A Novel *Bacillus pumilus* Strain producing Thermostable Alkaline Xylanase at and above 40°C

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Abstract

One strain from 45 analysed which could produce alkaline xylanase at and above 40°C was identified and characterized as *Bacillus pumilus* based on morphological characters and biochemical studies. The spent medium contained 27.9 UmL^{-1} xylanase activity and 1.5 $mgmL^{-1}$ protein and highest specific activity (33.2 Umg^{-1} protein) and was precipitated with 50% $(NH_4)_2SO_4$ saturation. The recovery of xylanase by $(NH_4)_2SO_4$ precipitation was 94.8%. The molecular weight of the purified xylanase was 55.4 KDa. The enzyme showed optimum activity at pH 9.0 and 60°C. The enzyme showed excellent stability at 50°C and pH 9.0 while showing substantial stability at pH values of 8.0, 9.0 and 10.0 and 50, 60 and 70°C.

Keywords: *Bacillus pumilus,* xylanase, alkaline pH, isolation, kinetic properties

Introduction

Xylan is the second most abundant renewable polysaccharide in nature [1]. Xylan is present in appreciable amounts in pulp and in agricultural residues. Xylanases are used to convert the xylan to xylose in the paper-pulp industry [2], treat agricultural wastes and recently, to improve the bread quality [3]. Xylanases are produced by different bacteria, fungi, Actinomycetes, herbivorus insects and some crustaceans [2]. However, most popular are bacteria [4-23] and fungi [20-23]. Several commercially available xylanases are active at neutral, acidic or alkaline pHs and their temperature optima range between 40-70°C. Enzymes which are active under alkaline and higher temperatures have great potential for industrial applications, as in bleaching processes without any need for changes in pH or temperatures. In previous investigations, there have been microorganisms isolated and identified as different strains of *Bacillus,* which are able to produce xylanase under neutral [10, 24] and alkaline [25, 26, 14, 16, 18, 19] conditions [27]. The *B. pumilus* strains reported so far, have produced xylanases showing optimal activities at pH 8.0 and 65°C [16], pH 6.5 and 40°C [28], pH 9.0 and 60°C [29] and pH 6.5 and 50°C [30]. The objective of this study is to select a bacterial strain which can produce xylanase at alkaline pH and temperatures above 40°C and to characterize the strain by microscopic, biochemical and cultural tests, and to determine the kinetic properties and stability of the xylanase, produced by the isolated and characterized strain.

Material and Methods

Selection of organisms and characterization

Among the xylanase producing bacterial strains available at the Biochemistry Laboratory, Faculty of Medicine, University of Jaffna, [31] the strains isolated from opened xylan agar plate medium (GS_7, GS_{15}, GS_{17}, GS_{20} and GS*) were used. Colonies obtained through repeated streaking were tested for xylanase production. The plates and slants containing 25.0 gL^{-1} nutrient agar (Oxoid) and 20.0 gL^{-1} Birchwood xylan (CarlRoth, Krlsruhe, Germany), were used at pH 8.6 for the storage of the strains. From 24 h old slants, the cells were transferred into the activation medium (2 loops/10mL), and incubated in shaker water bath at 40°C and 100 rpm for 18 hours. The activation and fermentation media were prepared at the required pH and incubated at different temperatures based on the experimental conditions and at 100 rpm. Fermentation medium contained 20 gL^{-1} Birchwood xylan, 20 gL^{-1} peptone (Oxoid), 2.5 gL^{-1}yeast extract (Oxoid), 0.005 gL^{-1} $CaCl_2.2H_2O$, 0.005 gL^{-1} $MgCl_2.6H_2O$, 0.005 gL^{-1} $FeCl_3$, 2.5 gL^{-1} K_2HPO_4, 1 gL^{-1} KH_2PO_4 0.1 gL^{-1} NaCl and 2 gL^{-1} $(NH_4)_2SO_4$ and based on the experiment, the pH was adjusted.

Production and assay of xylanase

Fermentation medium was inoculated with the five selected strains (20%, v/v) and incubated at 40°C in a rotory shaker (100 rpm). Samples taken at 42 h were centrifuged and the supernatant was the xylanase source. The enzyme activity was determined based on the release of reducing sugar from xylan using dinitrosalicylic acid (DNS) [32]. A mixture of appropriately diluted enzyme and 20 gL^{-1} Birchwood xylan at pH 9.0 was incubated at 60°C for 10 minutes and then the reaction was stopped by adding DNS reagent. One unit of xylanase activity is defined as the amount of enzyme that produces one mM of reducing sugar in one minute under the above conditions.

