International Journal of Multidisciplinary Research and Development

International Journal of Multidisciplinary Research and Development

Volume: 2, Issue: 10, 396-400
Oct 2015
www.allsubjectjournal.com
e-ISSN: 2349-4182
p-ISSN: 2349-5979
Impact Factor: 5.742

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Production and Partial Purification of Xylanase from *Bacillus pumilus*

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Abstract
Microbial enzymes produced from bacteria and fungi are widely used in the industries because they are cheaper to produce and their enzyme contents are more predictable and reliable as compared to those from plants and animal sources. Hemicellulose is the second most abundant plant fraction available in nature, formed by pentose sugars. Hemicelluloses are made of xylan (xylose, arabinose and glucuronic acid) backbone. Xylan is the major constituent of hemicelluloses Present study was planned with the objective to optimize the production parameters of bacterial xylanases and partial purification of Xylanase enzyme. *Bacillus pumilus* showed maximum xylanolytic activity at 50 °C, time 48 hour and pH 7.0 ± 0.2. The peak enzyme activity was observed while supplementing xylose, as carbon source and ammonium nitrate (inorganic) and tryptone (organic) as a nitrogen source. Enzyme was extracted by centrifugation at 10,000 rpm for 20 minutes. Crude enzyme assay was done by DNS method. After partial purification total enzyme activity was found 35.78 Uml^{-1} and specific activity was 12.33 Umg^{-1}. The pH and thermal stability of cellulose free xylanases are important issues for their potential industrial applications.

Keywords: Xylanase, *Bacillus pumilus*, Enzyme Activity.

1. Introduction
Wood and pulp fibers constitute renewable raw materials that can be processed with biological catalytic systems. Xylan, the second most abundant polysaccharide and a major component in plant cell wall consists of β-1, 4-linked xylopyranosyl residues. The plant cell wall is a composite material in which cellulose, hemicellulose (mainly xylan) and lignin are closely associated. [1] Three major constituents of wood are cellulose (35-50%), hemicellulose (20-30%)- a group of carbohydrates in which xylan forms the major class- and lignin (20-30%). Xylan is a heteropolysaccharide containing substituent groups of acetyl, 4-Omethyl-D-glucuronosyl and α-arabinofuranosyl residues linked to the backbone of β-1, 4, -linked xylopyranose units and has binding properties mediated by covalent an d non-covalent interactions with lignin, cellulose and other polymers. The energy content of both xylan and cellulose, based on the total plant biomass on Earth, is equivalent to almost 640 billion tons of oil [2]. The pH and thermal stability of cellulase free xylanases are important issues for their potential industrial applications [3]. There has been much industrial interest in xylanases for bioethanol production. Because of their biotechnological characteristics, xylanases are most often produced from microorganisms for commercial applications. The optimum temperature for the activity of endoxylanase from bacterial and fungal sources varies from 40 to 100 °C [4, 5] and commonly has a broad optimal pH range of 3.6–10.0. Applications of Xylanase [5] can be found in the food and beverage industries (bakery goods, coffee, starch, plant oil, and juice manufacture), feedstock improvement, and the quality improvement of lignocellulosic residues. Thus, the use of Xylanase is beneficial for society as well as for the environment. Xylanase enzymes play an important ecological role in the process of pulp bleaching, since they can be used to minimize the need for pollutant and environmentally aggressive chemicals (particularly chlorine-containing compounds) to degrade the lignins in pulp [5, 6, 7] .
The largest commercial bioconversion process utilizing wood is currently the conversion of underutilized hardwoods to provide substrates for growth of the shiitake mushroom Lentinula edodes [8]. The cost of an enzyme is one of the main factors determining the economy of a process; reducing the cost of enzyme production by optimization of the fermentation medium is the goal of basic research for industrial application. For commercial application, xylanase should ideally be produced quickly and in large quantities by the optimization of barriers production parameters such as temperature, time, pH, carbon and nitrogen sources. These parameters seem to enhance product recovery as well as high enzyme productivity. Xylanase are predicted to have a future market as 'Bulk Enzymes' in the industry. To satisfy the industrial need for

Xylanase, it is necessary to produce and purify the enzymes in large quantities and thus it is imperative to explore new microbial strains that are active at high temperatures [9].

