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Production, purification and characterization of cellulase free-xylanase by *Bacillus coagulan*s B30 using lignocellulosic forest wastes with different pretreatment methods

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Abstract

Bacillus coagulans B30 isolated from forest soil of northern Himalayan of India, explored for its cellulase free-xylanase production using physio-chemically pretreated forest biomass as a substrate. Maximum xylanase production was observed in microwave pretreated mixed biomass producing 281.63U/g of xylanase under solid state fermentation. Xylanase was purified having molecular weight of 66.54 kDa by using SDS Page. Purified xylanase showed high temperature (50□C) and pH (9.00) optimum.

Keywords: xylanase, pretreatment, purification, *Bacillus coagulans* B30, solid state fermentation

1. Introduction

Microbial enzymes Cellulase free-xylanase is of paramount significance in industries viz. paper and pulp industry to avoid hydrolysis of cellulose fibres. Treatment with cellulase free-xylanase at elevated temperature disrupts the cell wall structure, facilitates lignin removal in the various stages of bleaching and paper. Since xylanase production was commercially explored due to the use of purified xylan as a substrate during their production. Utilization of lowest cost and abundantly available forest residues as substrate for its production is one of the ways which substantially can reduce the enzyme production cost. These could be excellent cost effective substrate for large scale production of xylanase in solid state fermentation SSF [1].

Forest wastes are believed to be as one of the abundant resource of sugars although limited work has been reported on them because of their complex structure for biodegradation. Therefore several different pretreatment techniques are used to facilitate the accessibility of lignocellulosic material for biodegradation [2].

In order to obtain higher titers of enzymes, these technique focus on disrupting the protection of cellulose and hemicelluloses by lignin as well as dissociation between cellulose and hemicelluloses to preserve hemicelluloses fractions, to increase their structural porosity and to turn it cost effective [3]. The relationship among structural and functional reflects the complexity of lignocellulosic biomass matrix. The variability of these characters explains the enzyme production among the different biomass. Since different forest residues have been used in the present study, one of the objectives was to evaluate best carbon source verses pretreatment for enhanced xylanase production.

Xylanase is produced by a variety of microorganisms including bacteria [1], fungi [4] and actinomycetes [5]. However large scale cultivation of fungi and actinomycetes is often difficult because of their slow generation time, co-production of highly viscous copolymer and poor oxygen transfer [6]. Bacteria especially *Bacillus* sp. are used more extensively in industrial fermentation because they yield robust enzyme.

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The main aim of the present study was to replace conventional xylan substrate with inexpensive lignocellulosic forest material to produce industrially important xylanase by a hyperxylanase producing isolate *B.coagulans* B30.

2. Materials and methods

2.1 Isolation and identification of xylanase producing bacteria

Xylanase producing bacteria was isolated from forest soil samples collected from Shimla (Lattitude and Longitude of 31.1033°N and 77.9722°E), northern part of India, followed by serially dilution and spreading samples on petriplates containing Tryptone glucose yeast medium (oat spelt xylan at a concentration of 0.5%). The plates were incubated at 35ºC for 48 h. The isolate showing colonies with widest clear zone (B30) on the plates was a potential strain, selected for further purification study and was subjected to phenotyping [7] and genotyping using 16S rRNA gene test [8]. The isolate B30 was identified as *Bacillus coagulans* using BLASTN analysis. The isolate B30 was deposited in Genebank at IMTECH, Chandigarh, India under accession number MTCC 10305.

To detect the xylanase and cellulase production, initially the quantification was done in submerged fermentation and then xylanase [9] and FPase, CMCase [10] and β-glucosidase [11] assays were performed accordingly.