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Selection of strains which produce alkaline xylanase at high temperatures

Strains GS_7, GS_{15} GS_{17}, GS_{20} and GS* grown in the activation media at different pH values (7.5, 8.0, 8.5, 9.0 & 10.0) and at 40°C, were inoculated into the fermentation medium at the respective pH values (7.5, 8.0, 8.5, 9.0 & 10.0) and incubated at 40°C and at 100 rpm while maintaining the pH. The bacterial strains GS17 and GS* (18 h old, 20% v/v) activated at optimum pH value and at different temperatures (35, 40, 45, 50, 55 & 60°C) at 100 rpm, were inoculated into fermentation medium at the optimum pH value and incubated at the respective temperatures at 100 rpm while maintaining the pH. The pH was maintained by the manual addition of either 0.1 N HCl or 0.01 N NaOH.

Effect of Temperature on xylanase activity

The effect of temperature on the xylanase activity was studied from 40 to 95°C.

Characterization of the selected strain

Microscopic studies and Biochemical tests

The strain GS_{17} was subjected to gram staining [33] and motility test by hanging drop [34]. Oxygen requirement test, test for anaerobic growth, Catalase test, Oxidase test, Triple Sugar Iron Agar test and Lactose Fermentation test were done on the strain GS_{17}[34].

Identification of genus of selected strain

Colony morphology such as form, elevation, margin, opacity, diameter after 40 h growth (in mm), colour and surface of the strain GS_{17}, were studied to identify the Genus of the bacterial selected strain.

Determination of species of strain GS_{17}

Shape and arrangement of endospore were observed under oil-immersion microscope after gram staining. Production of acid from different carbohydrates such as glucose, xylose and mannose were tested. Production of urease, hemolysis of blood agar, indole test, nitrate reduction test, decomposition of tyrosine, hydrolysis of starch, citrate utilization test and Voges-Proskauer (VP) test were done on the selected strain [34]. Growth of the selected strain was tested at 5, 15, 25, 35, 40, 45, 50, 55 and 60°C at pH 9.0, and 100 rpm. Effect of different concentrations of NaCl on the growth of the selected strain was tested.

Enzyme Purification

Cells were removed by centrifugation from the 42 h old culture and the enzyme in the supernatant was precipitated with $(NH_4)_2SO_4$ (50% saturation). The residue obtained after centrifugation was dissolved in minimal volume of water and dialyzed against deionized water with five changes and finally equilibrated with 0.01M Tri-HCl buffer (pH 8.6). The dialyzed samples were centrifuged and the supernatants were further purified with DEAE fast Flow (Amersham Biosciences, Uppsala, Sweden), equilibrated with 0.01 M Tris- HCl buffer (pH 8.6) and eluted with 0.8 M NaCl. Fractions that exhibited xylanase activity were pooled and subjected to gel filtration with Sephadex G-75 (Amersham Biosciences, Uppsala, Sweden) at a flow rate of 10 mL/h. Protein [35] and xylanase activity [34] were measured.

Kinetic studies

The kinetic properties of the purified enzyme, such as optimum pH and temperature for enzyme activity, the apparent Km and Vmax were determined using Birchwood xylan as substrate.

Stability of the purified enzyme

The temperature and pH stabilities of the enzyme were determined.

Results

Selection of a bacterial strain which can produce alkaline xylanase at high temperatures

Among the five bacterial strains, strain GS15 showed highest growth at pH 8.0 and 8.5, while GS17 showed highest growth at pH 9.0, 9.5 and 10.0. Most of the organisms showed better growth in the pH non-maintained media than the pH maintained media (Tables 1 & 2) except GS_7 (at pH 8.0), GS_{15} (at pH 8.0), GS_{17} (at pH 8.5, 9.0 and 9.5) and GS* (at pH 8.5). Strains GS_{17} and GS* were able to grow at pH 10.0. Since GS_{17} and GS* grew and produced xylanase under alkaline conditions, they were selected.

Strains GS_{17} and GS* started to produce xylanase at 14 h at all the tested temperatures. At the commencement of the xylanase production, GS_{17} produced highest activity (8.85 UmL^{-1}) at 40°C while GS* at 35°C (6.25 UmL^{-1}). The strains GS_{17} and GS* produced xylanase activity at 14 h of fermentation (1.21 and 0.16 UmL^{-1} respectively) at 55°C. Strain GS_{17} produced 0.22 UmL^{-1} of xylanase activity at 60°C while the strain GS* did not produce xylanase activity at 60°C. The strains GS_{17}, and GS* produced highest xylanase activities [24.52 UmL^{-1} (42 h) and 19.26 UmL^{-1} (48 h)] at 40°C. With temperatures above 40°C, enzyme production decreased. Both the strains produced xylanase activity up to 55oC (Table 3).

