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Production of cellulase free thermostable xylanase from *Pseudomonas* sp. XPB-6

Sharma P.K. and Chand D.
Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla, INDIA

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Abstract

Pseudomonas sp. XPB-6 grows better in the medium containing xylan, glucose and meat extract, which was further modified by optimizing the concentrations of individual components. The organism was able to utilize most of the sugars for growth; however dextrose at 4% concentration proved to be the most suitable carbon source for production of xylanase activity from Pseudomonas sp. XPB-6. Different inorganic and organic nitrogen supplements were also tested and maximum production was achieved with 0.5% meat extract. The optimum temperature, pH substrate concentration and inoculum size for the growth and xylanase production were 30°C, 7.0, 1% and 6% respectively. The optimum time for growth and enzyme production was 24 hours. Maximum xylanase activity was reported with 100 mM sodium phosphate buffer (pH 7.5) at 55°C and 5 minutes incubation temperature. The enzyme was fairly stable at 25°C and 75°C. While studying the effect of various metal ions (addition of 1mM $CuSO_4.5H_2O$, $CdCl_3.2H_2O$, $ZnSO_4.7H_2O$ and $CaCl_2.2H_2O$) drastic decrease in xylanase activity was observed.

Key words: Xylanase, *Pseudomonas* sp. XPB-6, xylan, thermostability.

Introduction

Xylanases, a combination of hydrolytic enzymes, catalyze the hydrolysis of xylan, which are genetically single chain glycoproteins, ranging from 6–80 kDa and active at temperature between 40 and 60°C[1]. Xylanases are considered to be able to effectively hydrolyze xylan, the principal type of hemicellulose containing a linear polymer of β-D-xylopyranosyl units linked by (1-4) glycoside bonds which act cooperatively to convert xylan to its constituent simple sugars. These enzymes include β-1,4-endoxylanases (xylanases; EC 3.2.1.8), which cleave internal glycosidic bonds within the xylan backbone; arabinofuranosidase (EC 3.2.1.55), which hydrolyzes arabinose side chains; R-glucuronidase (EC 3.2.1.131), which removes glucuronic acid side chains from xylose units; xylan esterases (EC 3.1.1.6), which release acetate groups and finally xylosidase (EC 3.2.1.37), which hydrolyzes xylobiose to xylose[2]. There is a considerable degree of synergy among these enzymes[3]. Many xylanases do not cleave glycosidic bonds between xylose units that are substituted. Thus, side chains must be cleaved before the xylan backbone is completely hydrolyzed [2]. Conversely, several accessory enzymes will only remove side chains from xylooligosaccharides and therefore require xylanases to partially hydrolyze the plant structural polysaccharide, before side chains can be cleaved[3]. These enzymes have potential in the biodegradation of lignocellulosic biomass to fuels and chemicals, in improving rumen digestion and for use in the prebleaching of kraft pulp, mainly because of a desire to move away the use of chlorine as a bleaching agent [4,5]. Xylans do not form tightly packed structures and hence are more accessible to hydrolytic enzymes. Consequently, the specific activity of xylanase is two to three times greater than the hydrolases of other polymers like crystalline cellulose[6]. Xylanases from different sources differ in their requirements for temperature, pH etc. for optimum functioning. The complete enzymatic hydrolysis of xylan into its constituent monosaccharides requires the synergistic action of a consortium of xylanolytic enzymes. Xylanases have expanded their use in many processing industries, such as pulp and paper, food and textile[7]. Xylan degrading enzymes have wide industrial applications, either alone or in association with other enzymes, in various processes like pretreatment of forage crops and lignocellulosics biomass, improvement of nutrient utilization of cereal-based diets in pig and poultry, flour improvement for bakery products, saccharification of agricultural, industrial and municipal wastes, processing pulp and fibres, enhanced bleaching of cellulose pulps while decreasing consumption of chlorine containing chemicals and as an alternative to treatment with sulphuric acid of the textile-cellulosic waste[8]. Use of abundantly available agro-residues in fermentation processes serves the dual purpose of cost effective enzyme production and environment security[9]. Xylanases are produced by diverse genera and species of bacteria, actinomycetes and fungi. While several *Bacillus* species secrete high levels of extracellular xylanase, filamentous fungi secrete high amounts of extracellular proteins where xylanase secretion often accompanies cellulolytic enzymes for example as in species of *Trichoderma*, *Penicillium*, and *Aspergillus*[10].

Keeping in view the applications of xylanase the present work entitled "Production of cellulase free xylanase from *Pseudomonas* sp. XPB-6" has been carried out. The xylanase of

Pseudomonas sp. XPB-6 shows good enzyme activity, which can be explored in various industrial processes as already given.

Material and Methods

Microorganism: In the present study a xylan metabolizing bacterium XPB-6 was isolated in the Department of Biotechnology, Himachal Pradesh University, Shimla from the soil samples of Mandi (Himachal Pradesh, INDIA) has been explored for its xylanolytic activity. This bacterium has been identified as *Pseudomonas* sp. XPB-6 at Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh (INDIA).

Chemicals: All the chemicals were of analytical grade. Media components were from HiMedia (Mumbai) and the inorganic salts were of analytical grades.

Quantitative determination of xylanolytic activity: A loopful of bacterial cells were inoculated in seed medium containing peptone 0.5 %, yeast extract 0.2 %, beef extract 0.2 %, dextrose 0.25 % and was incubated at 30˚ C for 24 hours at 160 rpm. The precultures were added to 50ml production medium containing 0.5% peptone, 0.2% yeast extract, 0.2% beef extract and 0.25% xylan and were incubated at 30˚C for 24 hours at 160 rpm. The culture contents were centrifuged at 10,000 g for 20 min, at 0-4ºC and supernatant thus collected was further assayed for xylanase activity as per the standard protocol mentioned below.

Assay of xylanase activity: Xylanase activity was assayed using birchwood xylan 0.5 % as substrate and the amount of reducing sugar released was determined by DNSA (Dinitrosalicylic acid) method [11].

Procedure: Reaction mixture was incubated at 50˚C for 10 minutes in water bath and then 3.0 ml of dinitrosalicylic acid reagent (DNSA) was added to the mixture in the test tube to terminate the reaction. The test tubes were incubated in boiling water bath for 20 minutes to develop maximum colour and then cooled to room temperature and absorbance was recorded against blank at 540 nm.

Standard curve: Calibration curve was prepared with D-xylose (20-200 µg /ml).

Enzyme activity: One unit of xylanase activity was expressed as the amount of enzyme required to produce 1 µmol of reducing sugar (xylose equivalent) in 1 minute.

Assay of cellulase activity: Cellulase activity was determined as per procedure given in section 2.2.1 by replacing xylan with cellulose (cotton linter, filter paper and CMC).

