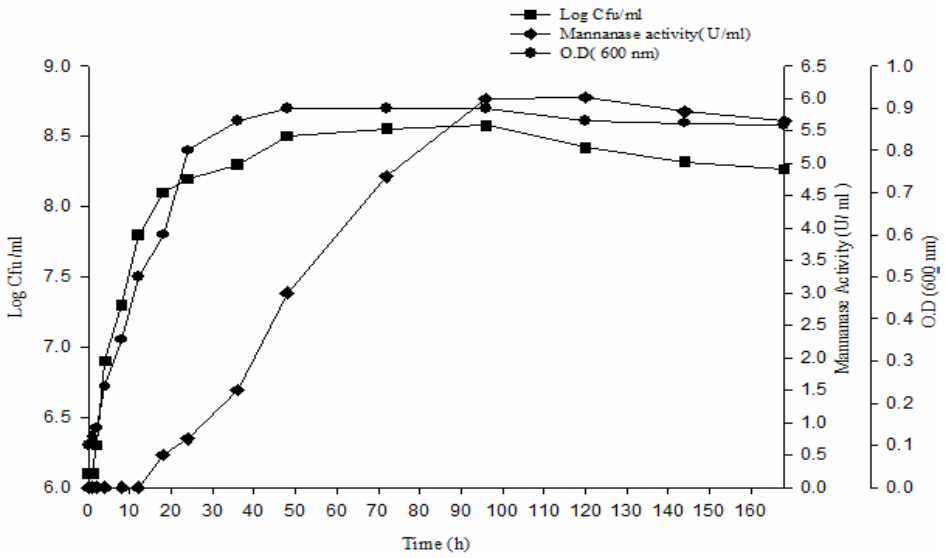

Fig.1 Growth curve of *B. nealsonii* PN-11 and time course for production of mannanase

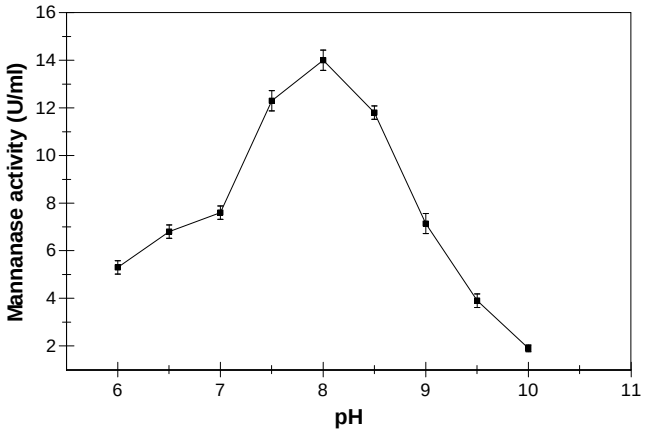

Fig.2 Production of mannanase from *Bacillus nealsonii* PN-11 at different pH ranges

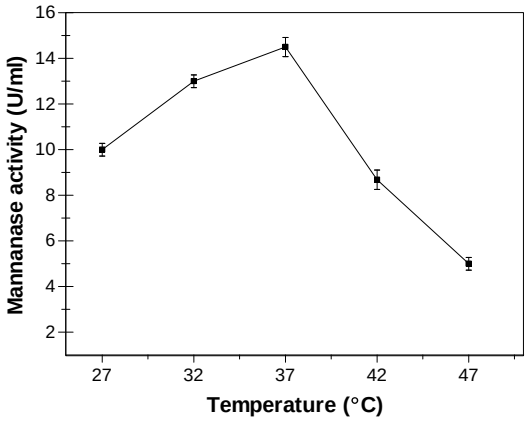

Fig.3 Production of mannanase from *Bacillus nealsonii* PN-11 at different temperature ranges

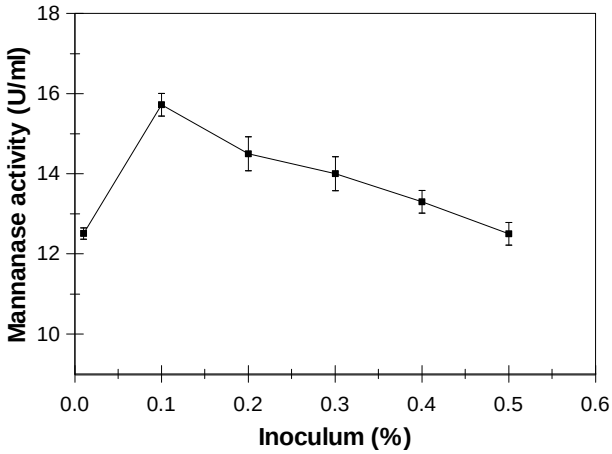

Fig.4 Production of mannanase from *Bacillus nealsonii* PN-11 at different inoculums sizes