2.2 Xylanase production in SSF using different forest waste:

The lignocellulosic hard and soft wood waste alongwith needles of *Quercus leucotricophora*, *Dadracalamus strictus, Cedrus deodar, Eucalyptus* sp., *Acacia catechu, Populus deltoids, Pinus roxburguli* (needles), *Pinus roxburghii* (wood), *Bombax ceiba, Dalbergia siso, Toona ciliate* and mixed biomass (in the ratio of 1:1 of all spp.) were collected from local forests/ wood industry. The biomass was dried in oven overnight at 50°C and was grinded to attain mesh size of 1.0-1.5 mm.

2.3 Pretreatments of lignocellulosic forest waste

2.3.1 Microwave irradiation

Microwave irradiation method used as pretreatment method in the present study where 200 g of each selected biomass was taken in beaker and was microwave (Godrej) irradiated for 3 min at 250V, 50 Hz and kept in air tight containers for further use.

2.3.2.Alkali (2.5% NaOH) pretreatment

200 g of each selected biomass was dipped in 2.5% NaOH. After 4 h, it was thoroughly washed with tap water until it becomes neutral. After washing it was dried at 60ºC overnight and stored in air tight containers.

2.3.3.Alkali (2.0% NaOH) + steam pretreatment

200 g of each selected biomass was dipped in 2.0% NaOH. After 2 h, it was thoroughly washed with tap water until it becomes neutral followed by steam pretreatment at 121ºC at 15 psi for 1 hr in an autoclave. After drying at 60ºC overnight, it was stored in air tight containers.

2.3.4.Acid (2.5% H_2SO_4) pretreatment

200 g of each selected biomass was dipped 2.5% H_2SO_4. After 4 h it was thoroughly washed with tap water until it becomes neutral. After washing it was dried at 60ºC overnight and kept in air tight containers.

2.3.5.Acid (2.0% H_2SO_4) + steam pretreatment

200 g of each selected biomass was dipped in 2.0% H_2SO_4. After 2 h it was thoroughly washed with tap water until it becomes neutral followed by steam pretreatment at 121°C at 15 psi for 1 h in an autoclave, dried at 60 ºC and kept in air tight containers.

2.4.Inoculum preparation

100 ml nutrient broth was seeded with 10% culture of *Bacillus coagulans* B30 (O.D. 1.0) in 250 ml Erlenmeyer flasks and was kept at 35°C at 120 rpm for 24 h.

2.5.Production of xylanase using lignocellulosic residue

To 5 g of each untreated and pretreated biomass, 10 ml of moistening agent *viz.* Tryptone glucose yeast extract (TGY) medium was added (in ratio of 1:2.4 i.e. substrate: moistening agent) in 250 ml Erlenmeyer flask and autoclaved.

After autoclaving, flasks were inoculated with 2 ml of *B. coagulans* B30 (O.D. 1.0) and incubated at kept 35±2°C for 10 days in static phase.

2.6.Extraction of xylanase by Repeated Extraction method [12]

To 5 g of each untreated and pretreated biomass, 50 ml of phosphate buffer (0.1M, pH 6.9) with 0.1% Tween-80 was added in 250 ml Erlenmeyer flask. The contents were kept in the shaker for 1 h at 120 rpm and then filtered through muslin cloth. The process was repeated twice with 25 ml of phosphate buffer. After filtration, contents were centrifuged at 5,400 rpm for 10 min at 4°C. The supernatant was collected for further studies.
Among all pretreated lignocellulosic biomass/substrate, the best substrate i.e. microwave irradiated mixed biomass was selected for further purification studies for *B. coagulans* B30.

2.7.Purification of xylanase

The crude xylanase was precipitated with 40-60% saturation of ammonium sulfate and allowed to settle overnight at 4ºC. The precipitates were collected by centrifugation at 12,000 rpm for 15 min at 4ºC. Pellet of enzymes were dissolved in phosphate buffer (0.1M, pH 6.9) and subjected to dialysis overnight against the same buffer. Dialyzed enzyme solution was loaded onto a Sephadex G-100 column (40 x 1.5cm) equilibrated with phosphate buffer, pH 6.9. The enzyme was eluted with linear gradient at a flow rate of 3ml/ 7min. The eluted fractions were assayed for enzyme activity. This partially purified enzyme solution used for investigating the effect of temperature and pH on enzyme activity.