Optimization of cultural conditions for xylanase production: The optimization of cultural conditions was carried out with stepwise modifications of the production medium selected. The bacterial isolate XPB-6 was grown in different media (pH 7.0, 160 rpm) at 30˚C for 24 hours.The investigation of effect of pH on xylanase production, the organism was cultivated in the selected production medium at varying pH viz. 5.0-10.0, temperatures ranging from 25˚C to 55˚C, various synthetic and natural carbon sources were tested in the proportion of 1.0% (w/v) to assess their suitability to promote xylanase yield from the selected microorganism. The selected carbon source (dextrose) was used at different concentrations (0.5% to 5.0% w/v), nitrogen source, inducer concentration, inoculum size and growth profile for enzyme production was also optimzed.

Optimization of reaction conditions: The buffer system and the pH optima, buffer concentration, reaction temperature, time of incubation, effect of substrate concentration, effect of enzyme concentration, effect of metal ions, thermostability of xylanase , time course of enzyme reaction, and Shelf life of enzyme was studied according to the assay given by Miller[11].

Results and Discussion

Pseudomonas sp. XPB-6 very efficiently produces cellulase free xylanolytic enzymes. In this paper optimization of cultural and reaction conditions, purification and potential application of its xylanase for biobleaching from agro based pulp and clarification of fruit juices is discussed. The results of the experiments performed in this study are presented and discussed in the following sections: optimization of culture conditions for xylanase production by *pseudomonas* sp. xpb-6.

Screening of media for xylanase production: Among different media (pH 7.0) used, the maximum production of enzyme with an enzyme activity of 20.69 IU/ml was observed in starch dextrose yeast extract medium. While lowest activity (0.016 IU/ml) was observed in mineral salt medium containing KH_2PO_4 and $(NH_4)_2SO_4$.

Effect of pH on xylanase production: Xylanase production has been shown to be markedly dependent on pH in different species of microorganisms. Cultivation of microorganism at an unfavourable pH may favour limited growth rate and xylanase production by reducing accessibility of the hemicellulosic substrate[12].

The microorganism was grown in 50 ml of production medium with a pH range of 5.0-10.0 at 30˚C under shaking conditions for 24 hours. Maximum xylanase production was observed at pH 7.0 (21.96 IU/ml). The enzyme production decreased with increase in pH after 7.0 as shown in figure-1.

***Pseudomonas* sp. XPB-6:** Similar results have been reported for production of xylanase from *Bacillus* sp. RPP1[9]. Most xylanases known so far have their optimum pH around neutrality[13]. Even xylanases produced by most alkaliphiles reported to date have their optimum production pH around neutral pH while enzymes showing optima around pH 9 have low stability at these pH values[14,15,16].

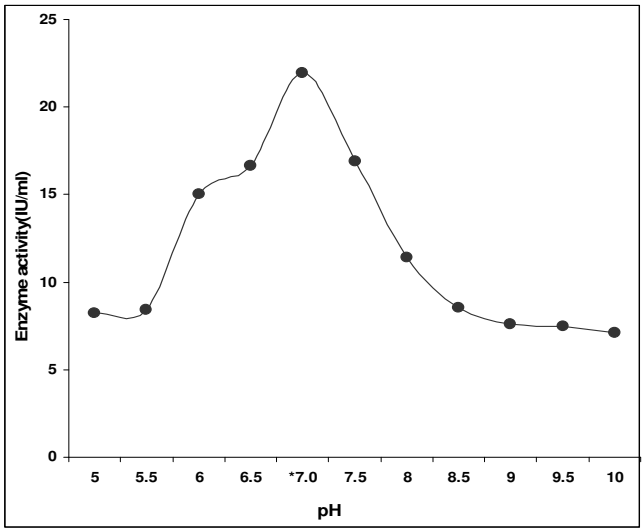

Figure-1
Effect of pH on xylanase production of xylanase from

Effect of production temperature: The microorganism produced appreciably high levels of xylanase at 30˚C as compared to other temperatures tested for xylanase production (Figure-2). The enzyme production considerably decreased with increasing temperature after 30˚C. *Aspergillus niger* USM A1I produces maximum of xylanase at 28˚C with an activity of 14.4 IU/ml[17]. Another fungus produce xylanse at 30 ˚C[18].

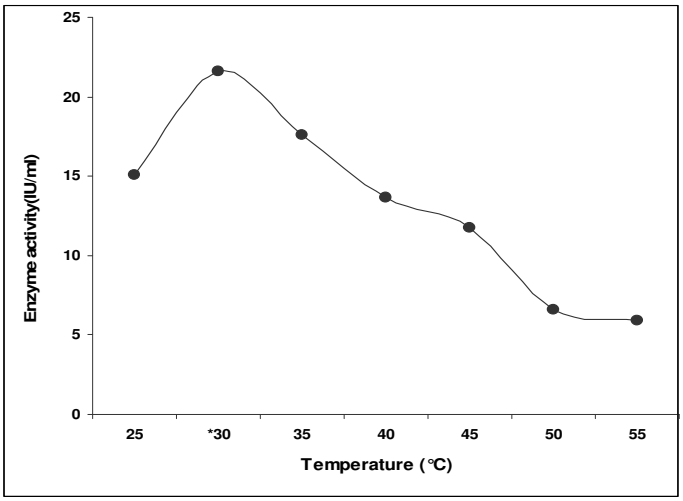

Figure-2
Effect of temperature on xylanase production by *Pseudomonas* sp. XPB-6

Effect of carbon sources on xylanase production: After 24 hours of incubation of selected microorganism with various synthetic and natural carbon sources, maximum activity was observed in dextrose (figure-3).

***Pseudomonas* sp. XPB-6:** In case of natural carbon sources, saw dust induced the highest level of xylanase activity. Bakri *et al*.,[19] observed maximum enzyme production in medium containing wheat straw (52.81 IU/ml). Ghosh *et al*.,[20] reported that xylose, the ultimate breakdown product of xylan, serves as a good inducer of this enzyme. However, other sugars like glucose and CM-cellulose were found poor inducer of xylanase production (0.26 IU/ml).

Low molecular mass degradation products of xylan and cellulose hydrolysis penetrate into the cells and induce the production of hydrolytic enzymes[21].

Different concentrations of dextrose (27.75-277.5mM) were used in the production medium and 222mM of dextrose showed the maximum xylanase production as shown in figure-4. Further increase in concentration led to decrease in xylanase production possibly due to metabolic inhibition.

Effect of substrate concentration: To study the effect of concentration of substrate, different concentrations of xylan was used. Xylan at 1.0% concentration gave maximum activity as shown in figure- 5. Further increase in xylan concentration resulted in a significant decrease in xylanase induction. This may be due to the fact that high concentration of substrate led to increase in medium viscosity, which influenced the medium components and oxygen transfer.

***Pseudomonas* sp. XPB-6:** Similar results have been reported for production of inducible xylanase from *Aspergillus niger* SS7. A very low xylanase production of 0.26, 0.29, 0.52, 0.71 and 2.43 IU/ml was detected in the medium containing glucose, sucrose, maltose, xylose, starch and cellulose while in the medium containing xylan, the amount of xylanase reached a level of 34.19 IU/ml after 120 hours[19].