2. Materials and methods

The bacterial strain *Bacillus pumilus* (MCCB0011) was obtained from Microbial Culture Collection Bank (MCCB), Department of Microbiology and Fermentation Technology (MBFT), Jacob School of Biotechnology and Bioengineering, SHIATS, Allahabad. The experimental part was carried out at above mention department. The culture was maintained in solid nutrient agar medium and subcultured time to time. To evaluate the activity of xylanase, the optimization of growth parameters at varying temperature, pH, incubation time, carbon and nitrogen sources was carried out and manifesting standard value for each parameter. At each step xylanase activity was assayed to know the optimum value.

2.1 Effect of incubation time

The optimum incubation time for xylanase production was determined by inoculating 50 ml broth media with the bacterial isolate and incubating it at 50±1 °C. The samples were withdrawn at different time intervals for 24, 48, 72 and 96 hour. The enzyme activity was determined for each time by the standard assay method.

2.2 Effect of temperature

The optimum temperature for xylanase production was determined by inoculating 50 ml broth media with the bacterial isolate and incubating it at different temperatures (30, 40, 50, 60 and 70) °C for 24, 48, 72 and 96 hour. The enzyme activity was determined for each temperature by the standard assay method.

2.3 Effect of pH

To determine optimum pH for maximum xylanase production, 50 ml broth media adjusted with buffers at different pH (5, 6, 7, 8, 9, 10 and 11) was inoculated with the bacterial isolate and incubated at 50 °C±1 °C for 48 hour. After incubation, the enzyme activity was assayed by standard assay procedure.

2.4 Effect of different carbon sources

Each 50 ml broth media was inoculated with the test organism supplemented with the additional carbon sources such as xylose, glucose, sucrose, galactose and arabinose at 1% (w/v) level and incubated at 50±1 °C for 48 hour and pH 7.0. The standard assay was performed to determine maximum xylanase production with the carbon sources.

2.5 Effect of different nitrogen sources

Each 50 ml broth media was inoculated with the test organism supplemented with the additional inorganic nitrogen sources such as ammonium chloride, sodium nitrate, ammonium sulphate, potassium nitrate and organic nitrogen source such as peptone, tryptone and yeast extract incubated at 50±1 °C for 48 hour at pH 7.0. The standard assay was performed to determine maximum xylanase production with the nitrogen sources.

2.6 Xylanase production from optimized media

The standardize media was prepared according to the parameter after optimization. The fermented broth media (100 ml) incubated at optimized conditions was subjected to extraction of the enzyme. The cells were removed by centrifugation at 10,000 rpm for 20 minute at 4 °C. The clear supernatant was obtained used as crude enzyme and used for further purification steps. The enzyme activity was determined by standard assay method.

3. Statistical Analysis

The optimization of growth parameters such as temperature and incubation time on xylanase production was analyzed using two way classification and conclusion was drawn on the basis of analysis of variance technique [10] at 5% level of significance. The effect of pH on the production of xylanase was analyzed using linear regression analysis and further the test of significance (t-test at 5% level of significance) was performed. The effect of carbon and nitrogen sources on xylanase production was analyzed using two way classification and conclusion was drawn on the basis of analysis of variance technique at 5% level of significance.

4. Result and Discussion

4.1 Effect of Temperature on Xylanase Production at various Time intervals

The enzyme activity for the bacterial strain was studied on the basis of two parameters i.e. time and temperature. It was observed in the present study that the maximum enzyme production from the bacterium was found to be maximal after 48 hour of time at 50 °C. Xylanase from a thermoalkalophilic bacterium showed optimum activity at 50 °C [11]. Optimum activity of xylanase obtained from both *Bacillus circulans* and *Bacillus amyloliquefaciens* was at 50 °C [12, 13] the industrial importance of an enzyme will be more if the temperature input for its optimal activity is less. The effect of time and temperature on xylanase production was found to be statistically significant. (F_{cal} 15.96 > F_{tab} 3.49 Due to time) (F_{cal} 45.28 > F_{tab} 3.26 Due to temperature) at 5% level of significance.