Though both strains GS_{17} and GS* produced xylanase at higher temperatures, the xylanase production by strain GS_{17} at 39 hours and at pH 9 was higher than by the strain GS*. GS* did not produce xylanase at 60°C at pH 9.0 while GS_{17} produced 2.12 UmL^{-1} (39h) of xylanase activity at 60°C. As GS17 gave highest xylanase enzyme activity than GS*, at alkaline pH and higher temperatures, $GS_{17}$was selected for further studies.

Table 1: The effect of pH on the growth of the bacterial strains GS7, GS15, GS17, GS20 and GS*, at 40°C and 100rpm in fermentation medium

pH	Highest growth (600nm)									
	GS_7		GS_{15}		GS_{17}		GS_{20}		GS*	
	T	C	T	C	T	C	T	C	T	C
8.0	1.94 (60)	1.89 (54)	2.42 (66)	2.36 (66)	2.35 (54)	2.43 (66)	1.71(60)	1.74 (66)	2.36 (66)	2.41(60)
8.5	1.8 (42)	1.84 (60)	2.34 (60)	2.45 (60)	2.31(60)	2.14 (60)	1.61(54)	1.69 (60)	2.22 (60)	2.12 (60)
9.0	1.67 (54)	1.82 (54)	1.84 (60)	2.42 (66)	2.37 (60)	2.09 (60)	1.41(66)	1.62 (60)	2.11 (66)	2.09 (66)
9.5	1.31(54)	1.48 (60)	1.26 (60)	1.52 (66)	1.58 (54)	1.41(66)	1.16 (60)	1.45 (66)	1.46 (60)	1.68 (66)
10.0	–	–	–	–	1.29 (66)	1.21(42)	–	–	1.22 (60)	1.35 (66)

T – pH was maintained at the respective pH values C – pH was not maintained
The time at which highest optical density obtained was given in parenthesis.

Table 2: The effect of pH on xylanase production by different bacterial strains (GS_7, GS_{15}, GS_{17}, GS_{20} and GS*) at 40°C and 100rpm in fermentation medium.

pH	Highest xylanase activity (UmL^{-1})									
	GS_7		GS_{15}		GS_{17}		GS_{20}		GS*	
	T	C	T	C	T	C	T	C	T	C
8.0	21.28 (66)	21.23 (66)	15.84 (42)	16.38 (42)	35.68 (54)	33.29 (54)	32.75 (60)	33.24 (60)	39.53 (54)	36.59 (54)
8.5	18.35 (60)	21.24 (60)	16.42 (42)	17.88 (42)	38.65 (54)	32.44 (54)	30.64 (66)	33.24 (66)	31.54 (54)	28.32 (60)
9.0	21.6 (60)	22.96 (60)	22.40 (42)	23.97 (42)	26.40 (54)	19.46 (54)	22.40 (54)	24.96 (54)	23.75 (60)	20.56 (60)
9.5	9.54 (54)	13.68 (60)	7.88 (42)	11.32 (42)	18.22 (54)	14.95 (54)	8.69 (54)	15.64 (54)	14.26 (60)	13.02 (66)
10.0	–	–	–	–	8.48 (54)	6.88 (54)	–	–	8.16 (60)	7.36 (66)

T – pH was maintained at the initial level C – pH was not maintained
Highest xylanase activity obtained is present and the time at which the highest activity obtained is given in parenthesis.

Table 3: The effect of temperature on the xylanase production by the strains GS_{17} and GS* grown at different temperatures at 100rpm in fermentation medium The pH of the medium was maintained at 9.0, by the manual addition of 0.1N NaOH.

Temperature (°C)	Activity at 14 h (UmL-1)		Highest activity (UmL-1)	
	GS17	GS*	GS17	GS*
35	7.35	6.25	22.48 (34)	18.42 (48)
40	8.85	5.96	24.52 (42)	19.26 (48)
45	7.54	4.16	18.84 (39)	15.94 (42)
50	4.56	2.24	12.42 (34)	11.69 (48)
55	1.21	0.16	7.85 (39)	4.48 (54)
60	0.22	–	2.12 (39)	–

Characterization of strain GS_{17}

Microscopic and biochemical studies

The strain GS_{17} was stained as blue-violet in colour rod with spores indicating that it is a gram positive rod. Strain GS_{17} moved rapidly across the microscopic field with twisting indicated true motility. The hanging drop method used here is a type of wet mount slide preparation that permits the observation of living, unstained cells in a fluid medium. Gram-positive motile non-branching spore forming rods belong to Family *Bacillaceae* [36-38].