Effect of nitrogen sources on xylanase production: Nitrogen can act as an important limiting factor in the microbial production of enzymes. Among various organic and inorganic nitrogen sources studied, 0.5% meat extract has been observed to be the best source for xylanase production as shown in figure-6. All other nitrogen sources used except yeast extract and ammonium sulphate repressed production of xylanases (figure-7). This might be due to change in C: N ratio that could have affected the overall enzyme production. Laxmi *et al*., [22] studied the impact of inorganic (ammonium sulphate, ammonium nitrate, ammonium chloride, sodium nitrate, potassium nitrate and urea) and complex nitrogen (yeast extract, peptone, beef extract, tryptone, soya bean meal, corn steep liquor and pea nut meal) sources on xylanase production by *Aspergillus* sp. RSP-6, by supplementing the 0.2 % of each selected nitrogen source individually in the fermentation medium. Among all nitrogen sources studied, yeast extract supported the maximum enzyme production followed by beef extract and pea nut meal.

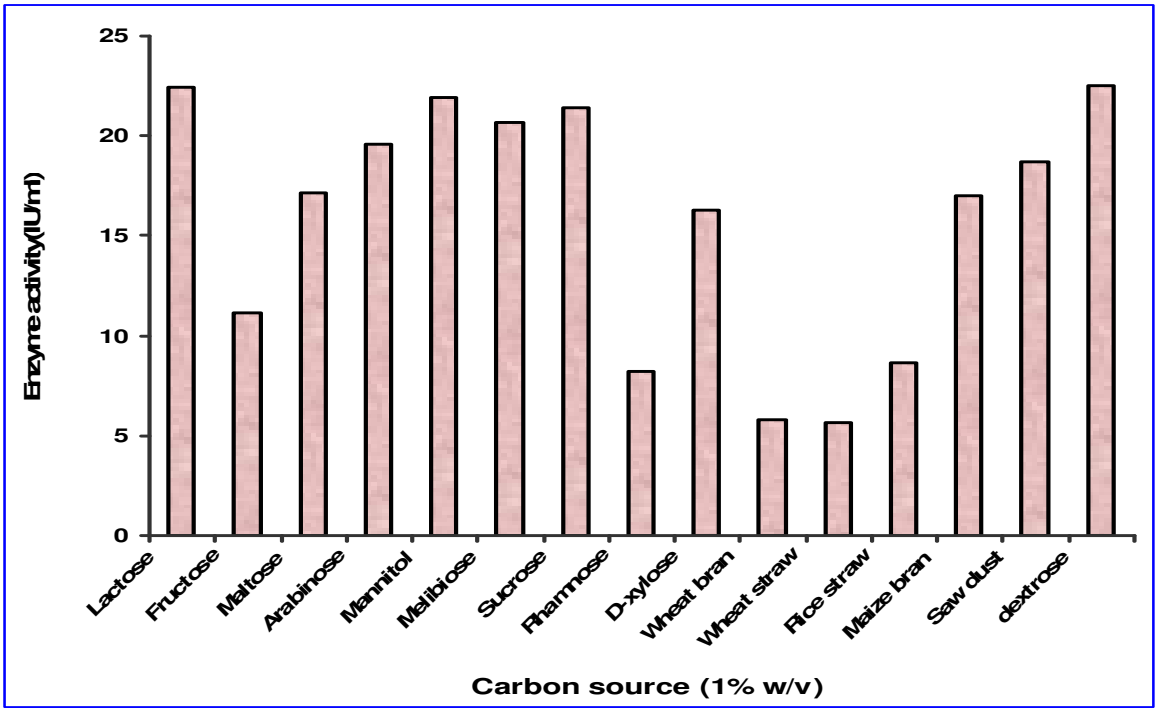

Figure-3
Effect of carbon sources on xylanase production from

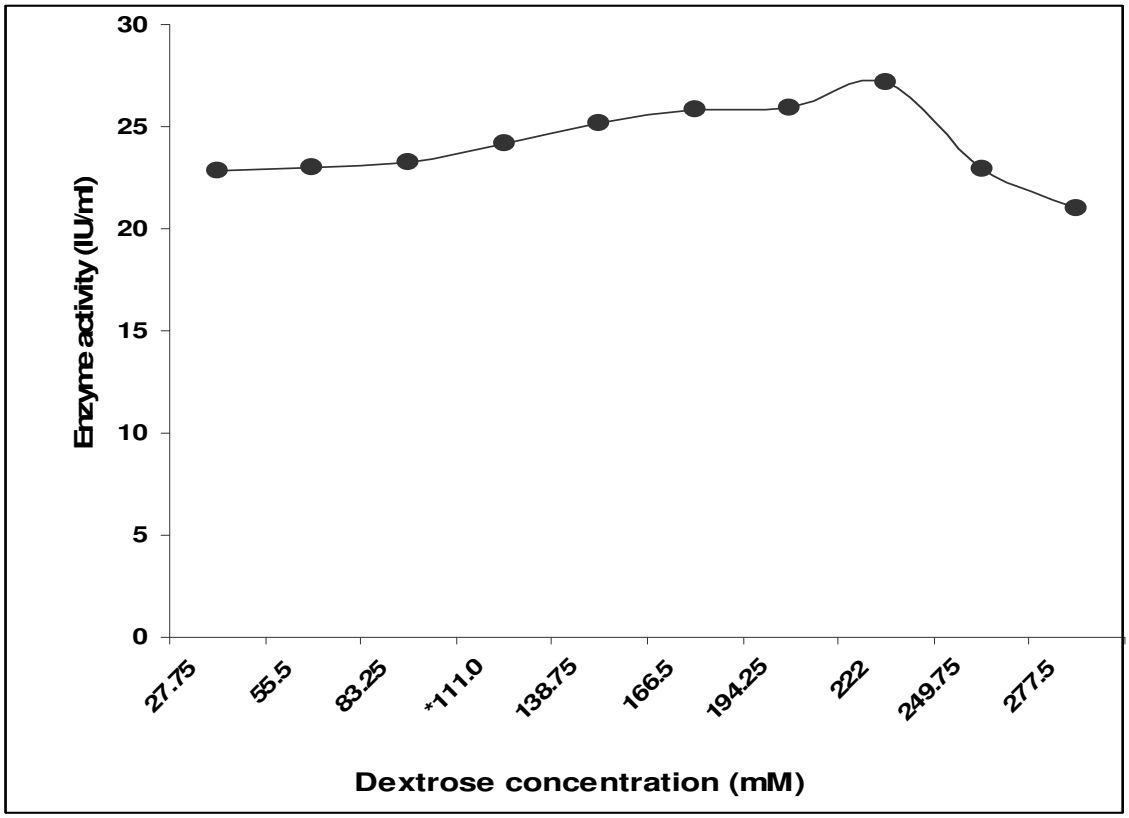

Figure- 4
Effect of dextrose concentration on xylanase production by *Pseudomonas* sp. XPB-6