Table 1: Effect of Temperature and Incubation Time on Xylanase Production

Temperature (°C)	Enzyme activity (Uml^{-1}) (Hours)			
	24	**48**	**72**	**96**
30	1.84	2.25	1.79	1.47
40	3.05	3.65	2.68	2.44
50	4.95	**5.82**	4.37	3.61
60	3.31	4.10	3.60	2.75
70	2.55	3.10	2.38	1.64

F_{cal} = 15.96 > F_{tab} (5%) = 3.49 (due to time)
F_{cal} = 45.28 > F_{tab} (5%) = 3.49 (due to temperature)
CD = 0.46

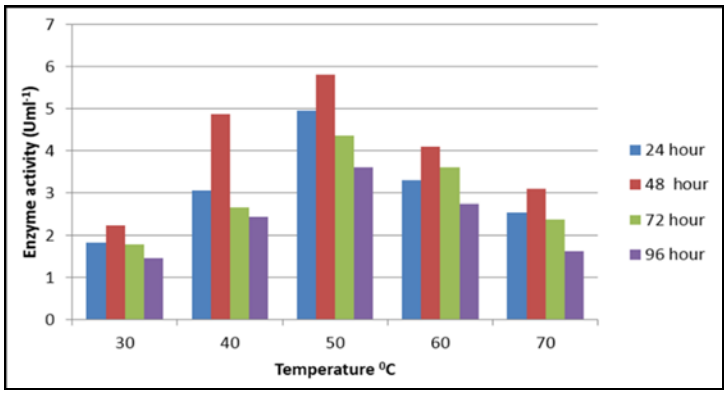

Fig 1: Effect of Different Temperature and Incubation Period on Xylanase Production

4.2 Effect of pH on Xylanase Production

There was a very vast influence of pH on xylanase production. This was observed in the present study. The enzyme activity

for different value of pH ranging from 5.0-11.0 was determined by keeping the optimum time and temperature combination. The maximum pH activity of xylanase production was at pH 7.0. Since there was no constant change in the xylanase activity with the increase or decrease of pH, the data was found to be statistically significant. (t_{cal} 0.053 < t_{tab} 2.447 at 5% level of significance). The persistence of activity in a large range of pH was desirable quality of an industrial enzyme. Moreover [14] described an optimum pH of 7.0 for active enzyme production by *Bacillus coagulan RI 69* which was in agreement with the present study.