Biochemical tests were carried out to confirm the genus of the strain and to identify the species. Strain GS_{17} had shown good growth under aerobic condition but did not grow under anaerobic condition. This indicated that the strain GS_{17} is a strict aerobe. The strain GS_{17} produced O_2 from H_2O_2. This showed that *Bacillus* GS_{17} is a catalase producer. The strain GS_{17} did not produce cytochrome oxidase.

Triple Sugar Iron (TSI) agar medium measures the ability of bacterium to ferment three sugars, glucose, sucrose and lactose. It is used primarily to distinguish the morphologically similar bacteria of Enterobacteriaceae, all of which ferment glucose to an acid end product [39]. Strain GS_{17} was observed as a fermenter of lactose or sucrose and glucose. These results indicated that this is a glucose fermenter. The ability to ferment glucose in *Bacillus* differs within species. Therefore this test is more useful in identifying species of the strains.

Mac Conkey agar is mainly used to identify lactose fermenting and gram-negative enteric pathogens and inhibit the growth of gram-positive organisms [40]. Strain GS_{17} did not grow in Mac Conkey agar medium indicating that it does not ferment lactose. This test is specific to identify *Lactobacillus.* Therefore based on this test, the strain GS_{17} does not belong to *Lactobacillus* of family *Lactobacillaceae.*

Identification of genus of selected strain

The colonies of strain GS_{17} have a circular form, entire margin white in color, are moist with shiny surface convex elevation of 1.5 to 2.0 mm in diameter after 40 h of growth and opaque single colonies. Based on the identification studies carried out so far by observing the morphology, culture and biochemical tests, it is confirmed that the strain GS_{17} belongs to the genus *Bacillus.*

Determination of species of selected strain GS_{17}

To determine the species of a bacterial strain, it is important to know its endospore formation, acid production from sugars, urea hydrolysis, blood haemolysis, indole production, nitrate reduction, tyrosine utilization, starch hydrolysis, citrate utilization, glucose fermentation, growth temperature and growth in NaCl. To identify the species of the selected strain, GS_{17} which belongs to the genus *Bacillus,* Bergey's Manual of Systematic Bacteriology [41] was used as the main guide.

When the strain GS_{17} was Gram-stained, it showed ellipsoidal shape spores arranged in central fashion. This pattern of spore arrangement is found in 13 species among 23 species of Group 1 described by Fisher [41]. Therefore GS_{17} could be one of *subtilis, pumilus, licheniformis, ceresus, anthracis, thuringiensis, megaterium, firmus, laterosporus, fastidiosus, popilliae, lentimorbus* and *amyloliquifaciens.* The characteristics of the strain GS_{17}were compared with the 13 species above. When GS_{17} was Gram-stained and observed under o il-immersion microscope, spherical shaped endospore was observed. Spores containing vegetative cells were not observed as swollen.

When the strain GS_{17} was inoculated into serum-water glucose medium, serum-water xylose medium and serum-water mannose medium and incubated at 37°C for 24 h, the colour changed to red. This indicated that GS_{17} can produce acid from glucose, xylose and mannose. Acid production from glucose is a common observation [41]. Among the identified 13 *Bacillus* species, *B. subtilis, B. pumilus, B. licheniformis* and *B. megaterium* can produce acid from xylose and *B. cereus* and *Bacillus anthracis* do not produce acid from mannose. Other species of *B. subtilis, B. pumilus, B. licheniformis, B. laterosporus* and *B. amyloliquifaciens* can produce acid from mannose. Therefore GS_{17} could be included in one of the above said species which give positive results for this test [41-42].

Strain GS17 is not a urease producer

When strain GS_{17} was inoculated onto blood agar, around the bacterial colony, a transparent clear zone was observed. This indicated that GS_{17} is a β-haemolytic organism. Among the identifited 13 species, *B. cereus* is the only organism of a β-haemolytic nature and the other 12 species are variable in nature.

When strain GS_{17} was inoculated into tryptone water and mixed with Kovac's indole reagent, no colour change was observed [43]. Among the selected group of organisms, with the exception of *Bacillus fastidiosus,* the other organisms did not produce indole. Among *Bacillus cereus,* some organisms can produce indole and some organisms cannot. Therefore GS_{17} could not be *Bacillus fastidiosus.*

GS_{17} did not convert nitrate into nitrite indicating its inability to produce nitrate reductase. *B. subtilis, B. licheniformis, B. cereus, B. anthrasis, B. thuringiensis, B. megaterium, B. firmus, B. laterosporus* and *B. amyloliquifaciens* can produce nitrate reductase, but *B. pumilus, B. popillae, B. lentimorbus* and *B. fastidiosus* cannot produce nitrate reductase. Therefore GS_{17} will be included in the species of *B. pumilus, B. popillae, B. lentimorbus* and *B. fastidiosus.*