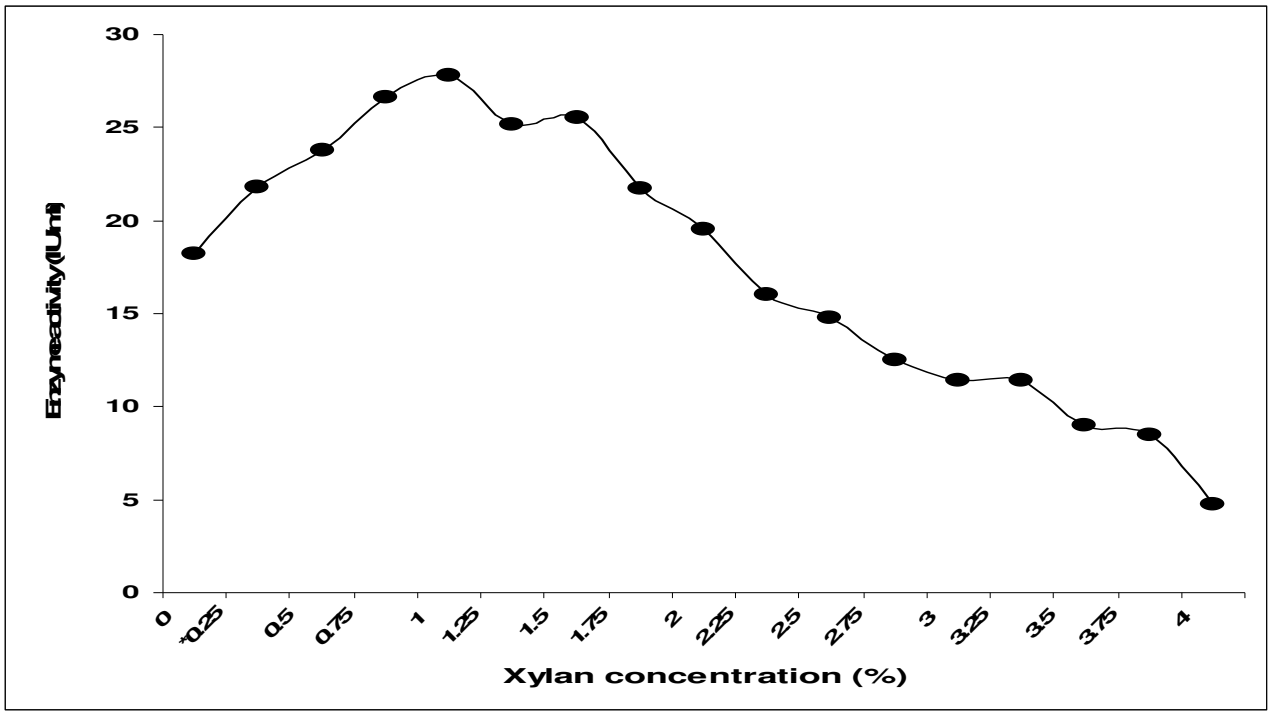

Figure-5
Effect of xylan concentration on enzyme production by

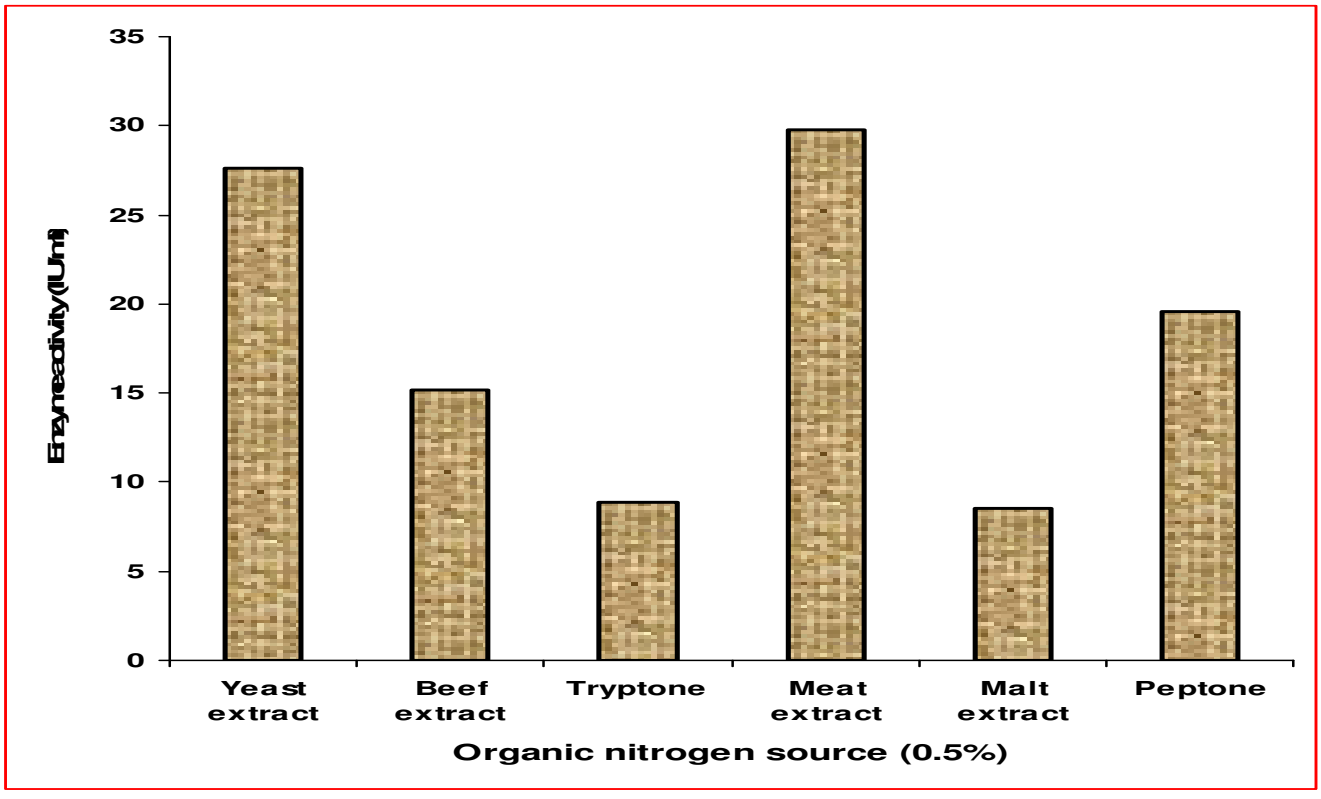

Figure-6
Effect of organic nitrogen sources on production of xylanase from *Pseudomonas* sp. XPB-6