2.8.Molecular weight and purity

The molecular weight and purity of purified xylanase was determined by using 10% of SDS-polyacrylamide gel following the procedure of Lammeli [13]. The stacking and resolving gels used were 5 and 10% polyacrylamide, respectively. The gel was stained with Coosassie brilliant blue. The protein content was estimated by Lowry's method.

2.9. Characteristics of purified enzyme

2.9.1.Effect of temperature and pH on enzyme activity

Effect of pH on enzyme activity was determined at different pH values (pH 3-11) with 10mM citrate buffer (pH 5.0 -6.0), 10mM of sodium phosphate buffer (pH 6.0-8.0), 10mM Tris HCl buffer (pH 8.0-9.0) and 10mM sodium carbonate buffer (pH 8.0-11.0) and the reaction was carried out at 35ºC for 5 min and the residual activity was measured by standard assay [9]. The effect of temperature on enzyme activity was performed in eppendorf tubes (1.5 ml) containing 0.2 ml of purified enzyme in 10mM Tris-HCl (pH 9.0) and incubated at 30, 40, 50, 60 and 70°C for 15 min. Tubes were removed, cooled immediately and enzyme activity was determined by the standard assay procedure.

2.10.Statistical analysis

Completely randomized design was used to test the effect of various biomass. Different predication models viz. linear, quadratic, cubic, power and exponential model were applied to estimate the xylanase activity on the basis of temperature and pH.

Model	Prediction equation
Linear	$Y= b_0+b_1x$
Quadratic	$Y=b_0+b_1x+b_2x^2$
Cubic	$Y=b_0+b_1x+b_2x^2+b_3x^3$
Power	$Y=box^{b1}$
Exponential	$Y=b_oexp(b_1x)$

3. Result and Discussion

3.1. Isolation and screening for xylanase producers

In total, 17 bacteria were isolated from soil sample. Among these, 7 were scored as xylanase producers by plate assay. Particularly one isolate, B30 exhibiting largest clear zone around the colonies on xylan agar plates and was selected for further studies for the production of xylanase using forest waste in solid state fermentation and its purification in order to employ it for different industrial processes.

3.2. Characteristics of xylanase producing *Bacillus coagulans* B30

Considering biochemical and physiological tests performed isolate B30 was identified as *Bacillus coagulans*. The strain grew well between 15 to 55ºC

with an optimum growth at 35 ºC and at a wide pH range of 3.0 to 11.0 with an optimum an optimum growth at pH 9.0. Taxonomic affiliation of 16S rRNA sequence of the isolate B30 was retrieved by using BLASTN tool of analysis (www.ncbi.nlm.nih.gov:80/BLAST).

This confirmed that isolate was *Bacillus coagulans* B30 (≈90% homology). It was deposited in IMTECH, Chandigarh, India and the accession number MTCC 10305 was obtained.

B.coagulans produced 3.99 IU/ml of xylanase in TGY medium, was also assayed for its cellulase activity i.e. FPase 0.0062 $IU.ml^{-1}$, CMCase 0.016 $IU.ml^{-1}$, β-glucosidase 0.00 $IU.ml^{-1}$ under submerged fermentation. The meager production of cellulase from *B.coagulans* 30 indicated its cellulase-free xylanase producing nature.

Major challenges met to exploit the commercial potential of xylanase are lower yield and higher cost of xylanase production. One effective approach to reduce the cost of enzyme production is to replace highly expensive xylan used for enzyme production by relatively cheaper substrate viz. lignocellulosic biomass containing plentiful hemicelluloses in them. Among them forest waste is least explored, widely abundant and inexpensive substrate to be used for xylanase production.