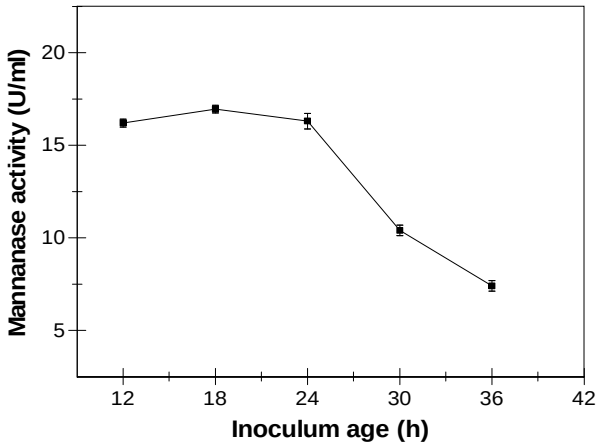

Fig.5 Production of mannanase from *Bacillus nealsonii* PN-11 at different inoculums age

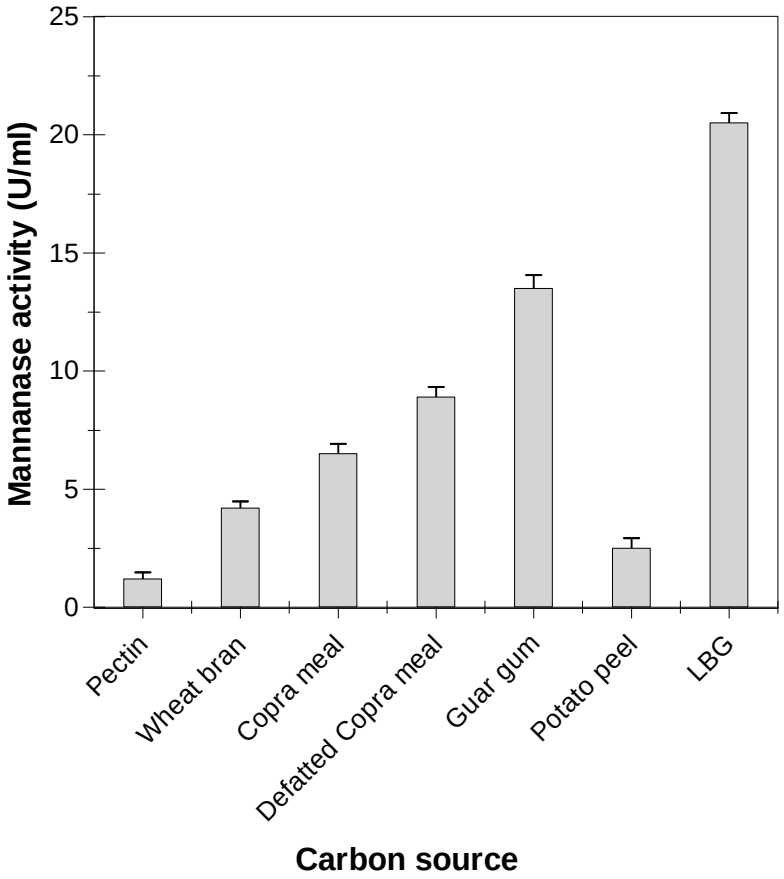

Fig.6 Production of mannanase from *Bacillus nealsonii* PN-11 at different carbon sources

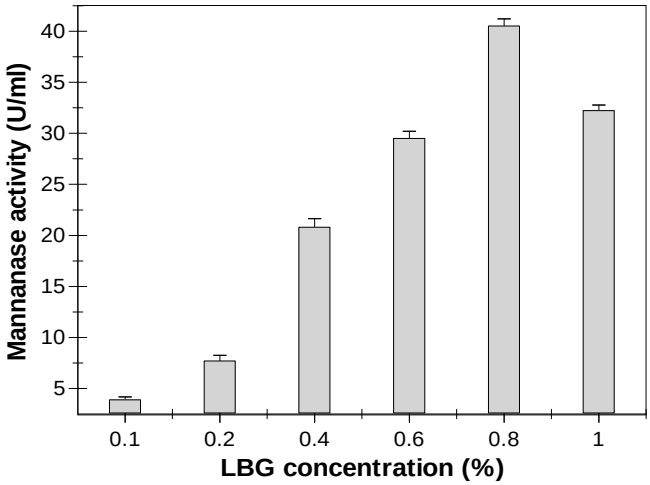

Fig.7 Production of mannanase from *Bacillus nealsonii* PN-11 at different LBG concentration