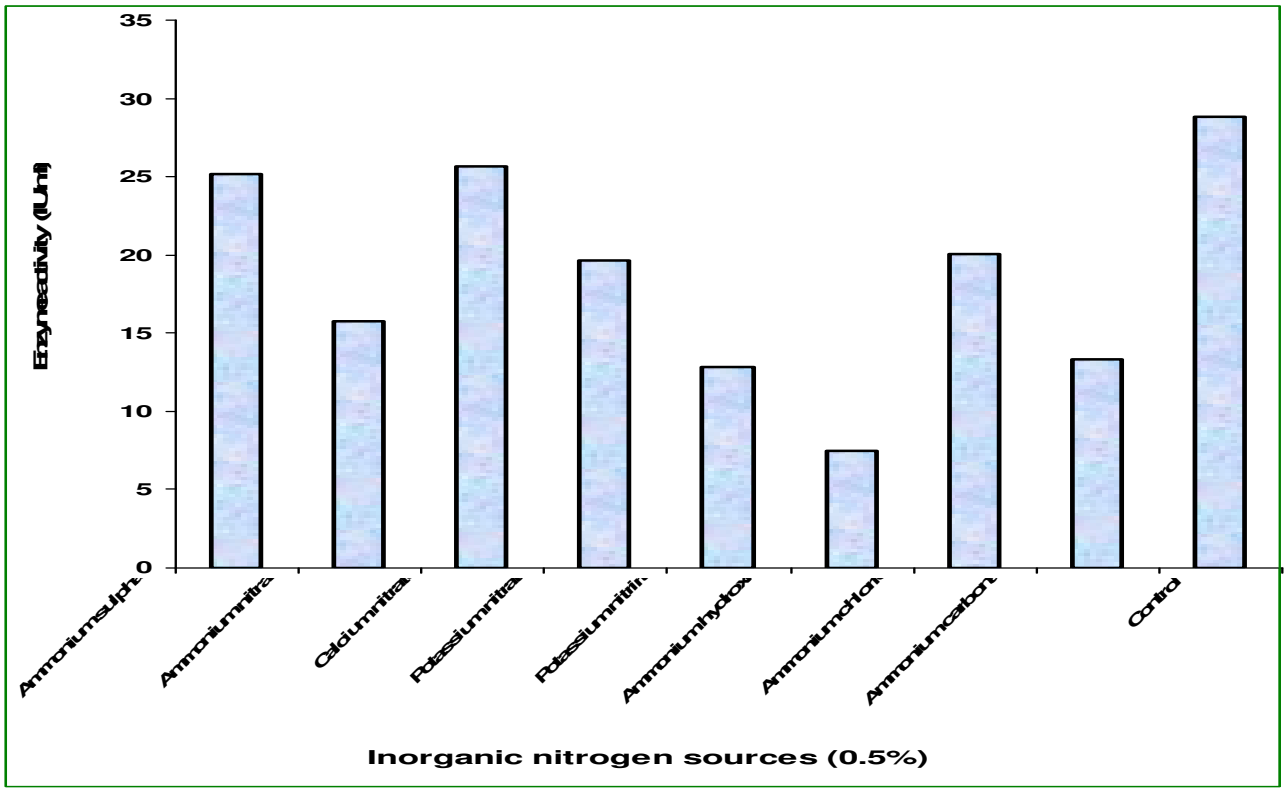

Figure-7
Effect of inorganic nitrogen sources on xylanase production from *Pseudomonas* sp. XPB-6

Further bacterium was grown with different concentrations of meat extract. Maximum enzyme activity was obtained in 0.25 % meat extract and further increase in meat extract concentration led to decrease in enzyme production (Figure-8). This may be due to metabolic inhibition. Nitrogen sources of varying nature were studied by Muthezhilan *et al*., [23] and Sanghi *et al*.[24]

Effect of inoculum size on xylanase production: To study the effect of inoculum size on growth and enzyme production by *Pseudomonas* sp. XPB-6, the precultures were used in the range of 2-16 % v/v and 6 % v/v inoculum has been shown to produce maximum enzyme and thus increase in xylanase activity (figure-9). Kuhad *et al*., [9] reported 2 % inoculum to give maximum xylanase production from *Bacillus* sp. RPP1.

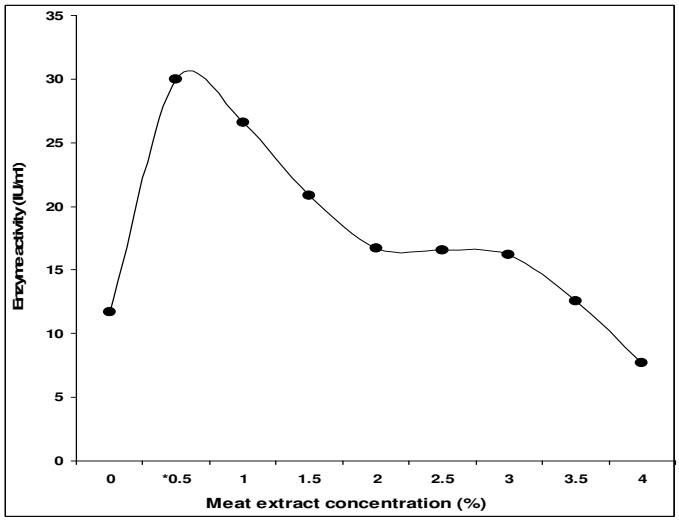

Figure-8
Effect of concentration of meat extract on xylanase production by *Pseudomonas* sp. XPB-6

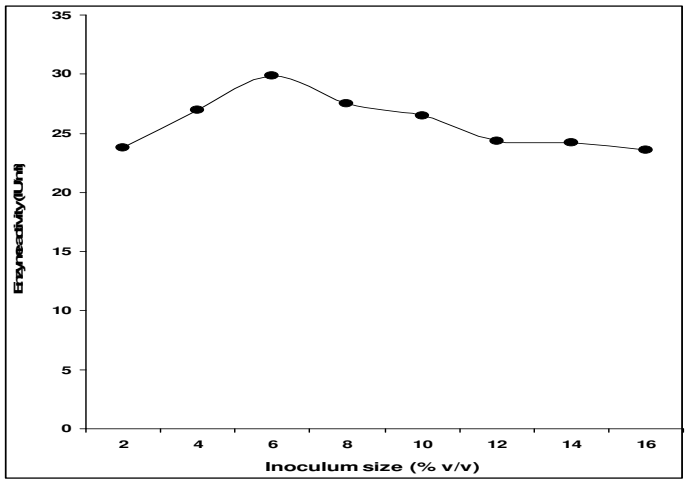

Figure-9
Effect of inoculum size on xylanase production by *Pseudomonas* sp. XPB-6

Growth and xylanase production profile: The time course of the production of xylanase enzyme which could play a major role in industrial applications was determined in shake flasks using the optimized medium. Enzyme assay was performed after every 4 hour and maximum activity was found at 24 hours of incubation (29.63 IU/ml) which remained constant and gradually after 24 hour as shown in figure-10. Purkarthofer *et al.*,[25] obtained maximum enzyme production after 7 days of incubation with pH optima of 6.5.