Table 2: Effect of pH on Xylanase production

pH	Enzyme activity (Uml^{-1})
5	2.15
6	3.55
7	5.46
8	4.80
9	3.93
10	3.35
11	2.88

r = 0.024 t_{cal} = 0.053 < t_{tab} (5%) = 2.447

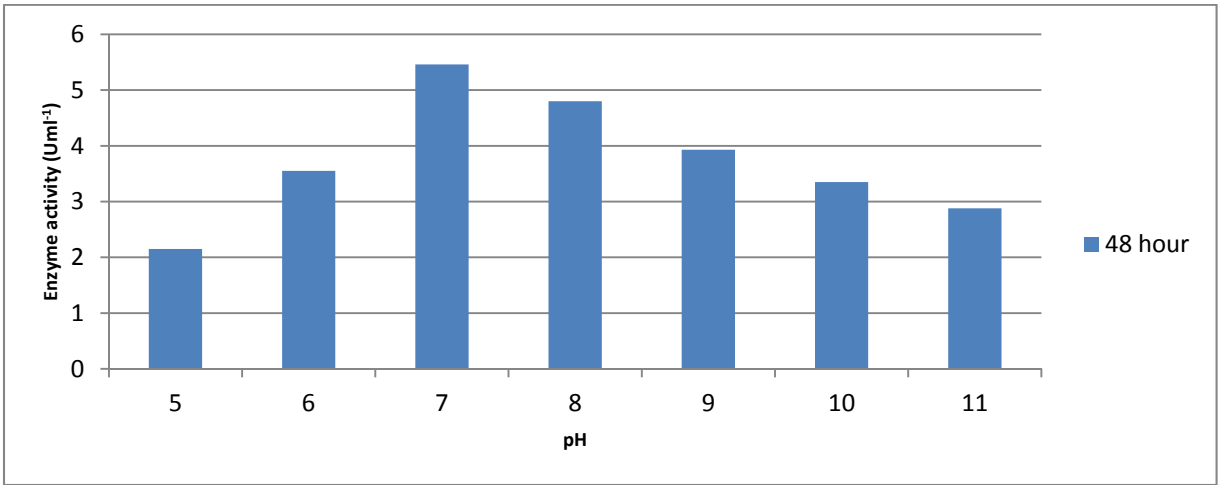

Fig 2: Effect of Different pH on Xylanase Production

4.3 Effect of Different Carbon sources on Xylanase Production

Various carbon sources such as Xylose, Arabinose, Glucose, Sucrose and Galactose (1%) were used to supplement the production media. The maximum xylanase production was found when the media was standardized *viz.* incubation time; temperature and pH were kept constant. Arabinose was also found to induce the production of enzyme. Since the data with respect to carbon sources are statistically significant, it can be concluded that variation in different carbon sources would significantly affect the xylanase production. (F_{cal} 34.43 > F_{tab} 6.26, at 5% level of significance). The findings in the present study were in agreement with [15] where xylose was observed as the best carbon source utilized by the organism.

Table 3: Effect of Different Carbon sources on xylanase production

Carbon sources (1%)	Enzyme activity (Uml^{-1})
Xylose	**5.85**
Arabinose	4.76
Glucose	4.08
Sucrose	3.63
Galactose	3.36