Strain GS_{17} did not utilize tyrosine. Among the selected 13 *Bacillus* species, *Bacillus cereus* can utilize tyrosine [41-42]. *B. subtilis, B. pumilus, B .licheniformis, B. laterosporus, B. popillae* and *B. lentimorbis* do not utilize tyrosine like the strain GS_{17}. The strain GS_{17} did not produce starch-hydrolyzing enzymes. Among the 13 species considered for classification, *B. pumilus, B. laterosporus, B. popillae* and *B. lentimorbus* do not hydrolyse starch while and other species can hydrolyse the starch. Therefore GS_{17} could be included in the species of *B. pumilus, B.laterosporus, B. popillae* and *B. lentimorbus.*

The strain GS_{17} did not utilize citrate as the carbon source. *B. subtilis, B. pumilus, B. licheniformis, B. thuringiensis* and *B. megaterium* utilize citrate as a carbon source and produce alkaline medium. *B. anthracis, B. firmus, B.popillae B. lentimorbus* and *B. laterosporus* do not utilize citrate as carbon source. The results indicated that GS_{17} could be either *B. subtilis, B. pumilus, B. licheniformis, B. thuringiensis* or *B. megaterium.*

The strain GS_{17} produced acetoin by the fermentation of glucose. *B. subtilis, B. pumilis, B. cereus, B. anthracis, B. thuringiensis, B. laterosporus* and *B. amyloliquifaciens* give positive results with this test and *B. megaterium* and *B. firmus* give negative results. Therefore GS_{17} could be included in the species of *B. subtilis, B. pumilis, B. cereus, B. anthracis, B. thuringiensis, B. laterosporus* and *B. amyloliquifaciens.*

The strain GS_{17} did not grow between 5 and 15°C at 20 h and grew at 25, 35, 40, 45, 50, 55 and 60°C (Table 4). Among the 13 *Bacillus species, Bacillus*

subtilis, Bacillus licheniformis, *Bacillus cereus, Bacillus licheniformis, Bacillus anthrasis, Bacillus thuringiensis, Bacillus amyloliquifaciens* and *Bacillus pumilus* can grow at 50°C [41]. The growth of *B. laterosporus* and *B. firmus* at 50°C is variable. *B. subtilis* and *B. licheniformis* can grow at 55°C. From these results it can be concluded that GS_{17} could be included in the species of *B. laterosporus, B. subtilis* and *B. pumilus.*

GS_{17} grew in the medium with NaCl up to 10 gL^{-1} (Table 5). The growth of *B. laterosporus, B. lentimorbus* and *B. popillae* is inhibited by 70 and 100 gL^{-1} NaCl [41-42]. All the other species, except the above, can grow in the fermentation media containing 70 gL^{-1} of NaCl. Hence strain GS_{17} could not be classified as *B. laterosporus, B. lentimorbus* and *B. popillae.*

Final confirmation of species of identified strain Bacillus GS_{17}

Characteristics of the selected strain GS_{17} were compared with 13 species of *Bacillus*. If the character of GS_{17} is similar to the known species its score would be 1. If the character is not similar or variable, it will not get any score. The total score was counted, divided by total number of characteristics, multiplied by 100 and presented as a percentage.

Among the selected *Bacillus* species based on these morphological findings and biochemical studies, Bacillus pumilus got the highest score of 86%, followed by *Bacillus subtilis* (81%, Table 6). Summary of the identification studies of strain GS_{17} is present in Table 6. Strain GS_{17}

Table 4: Growth of strain GS_{17} at 20h in activation medium at pH 9.0 and different temperatures while shaking at 100rpm

Temperature (°C)	OD (600nm)
5	0.0
15	0.0
25	0.62
35	1.89
40	2.16
45	1.61
50	0.94
55	0.38
60	0.14

Table 5: Growth of strain GS_{17} at 24h in fermentation medium containing different concentrations of NaCl at pH 9.0 and 40°C while shaking at 100rpm.

NaCl (gL^{-1})	OD (600nm)
0.0	1.021
1.0	1.164
2.0	1.284
4.0	1.346
6.0	1.491
8.0	1.664
10	1.678
25	0.634
50	0.132
70	0.021

Table 6: Cultural and biochemical characteristics of different species in *Bacillus* (Fisher, 1895; Barrow and Feltham, 1993).