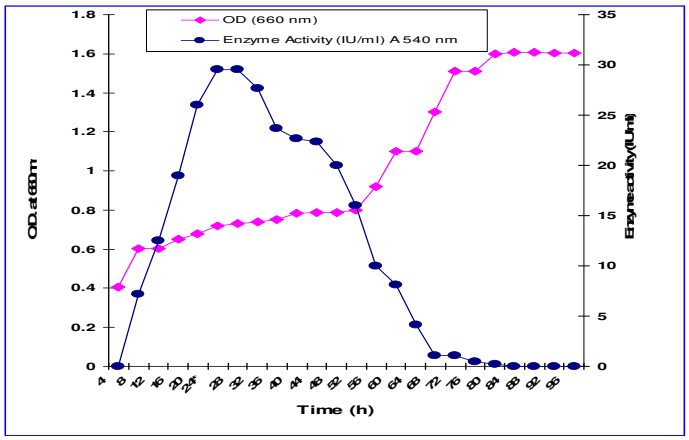

Figure-10
Time course of xylanase production and growth profile of *Pseudomonas* sp. XPB-6

Optimization of reaction conditions: Optimization of buffer system for xylanase assay: The most important parameter which affect enzyme activity are pH and buffer system, different buffers were used at 50mM concentration at various pH. Among various buffers tested, sodium phosphate buffer at 7.0 pH gave maximum activity (figure-11). Similar buffer system has been reported for thermostable xylanase from *Bacillus circulans*[26]. Comparable enzyme activity was also observed in potassium phosphate buffer. Very little activity was observed in glycine-NaOH and Borate buffer. Whereas acidic pH range (4.6-5.6) was reported for xylanase activity of *Asprgillus foetidus* MTCC 4898[27].

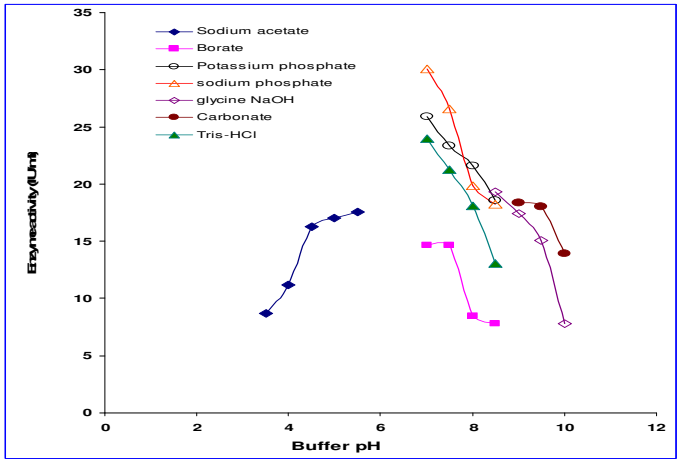

Figure-11
Optimization of buffer system

Buffer molarity: To investigate the effect of different molar concentrations of phosphate buffer, the xylanase was assayed in 25mM to 200mM of sodium phosphate buffer at pH 7.0. Maximum xylanase activity was recorded in 100mM sodium phosphate buffer at pH 7.0 (figure-12). Khasin *et al.*,[28] reported maximum enzyme activity at 50 mM in *Bacillus stearothermophilus* T-6.

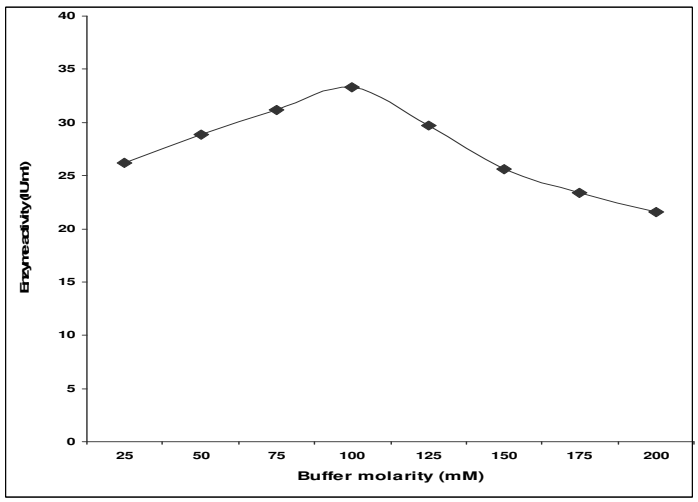

Figure-12
Effect of buffer molarity on xylanase activity

Effect of reaction temperature: Temperature plays a very crucial role in expressing the activity of any biological system. To determine the effect of reaction temperature on xylanase activity, the reaction was performed at different temperatures ranging from 25˚C to 60˚C. The optimum temperature for xylanase activity was found to be 55˚C as shown in figure-13. The results are in close agreement with that obtained by Knob and Carmona, [7] reported 50˚C as optimum temperature for xylanase from *Penicillium sclerotiorum.*

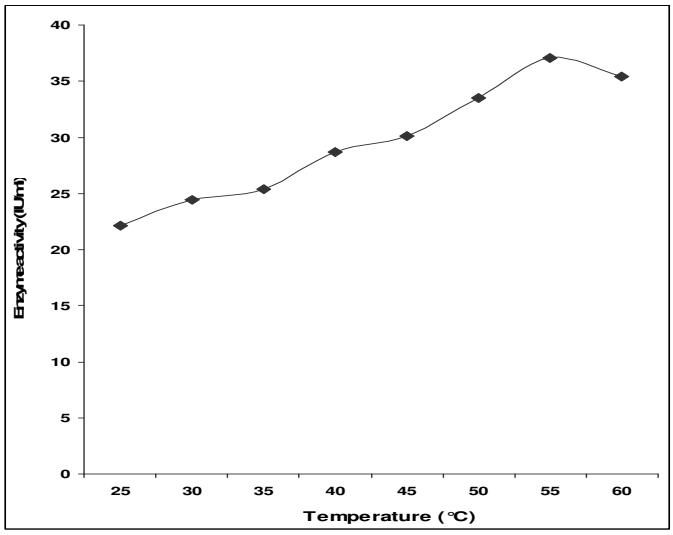

Figure-13
Effect of reaction temperature on xylanase activity

Effect of time of incubation: Time of incubation has a profound effect on enzyme activity. Specific time of incubation is required for substrates to bind to the enzyme's active sites. Thus after a specific time of incubation no increase in activity is observed because all the active sites of enzymes are blocked by the substrates. To know the effect of time of incubation on xylanase activity, reaction was terminated at a range of time intervals i.e. 1 minute to 30 minutes. The enzyme activity was found to be maximum with 5 minutes of incubation (40.53 IU/ml) as shown in figure-14.

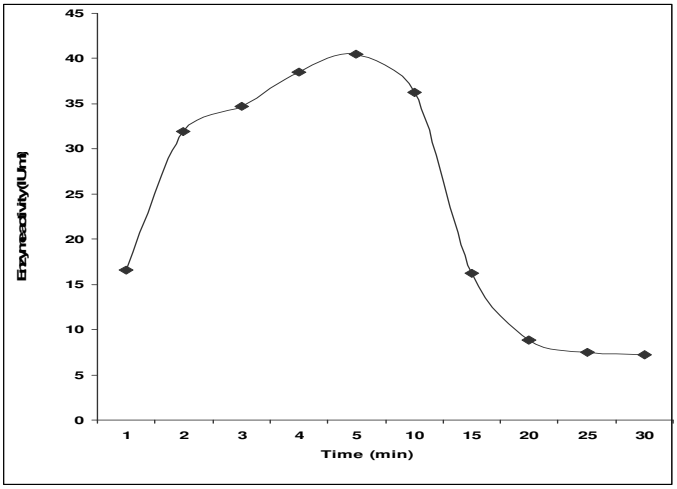

Figure-14
Time of incubation for enzyme reaction

Effect of substrate concentration: The enzyme reaction was carried out at different concentrations of xylan in the reaction mixture ranging from 0.25% to 2.0% amd maximum activity was recorded in the presence of 1.0% of xylan (figure-15). Higher concentration beyond 1.25% tends to suppress xylanase activity. This may be due to substrate inhibition. Similar results have been reported by Muthezhilan *et al*.,[23] for xylanase from *Penicillium oxalicum*.