F_{cal} = 34.43 > F_{tab} (5%) = 6.26 CD = 0.49

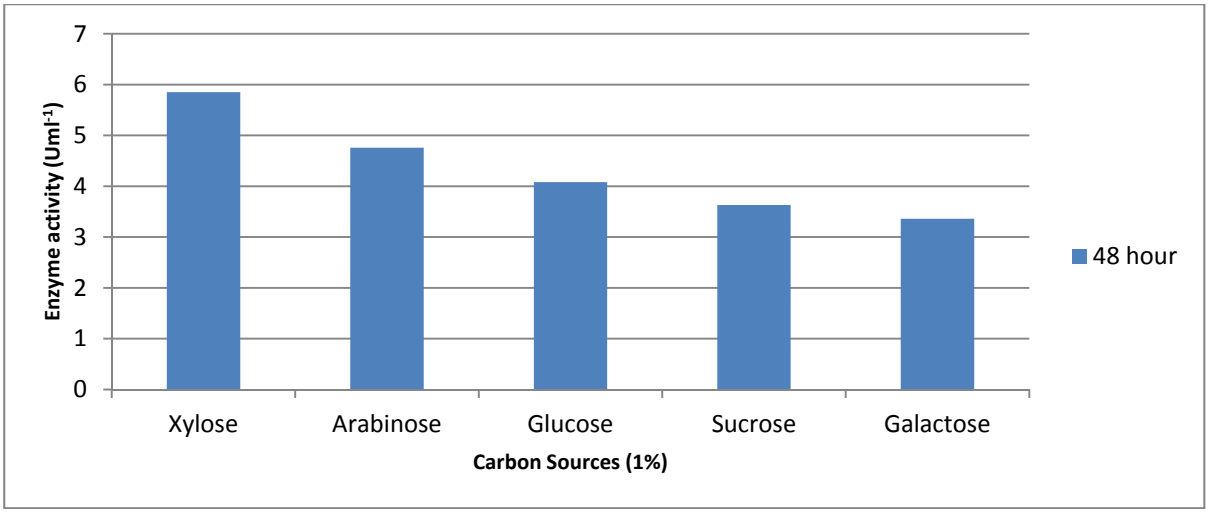

Fig 3: Effect of Different Carbon sources on enzyme activity

4.4 Effect of Nitrogen sources on Xylanase Production

Different organic and inorganic nitrogen sources were supplemented in the production media to determine the maximum enzyme activity. The maximum xylanase production was observed with ammonium nitrate (inorganic) and tryptone (organic). All other production parameters *Viz.*

temperature, incubation time, pH and carbon source were kept constant. Since the data with respect to nitrogen sources are statistically significant, it is clear that variation in different organic and inorganic nitrogen sources would significantly affect the xylanase production. (F_{cal} 28.81 > F_{tab} 4.39 at 5% level of significance). The utilization of tryptone as the suitable organic nitrogen source in the present study was in agreement with the reports of [16]. Therefore it was expected that the improvement of the nutritional value in the medium by the supplementation of organic and inorganic sources will also improve the growth of the isolate and subsequently in the enzyme production.

Table 4: Effect of nitrogen sources on Xylanase production

Nitrogen source	Enzyme activity (U ml^{-1})
Inorganic	
Ammonium chloride	3.80
Sodium nitrate	4.71
Ammonium nitrate	**5.18**
Ammonium sulfate	3.31
Organic	
Yeast extract	5.27
Peptone	5.70
Tryptone	**6.30**

F_{cal} = 28.81 > F_{tab} (5%) = 4.39 CD = 0.31

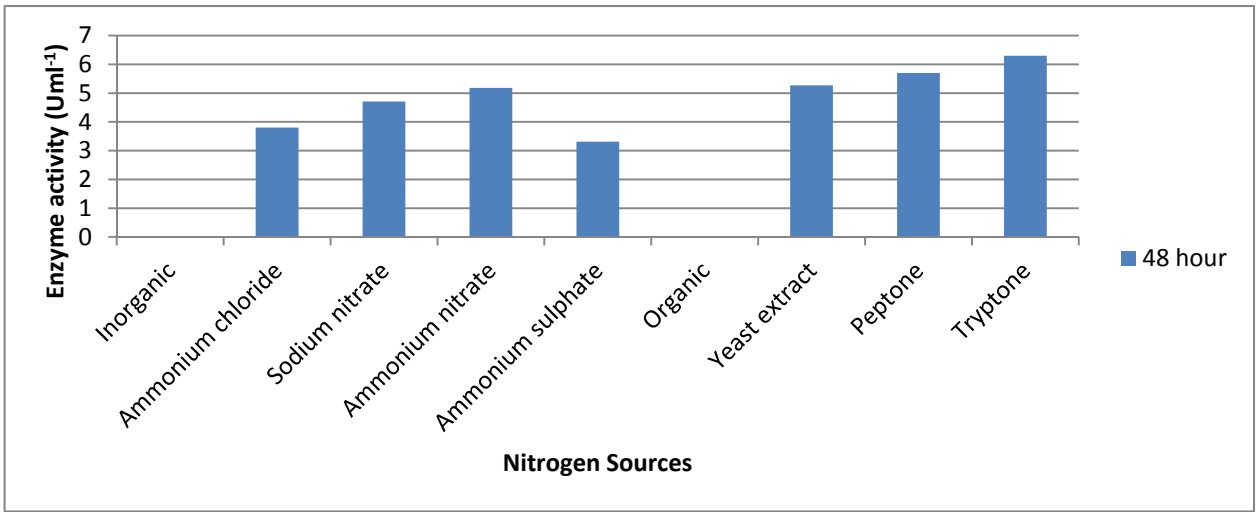

Fig 4: Effect of Different Inorganic and Organic Nitrogen sources on enzyme activity