3.3. Xylanase production:

Since *B.coagulans* B30 showed good capability for xylanase production under SmF with an apparent aim to increase titres of xylanase enzymes. In the present study, the potential of *B.coagulans* B30 to utilize various forest residues (needles and sawdust) as sole carbon sources and produce xylanase was explored under solid state fermentation (SSF). Among natural lignocellulosic waste, highest enzyme titres i.e. 64.47 U/g were produced from needles of *Pinus roxburghii* when used as a substrate followed by 49.82 U/g from *P. roxburgi* wood while *Q. leucotricophora* and *D. sisoo* wood had yield 36.63 U/g of enzymes.
A significant increase in enzyme activity was noticed when *B.coagulans* B30 was grown on pretreated lignocellulosic waste.

Among different pretreatments i.e. microwave irradiation, alkaline (2.5% NaOH), alkali (2.0% NaOH)+stream, acid (2.5% H_2SO_4), Acid (2.0% H_2SO_4) + stream pretreatment given to biomass, all of them have responded increasing xylanase activity as compared to untreated biomass. Maximum enzyme activity of 281.63U/g (16.78 IU/ml) was reported from microwave irradiated mixed biomass followed by 263.77 U/g (15.39 IU/ml) from *Eucalyptous* sp. sawdust thus placing this treatment at first rank. Acid + stream pretreatment *D.sisso* wood had yielded 244.14 U/g (13.18 IU/ml) of xylanase followed by 190.51 U/g (10.65 IU/ml) by acid pretreated mixed biomass. Alkali and alkali+ stream has been found comparatively less effective in forest biomass. A comparative account showing enhanced in xylanase activity of pretreated biomass over untreated shown in Table and Figure 1. Pretreated biomass has suspended before for xylanase production as compared to untreated one. This is because native biomass is complex in nature containing firmly interwoven cellulose and hemicellulose shielded by lignin from outside thus making hemicelluloses inaccessible to xylanase producing organisms. Different physiochemical treatments help to break the interbonds and disrupts the lignin barrier thus rendering hemicelluloses free as a substrate for xylan utilizing microbes. Similar studies using Alkali (2.5% NaOH) pretreatment [14], Alkali (2.0% NaOH) + steam pretreatment [15], Acid (2.5% H_2SO_4) pretreatment [16], Acid (2.0% H_2SO_4) + steam pretreatment [17] has been conducted with different lignocellulosic materials to increase xylanase yield.

As is clear from the results obtained in this study that microwave pretreated forest mixed biomass has emerged as a best substrate for enzyme production. Microwave irradiation of lignocellulosic biomass is found interesting because it is supposed to simplify three structural components. When the crystalline region is placed between electromagnetic field it acts polarized generating a charge on crystalline interphase [18].

Thus microwave pretreatment is proven to be an effective way of increasing the enzymatic digestibility of forest lignocellulosic waste for xylanase enzyme production.

Microwave irradiation is recommended as the best pretreatment in this study and it has an edge over other treatments due to pollution free approach and possibility of removing more lignin from lignocellulosic biomass with shorter pretreatment time as compared to alkali pretreatment. Microwave irradiation of mixed forest waste depending upon its highest yield was selected as substrate for further purification studies.

Keshwani and Cheng [19] observed modeling changes in biomass composition during microwave-based alkali pretreatment of switchgrass. Similar results were also observed by Nikolic et al. [20] for microwave pretreatment that could increase the maximum ethanol concentration by 13.4% under the SSF. Similarly it has been found that microwave pretreatment of switch grass (*Panicum virgatum*) gave the rich sugar yield (34.5%), the yield was higher than obtained from conventional heating [21].