	Gram stain ing	Motility	Growth in 7% NaCl	Anaerobic growth	Acid from mannose	Acid from xylose	Utilization of citrate	Urease activity	Production of indole	VP test	Nitrate reduction	Starch hydrolysis	Production of oxidase	Production of catalase	Chains of cells	Hydrolysis of tyrosine	Hemolysis	Swelling of cells	Growth at 45°C	Growth at 50°C	Growth at 55°C	Total scoring	% of suitability
Subtilis	+	+	+	-	+	+	+	-	-	+	+	+	-	+	d	-	d	-	+	+	+	17	81
Pumilus	+	+	+	-	+	+	+	-	-	+	-	-	-	+	+	-	d	-	+	+	-	18	86
Licheniformis	+	+	+	+	+	+	+	d	-	d	+	+	-	+	d	-	d	-	+	+	+	14	67
Cereus	+	+	+	+	-	-	+	d	-	+	+	+	d	+	+	+	β	-	+	+	-	11	52
Anthracis	+	-	+	+	-	-	-	-	-	+	+	+	d	+	+	d	-	-	-	+	-	8	38
Thuringiensis	+	+	+	+	d	-	+	-	-	+	+	+	d	+	+	d	d	-	+	+	-	11	52
Megaterium	+	+	+	-	d	+	+	d	-	-	+	+	d	+	+	d	d	-	+	-	-	10	48
Firmus	d	+	+	-	d	-	-	-	-	-	+	+	-	D	d	d	d	d	+	d	-	7	33
Laterosporus	+	+	-	+	+	-	-	-	-	+	+	-	-	+	-	-	d	+	+	d	-	12	57
Fastidiosus	+	+	d	+	d	-	d	+	d	d	-	d	d	+	d	d	d	-	+	-	-	6	29
Papillae	d	d	-	+	d	-	-	d	-	d	-	-	d	-	d	-	d	d	+	-	-	5	24
Lentimorbus	d	-	-	+	d	-	-	d	-	d	-	-	d	-	d	-	d	d	+	-	-	5	24
Amyloliquifaciens	+	+	+	+	d	-	d	-	-	+	+	+	-	D	d	d	d	-	+	+	-	10	48
Selected *Bacillus*	+	+	+	-	+	+	+	-	-	+	-	-	-	+	-	-	β	-	+	+	+	21	

showed clear characteristics of *Bacillus pumilus* compared to the other *Bacillus* species.

From these experiments, GS_{17} can be classified as belonging to the Kingdom: Procaryotae; Division: Bacteria; Order: Bacillales; Family: Bacillaceae; Genus: *Bacillus;* Species: *pumilus* [36-37, 40-42].

Characteristics of the purified enzyme

The $(NH_4)_2SO_4$ precipitated sample contained a total protein of 4.8 mg and the total xylanase activity of 156.8 U. When the DEAE – fast flow bound xylanase was eluted, it contained 18.9 UmL^{-1} enzyme activity and the protein concentration was 0.085 $mgmL^{-1}$. By this ion exchange purification, the specific activity of xylanase increased from 33.2 to 222.6 Umg^{-1}, which was 6.7 fold higher than that of the crude xylanase. When the pooled sample of purified xylanase was subjected to gel electrophoretic separation, and stained with coomassie brilliant blue, the sample gave a characteristic thick band (Figure 1). The molecular weight of the purified xylanase is estimated to be about 55.4 KDa.

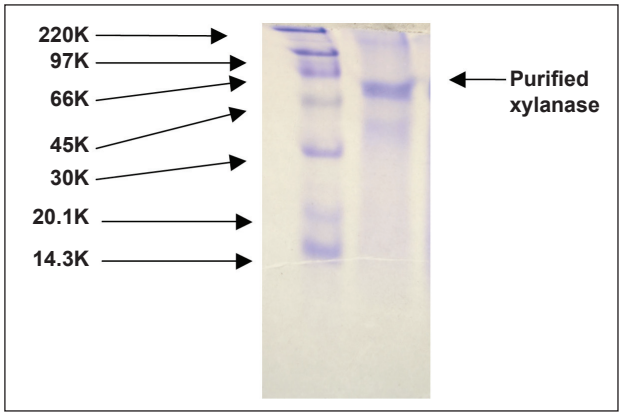

Figure 1: SDS-PAGE pattern of purified xylanase from *Bacillus pumilus* Lane A: marker proteins; Lane B: purified xylanase sample.

Kinetic properties

The Km value of the purified xylanase from the *Bacillus pumilus* was 6.7 gL-1. Xylanase activity was measured between 40-95°C. The optimum temperature was at 60°C. At 55 and 65°C, the enzyme exhibited 97.5% of the activity at 60°C (Figure 2). The activity of xylanase was measured in a pH range of 3-12 at 60°C. Enzyme expressed high activity in a pH range spanning from 7.0 to 12.0. The optimum was pH 9.0. At pH 8.0 and 10.0, the activity was 94 and 99.5%, respectively.