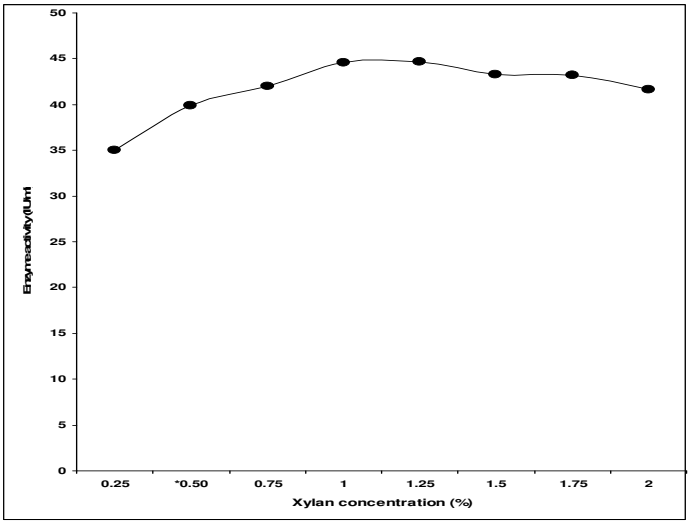

Figure-15
Effect of xylan concentration on xylanase activity

Effect of enzyme concentration: The enzyme reaction was carried out at different concentrations of supernatant in the reaction mixture ranging from 5µg to 30µg and maximum activity was recorded in the presence of 20µg of enzyme (figure-16). A decrease in activity was observed on increasing the concentration further.

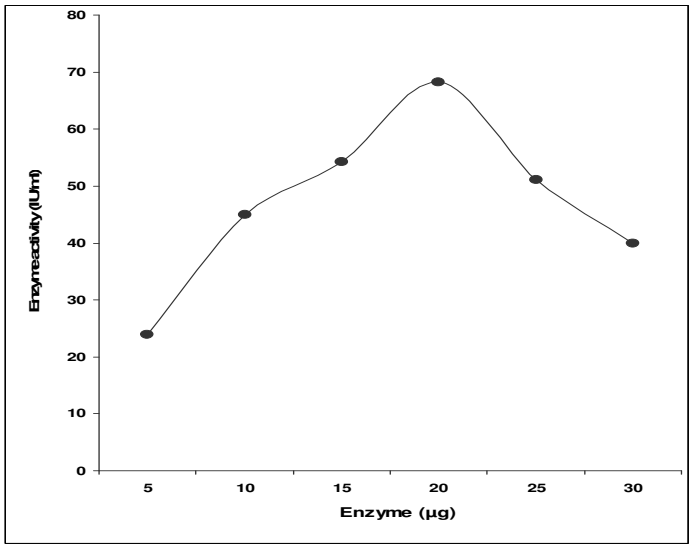

Figure-16
Effect of enzyme concentration on xylanase activity

Effect of metal ions: The effect of metal ions on xylanase activity was studied and the results are summarized in Figure-17. All the metal ions used at 1mM concentration had inhibited xylanase activity. After addition of 1mM of $CuSO_4.5H_2O$, $CdCl_3.2H_2O$, $ZnSO_4.7H_2O$ and $CaCl_2.2H_2O$, a drastic decrease in xylanase activity was observed. Dhillon and Khana,[26] also observed partial inhibition in the presence of 1mM $FeSO_4$, $MnCl_2$, $CuCl_2$ and $MgCl_2$. Purified *Xyl A* from *Bacillus circulans* AB 16 , $CdCl_2$(1mM) has shown to slightly increase yield but *Xyl B* showed inhibition, while $CoCl_2$ and $FeSO_4$ showed some enhancement in activity in *Xyl B*.

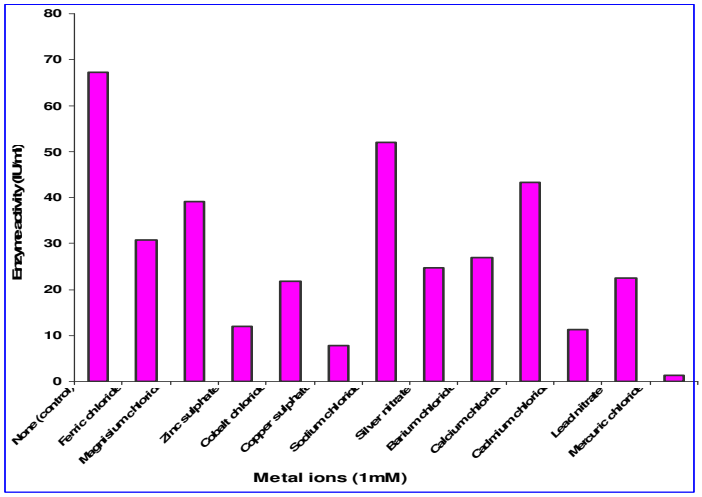

Figure-17
Effect of metal ions on xylanase activity

Thermostability of xylanase: Thermal stability is an interesting enzymes property due to the great industrial importance. The enzyme was preincubated at different temperatures and its activity was assayed after every 60 minutes till 300 minutes. The enzyme was fairly stable at 25˚C and 45˚C upto 120 minutes (figure-18). As the temperature was increased, the xylanase activity decreased considerably. Shah and Madamwar,[27] found that the xylanase from *Asprgillus foetidus* retained 100% activity at 30˚C after 3 hours while at 40˚C only marginal decrease was found. The enzyme was sensitive to 50˚C, retaining 71 % after 30 minutes exposure and only 30 % activity after 3.0 hours.

Time course of enzyme reaction at different temperatures: Xylanase reactions were followed upto 180 minutes at different temperatures (35˚C-65˚C). The results obtained have been shown in Figure-19. Sugar estimation was done after different time intervals viz. 5, 10, 30, 60,120,180 minutes.