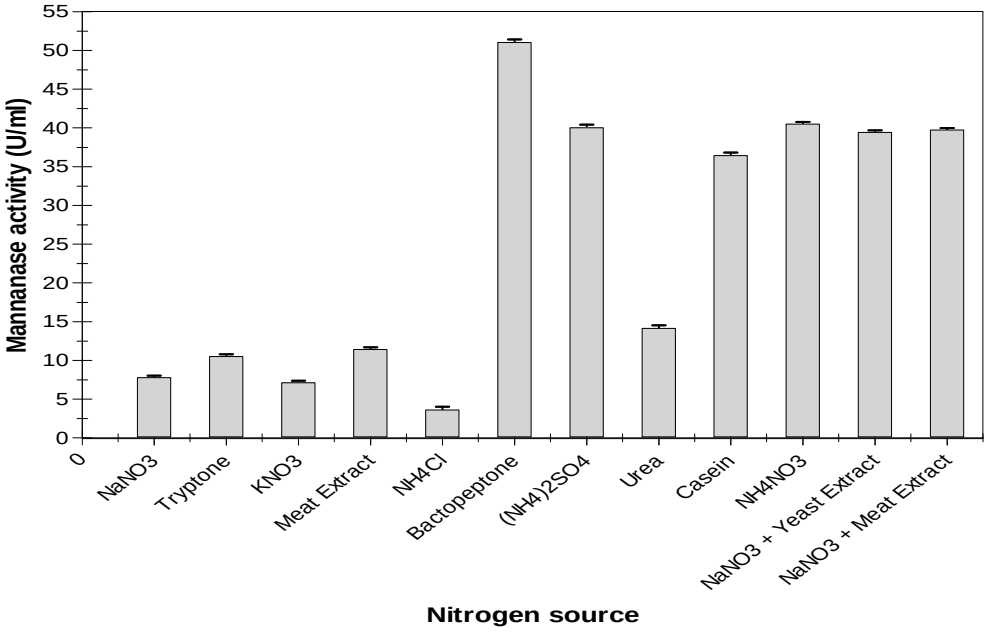

Fig.8 Production of mannanase from *Bacillus nealsonii* PN-11 at different nitrogen concentration

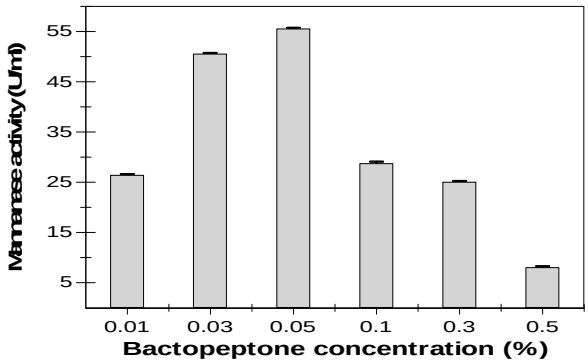

Fig.9 Production of mannanase from *Bacillus nealsonii* PN-11 at different bactopeptone concentration

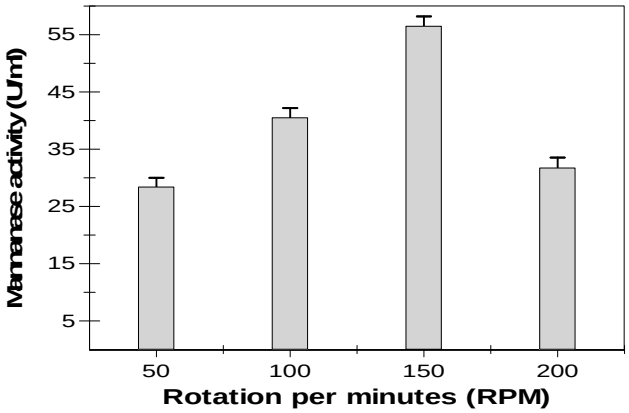

Fig.10 Production of mannanase from *Bacillus nealsonii* PN-11 at different agitation rates

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