The stability of xylanase

Effect of temperature on xylanase stability

Xylanase was preincubated at the optimum pH 9.0 and at 50, 60 and 70°C for 100 min. The enzyme retained more than 70% of its initial activity up to 100 min at all the temperatures tested (Figure 4). When incubated at

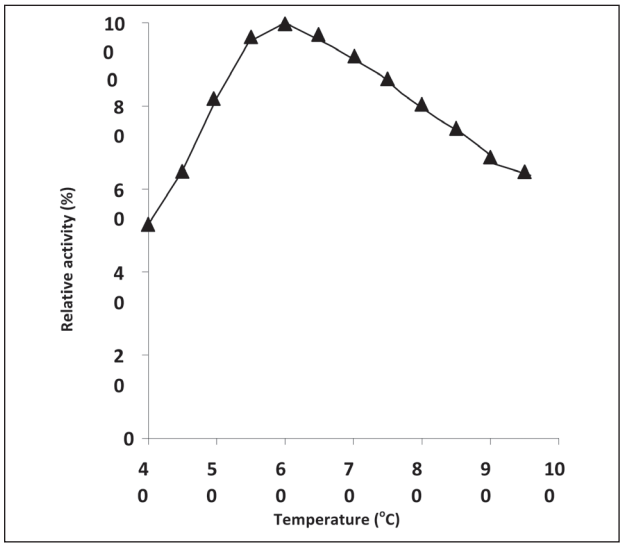

Figure 2: Effect of temperature on the activity of xylanase with Birchwood xylan (20gL^{-1}) -0.01M Tris buffer at pH 9.0.

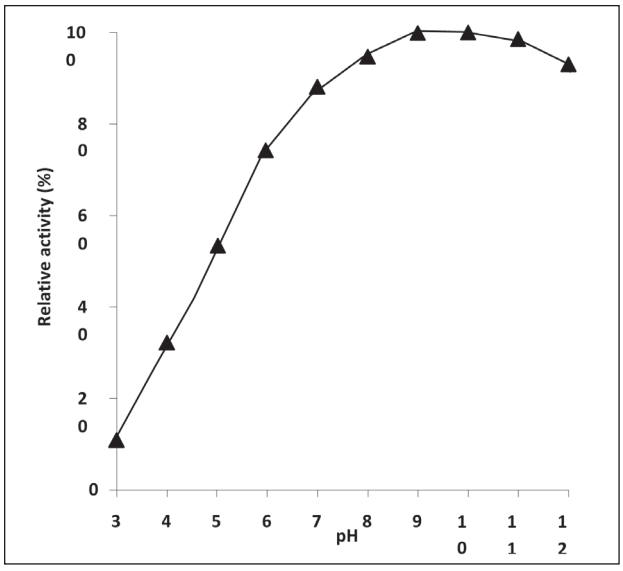

Figure 3: Effect of pH on purified xylanase activity at 60oC with Birchwood xylan (20gL^{-1}).

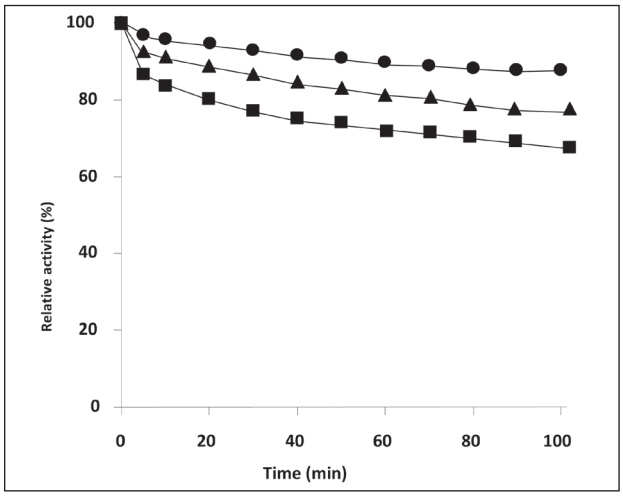

Figure 4: Stability of purified xylanase in 0.01M Tris buffer (pH 9.0) at different temperatures of (●), 50; (▲), 60 and (■), 70°C.

50°C the loss of activity was less than 10% at 100 min while the loss at 100 min at 60 and 70°C were 18 and 24% respectively.

Effect of pH on the stability of xylanase

The stability of xylanase was measured at pH 8.0, 9.0 and 10.0 at 60°C and found that more than 80% of the initial activity was retained at 60 min (Figure 5). The enzyme was most stable at pH 9.0 than at pH 8.0 and 10.