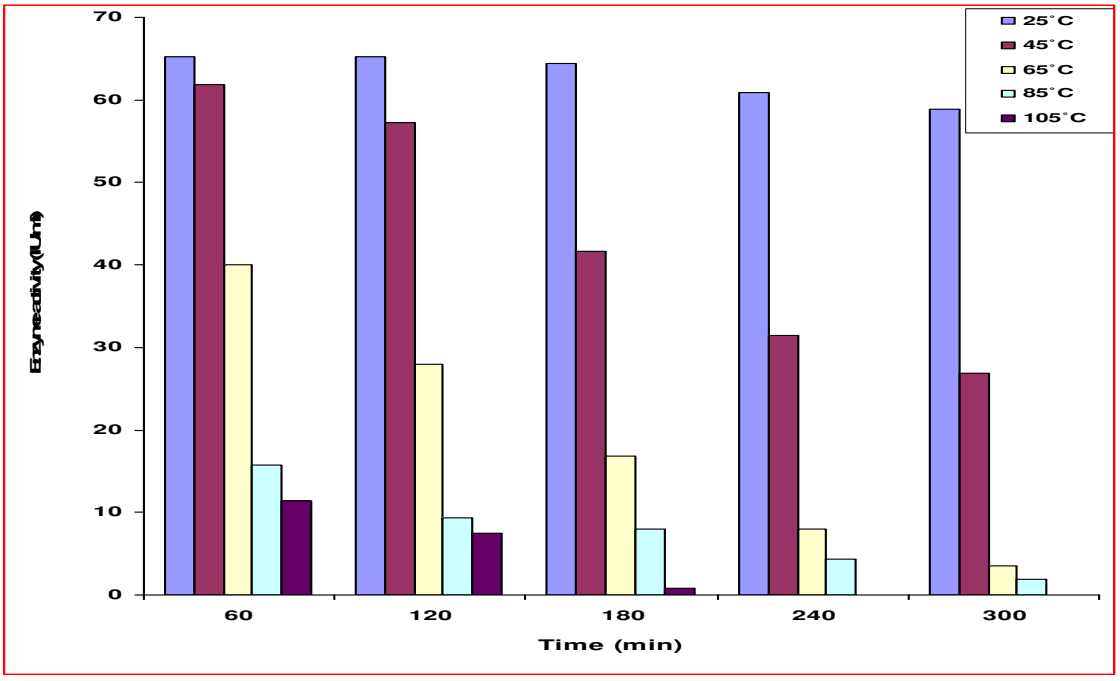

Figure-18
Thermostability of xylanase

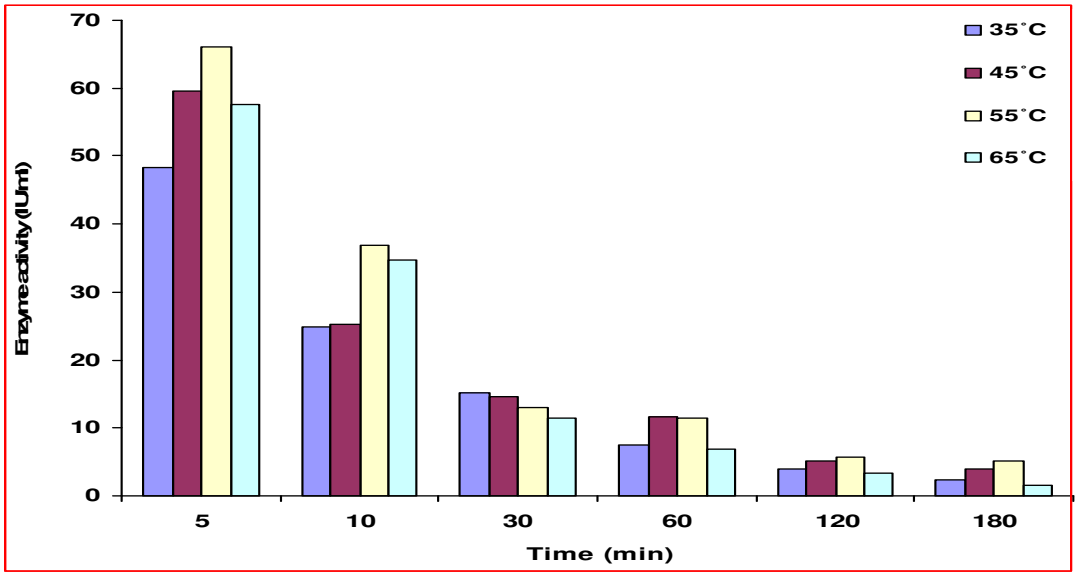

Figure-19
Time course of enzyme reaction at different temperatures

Maximum enzyme activity was observed at 55˚C after 5 minutes of reaction. After 60 minutes, the enzyme activity at all the temperatures remained almost constant. This may be due to the fact that all the catalytic sites of the enzyme have been blocked by the substrate and no more sites are there for substrate to act upon.

Shelf life of enzyme: Shelf life of xylanase of *Pseudomonas* sp. XPB-6 was studied at both room temperature and at 4˚C. The results obtained are shown in Figure-20. The enzyme was stable both at 4˚C for 25 and at 25˚C for 16 days. After that the enzyme activity gradually started decreasing.

Xylanase from *Bacillus subtilis* which showed maximum activity at 60˚C, did not lose its activity at pH 8.2 up to one year at 4˚C to 25˚C but was inactivated when frozen[29]. Shah and Madamwar,[27] observed full enzyme activity when stored in deep freeze up to 6 months. At refrigeration temperature, no loss of activity was found up to 2 weeks but after 4 weeks a marginal decrease (5-10%) was found. At room temperature decrease in activity was not found for up to 2 days but at the end of a week 10 % loss in enzyme activity was found.

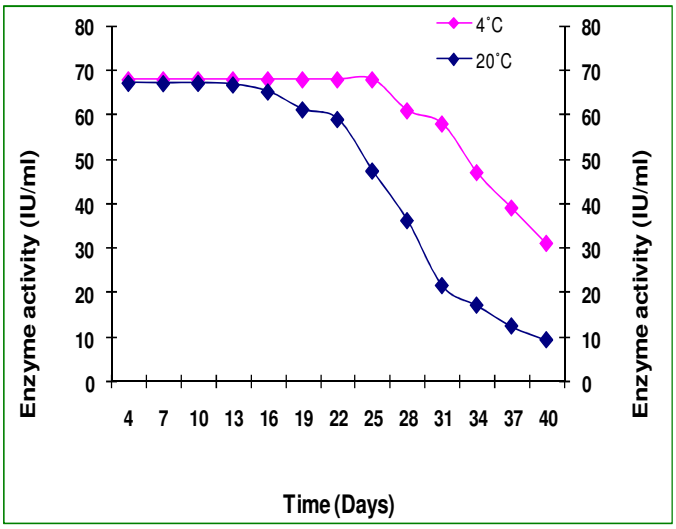

Figure-20
Shelf life of enzyme

Conclusion

The present study shows good xylanase activity from *Pseudomonas* sp. XPB-6. Maximum growth obtained in the medium containing xylan 1%, dextrose 4%, meat extract0.5%, temperature 30˚C, pH 7.0, substrate concentration 1% and inoculum size 6%. The optimum time for growth and enzyme production was 24 hours. Maximum xylanase activity was reported with 100 mM sodium phosphate buffer (pH 7.5) at 55˚C and 5 minutes incubation temperature. The enzyme was fairly stable at 25˚C and 75˚C, metal ions (1mM $CuSO_4.5H_2O$, $CdCl_3.2H_2O$, $ZnSO_4.7H_2O$ and $CaCl_2.2H_2O$) have shown drastic decrease in xylanase activity. The enzyme could be further used in its application for pulp and paper industry as it has maximum activity at 55 ºC and neutral pH without cellulase activity. The xylanase could also be used in fruit juice clarification either alone or in combination with pectinase to get maximum percent clarification.

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