3.4.Purification of xylanase

The extracellular enzyme was purified to homogeneity from the cultural filtrate when grown in a microwave irradiated biomass. The crude enzyme was first precipitated with ammonium sulfate and it was found that 40-60% of ammonium sulfate saturation was optimal for xylanase precipitation. The enzyme was eluted through Sephadex G-100 for gel elusion chromatography. A total of 72 fractions of 3ml each were collected from elution profile of xylanase. The fraction number which showed the highest enzyme activity by calorimetric method was shown in Figure 2. Samples were pooled and concentrated for enzymatic studies. Table 2 shows xylanase was partially purified with 1.73 fold with 72.22% yield and final specific activity of 20.52 by *B.coagulans* B30 from microwave irradiated mixed biomass. In gel exclusion chromatography xylanase enzyme was purified and 4.70 purification fold with 64.07% yield and final specific activity of 55.80 in gel exclusion column chromatography was observed. The molecular mass of purified xylanase was found to be approx 65.54 kDa (Plate 1 and 2).

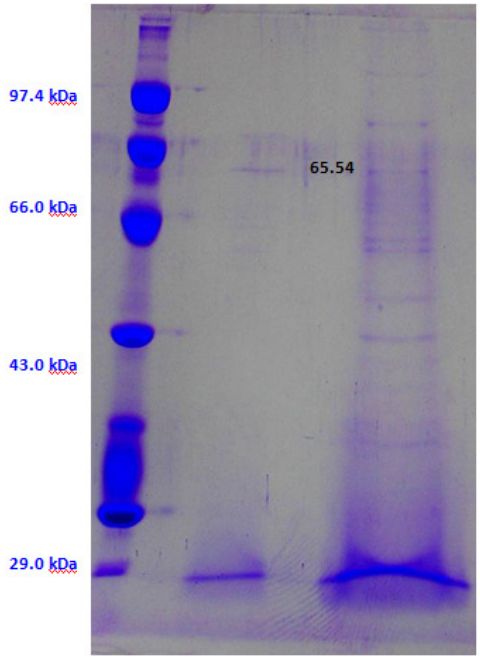

Plate 1. SDS – Polyacrylamide Gel Electrophoresis of purified xylanase by *B.coagulans* B30

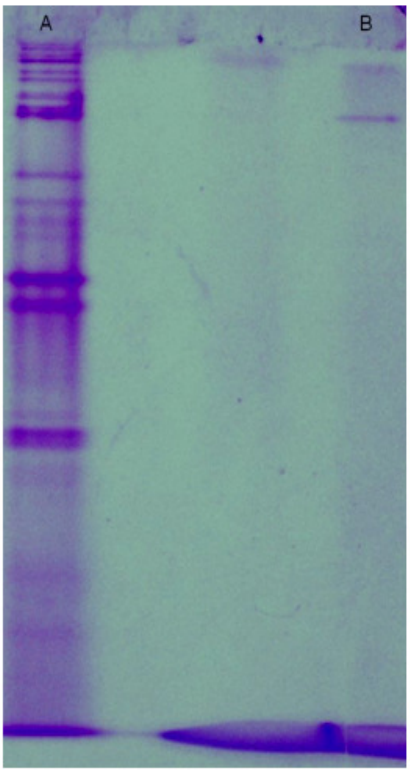

Plate 2. Native Gel Electrophoresis of purified xylanase by *B.coagulans* B30.

3.5.2 Effect of pH:

Different pH range varying from 3.0 to 11.0 (using buffers i.e. citrate buffer, phosphate buffer, Tris-HCl buffer and sodium carbonate buffer) were used to detect its effect on activity of purified xylanase from *B.coagulans* B30 using mixed biomass. The maximum activity was found at pH 9.0 with 11.99 IU/ml of activity indicating its alkaline nature (Figure 4). Khandeparker and Bhosle [23] studied the effect of pH on enzyme activity from *Enterobacter* sp., using wide range of pH 4 to11 and observed that optimal pH for purified xylanase was 9. In contrast Yin et al., [24] reported an optimal pH of 5.0 in *Bacillus* sp. YJ6 and its stability in between pH 5.0 -8.0 by measuring the activity of purified xylanase at different pH 3.0-11.0. Alkaline xylanase has special advantage in paper and pulp industries, in which active and stable xylanase in high pH and temperature ranges is mighty pressing for their high quality of final products [25].