5. Statistical analysis

Table 5. Effect of temperature and time on Xylanase Production

Source of Variation	Degree of freedom	Sum of square	Mean sum of Sq.	F_{cal}	$F_{tab\ 5\%}$	Result
Due to temperature (t)	4	18.84	4.71	45.28	$F_{4,12}$ 3.26	S
Due to time (m)	3	4.99	1.663	15.96	$F_{3,12}$ 3.49	S
Due to error	12	1.25	0.104			
Total	19		-			

Table 6. Effect of Carbon sources on Xylanase production

Source of Variation	Degree of freedom	Sum of Sq.	Mean sum of Sq.	F_{cal}	$F_{tab\ 5\%}$	Result
Due to replication (r)	1	1.16	1.16	20.17	-	-
Due to carbon source (c)	4	7.95	1.98	34.43	6.26	S
Due to error	4	0.23	0.0575	-	-	-
Total	9	-	-	-	-	-

Table 7. Effect of Nitrogen sources on Xylanase production

Source of Variation	Degree of freedom	Sum of Sq.	Mean sum of Sq.	F_{cal}	$F_{tab\ 5\%}$	Result
Due to replication (r)	1	1.33	1.33	-	-	-
Due to Nitrogen source (n)	6	13.17	2.19	28.81	4.39	S
Due to error	6	0.46	0.076	-	-	-
Total	13	-	-	-	-	-

5.1 Effect of pH on Xylanase Production

r = 0.024**,**
$t_{5\%}$ = 0.053 (Calculated value)**,**
$t_{tab\ 5\%}$ (n-2) = 2.447
b_{xy} = 0.306
b_{yx} = 0.083
a) Regression equation of x on y:
X = 0.306y + 6.86
b) Regression equation of y on x:
Y = 0.083x + 3.06

6. Purification of Xylanase

Crude enzyme was partially purified by Acetone precipitation (70% saturation) and Ammonium Sulphate precipitation. After overnight incubation at 4 °C precipitated enzyme was centrifuged at 10000 rpm for 20 minutes. The pellet was dissolve in .1 M phosphate buffer (pH 6.9) and dialyzed against the same buffer. At 70% saturation precipitation by acetone showed a specific activity 8.58. After precipitating with ammonium sulphate the partially purified enzyme showed specific activity 9.33 Umg^{-1}, with a total activity of

53.21 Uml^{-1}. In dialysis total enzyme activity was found 35.78 Uml^{-1} and specific activity was 12.33 Umg^{-1}

7. Conclusions

The optimum pH activity of xylanase was at pH 7.0 and the organism being capable of producing xylanase at wide range of pH (5-11) of xylanase, specially at alkaline pH is advantageous in application of the enzyme in biobleaching of Kraft pulps. The persistence of activity in a large range of pH was a desirable quality of an industrial enzyme. Optimizing the conditions for the production of Xylanase by *Bacillus pumilus* revealed maximum productivity at 50 °C and 48 hour. Temperature and time combination required for the peak enzyme activity. The data was found to be statistically significant. The industrial importance of an enzyme will be more if the temperature input for its optimal activity is less.

Supplementing the substrate with different carbon sources which affects the overall cellular growth and metabolism. Different carbohydrates (xylose, arabinose, glucose, sucrose and galactose) were used to observe their effect on enzyme production. Among the different carbon sources, xylose supported highest xylanase production followed by arabinose. Similarly a significant effect on the xylanase production with different organic and inorganic nitrogen in the substrate was observed. Ammonium nitrate (inorganic) supported highest xylanase production followed by sodium nitrate and Tryptone (organic) supported highest xylanase production followed by yeast extract were found to enhance the enzyme production.

In the present study, xylanase produced by *Bacillus pumilus* (MCCB 0011) was found to be potent since it could be active at a wide range of pH, Temperature, Carbon and Nitrogen (inorganic and organic) sources. Finally it was found that the selected strain of *Bacillus pumilus* should be beneficial tool for biobleaching in paper industry by replacing other environmentally hazardous methods.

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