3.6. Statistical analysis:

High value of R^2 (0.95) was found in cubic model followed by quadratic, thus the predication equation/ cubic model to predict xylanase activity on the basis of temperature are presented in Table 3:

$$Y = 78.173\text{-}1058\ X + 0.272\ X^2 - 0.002X^3$$

Table 1. Extracellular xylanase production by *B.coagulans* B30 using different physio-chemical pretreatments

	Xylanase activity (U/g)					
Biomass Δ	**Untreated biomass**	**Microwave treated**	**Alkali pretreated**	**Alkali+ stream pretreated**	**Acid pretreated**	**Acid + stream pretreated**
Pinus roxburghiii(needle)	*64.47	*156.65	*80.59	* 66.03	*156.79	*64.98
Pinus roxburghii (wood)	49.82	114.44	52.75	52.91	101.11	50.23
Toona ciliata	29.30	179.84	65.94	36.63	172.91	55.68
Eucalyptus sp.	32.23	263.77	57.15	39.56	117.23	33.70
Cedrus deodara	30.77	209.82	63.01	38.77	58.61	35.77
Populus deltoides	21.98	175.46	55.68	39.56	39.57	27.84
Quercus leucotricophora	36.63	221.81	51.28	42.49	175.84	48.35
Acacia catechu	24.91	93.25	65.94	33.7	38.10	41.03
Bombax ceiba	23.44	62.94	36.68	143.60	45.42	38.10
Dalbergia sissoo	36.63	48.95	41.63	61.54	43.96	244.14
Dandracalamus strictus	29.30	116.47	112.83	87.92	58.61	39.56
Mixed Biomass	24.91	281.63	84.29	28.52	190.51	29.09
S_E	3.73	18.7	9.48	4.37	8.79	11.32
$CD_{0.05}$	7.71	38.6	19.32	9.41	19.48	23.48
Mean	33.70	160.42	63.98	55.94	99.89	59.04

*U/g = μ moles of reducing sugar released/min/g of biomass; Δ pH= 7.1

Table 2. Purification stages of xylanase enzyme produced by *B. coagulans* B30 using microwave irradiated mixed biomass.

Steps	**Volume (ml)**	**Xylanase activity (IU)**	**Total activity**	**Protein concentration (mg/ml)**	**Specific activity (U/mg)**	**Purification fold**	**Recovery (%)**
Crude culture supernatant	250	16.01	4002.50	1.35	11.85	1.00	100
Partial purification $(NH_4)_2$ SO_4	14	20.01	280.14	0.97	20.52	1.73	72.22
Column chromatography/ Sephadex (G-100)	10	48.27	482.70	0.86	55.80	4.70	64.07

Specific activity = Enzyme Activity/mg protein concentration; Purification fold is increase in specific activity. Percent recovery is remaining protein concentration as % of the initial protein concentration.

Table 3: Parameters of various models to predict xylanase activity at different temperature

		Parameters			
Model	**R^2**	**Constant**	**b_1**	**b_2**	**b_3**
Linear	0.54	58.76	-0.20		
Quadratic	0.69	-13.38	3.25	-0.03	
Cubic	0.95	178.17	-10.57	0.27	0.002
Power	0.56	104.12	-0.20		
Exponential	0.57	63.74	-0.006		

Table 4: Parameters of various models to predict xylanase activity at different pH

		Parameters			
Model	**R^2**	**Constant**	**b_1**	**b_2**	**b_3**
Linear	0.41	38.17	1.91		
Quadratic	0.57	60.01	-5.75	0.59	
Cubic	0.64	105.63	-30.75	4.73	0.21
Power	0.32	36.19	0.18		
Exponential	0.42	39.85	0.03		

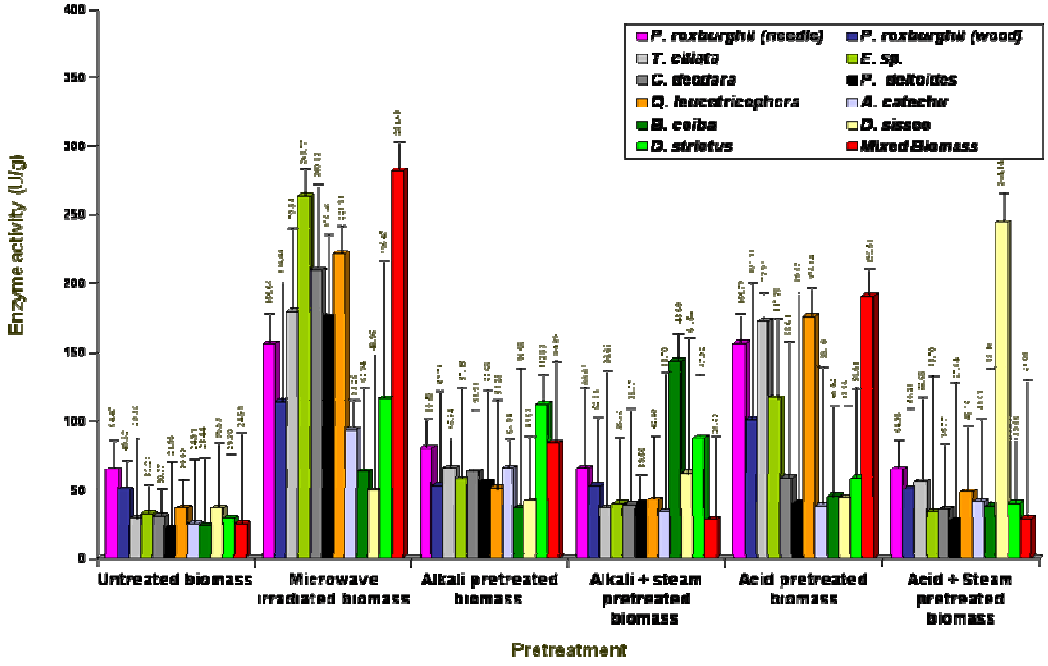

Fig. 1. An overview of xylanase activity from untreated and pretreated lignocellulosic residue from *B. coagulans* 30

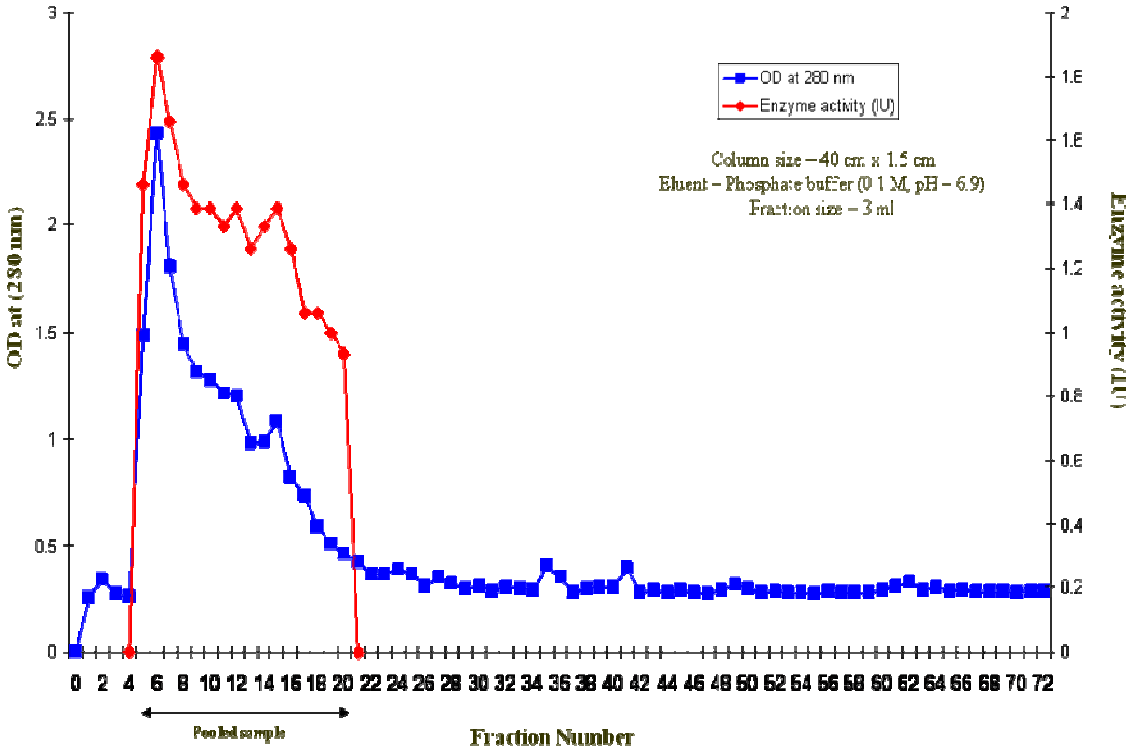

Figure 2. Elution profile of purified xylanase of *B. coagulans* B30 on Sephadex (G100) column

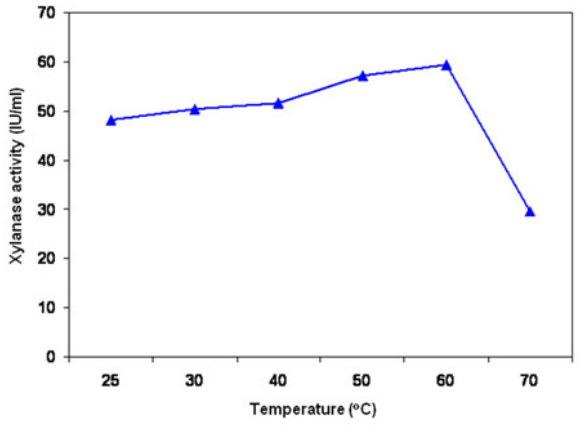

Fig 3 . Effect of temperature on enzyme activity of purified xylanase produced by *B.coagulans* B30

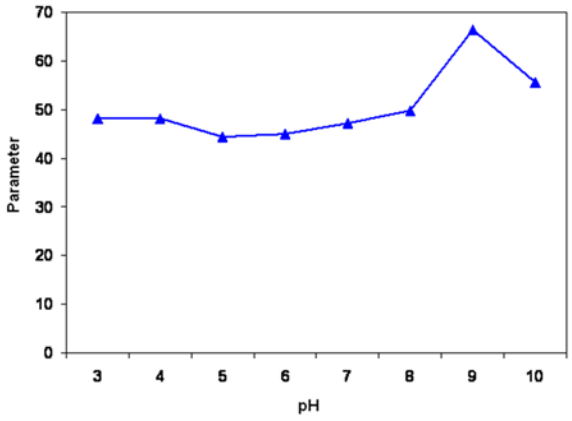

Fig 4. Effect of pH on enzyme activity of purified xylanse produced by *B.coagulans* B30.

In case of pH, high value of R^2 was found in cubic (0.58) and quadratic model (0.58) followed by quadratic to predict the xylanase activity (Table 4), thus the predication equation to estimate xylanase activity on basis of pH was:

$$Y = 105.64 - 30.75\ X + 4.74\ X^2 - 0.21\ X^3$$

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Compliance with Ethics Requirements

Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human and/or animal subjects (if exists) respect the specific regulations and standards.

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