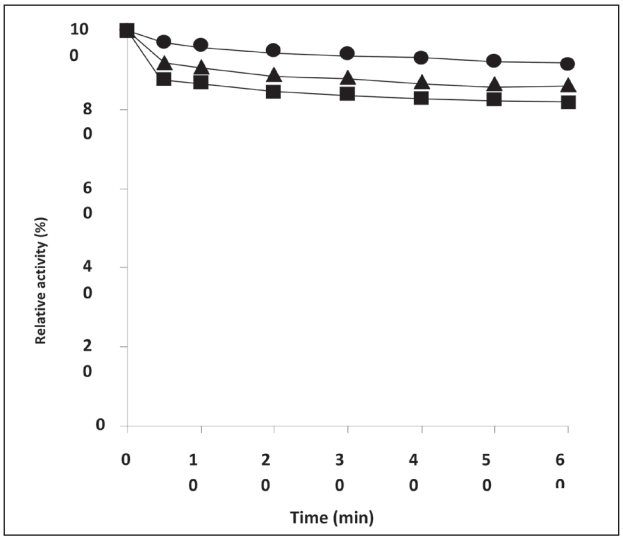

Figure 5: Stability of xylanase at different pH values of (▲), 8.0; (●), 9.0 and (■), 10.0 and at 60°C.

Discussion

Alkaline active xylanses have potential applications in industries, since xylan has better solubility under alkaline conditions. Among the 45 strains previously isolated [31], five strains which can produce xylanase at and above 40°C, and above pH 7.0 were selected. Out of the five strains, the strain GS_{17} is capable of growing and producing xylanase at and above 40°C. The biochemical tests revealed that the strain GS_{17} has 86% homology with *Bacillus pumilus*. Previous studies have reported the *Bacillus pumilus* strains which can produce xylanase at 37°C and pH 9.0 [17], and at 30°C [16]. However other bacterial strains reported have produced xylanase at an above 40°C up to 70°C [5, 7].

Purification of xylanase was carried out by different methods such as using ion exchange chromatography followed with gel filtration [26, 28, 45, 47] and/ hydrophobic interaction chromatography [45] and also using an anioinic polymer Eudragit S 100 [46]. With ammonium sulphate precipitation, ion exchange and gel filtration chromatographies 46% yield was obtained [28] and with an anioinic polymer Eudragit S 100 more than 89% yield was obtained with 4.2 fold purification [46]. In this study, the yield of xylanase and purification foldwere 94.8% and 6.7 respectively. The molecular weight of the xylanase produced by *Bacillus pumilus* was 55 kDa and the molecular weight reported by Panbangred *et al.* [28] was 24 kDa.

The Km value of the xylanase from the characterized *Bacillus pumilus* was 6.7gL^{-1} for Birchwood xylan. The Km values of the xylanases from different *Bacillus* strains were 8.9, 1.1, 33.3 and 71.4 [29]. The Km reported for different xylanases from different bacteria ranged from 4.53 (±0.58); 4.37 (±0.65) & 5.42 (±1.41) (for *Bacillus halodurans* S7 xylanase respectively with Birchwood, oat spelt and Beech wood xylans) [26] to 7.69 $mgmL^{-1}$ (with oat spelt xylan for Cryptococcus sp. X-1) [44].

Xylanolytic activity has been reported to lie between pH 5.2 to 9.5 [5-7; 13-14; 24-25] and 50-100°C [5-7; 13, 25]. Earlier studies have shown that *Bacillus pumilus* is an alkaline xylanase producer [28-29] and have shown the optimum pH as 6.5-7.0 [30], 6.5 [28] and 9.0 [29], while the strain isolated in this studies has shown the optimum pH at 9.0 and was active in the range from 8.0 to 12.0. The xylanases from *Bacillus pumilus* was reported to have optimum temperature as 40 [28], 50 [28] and 60°C [29], while the strain isolated in this studies has shown the optimum temperature at 60°C.

The xylanase from *Bacillus amyloliquefaciens* showed remarkable stability at pH 9.0 by retaining complete activity while losing 20% of its initial activity at pH 10.0 and 5 h [45]. Xylanase from *Bacillus pumilus* was stable in the pH range from 5.5 to 8.5 [29]. *Bacillus halodurans* xylanase showed a wide range of stability at 50°C from pH 5.0 to 10.5 after 12 h [26].

Temperature stability of Xylanase from *Bacillus pumilus* showed that the enzyme was stable and showed 80% of its activity at 65°C after exposing for 135 min while there was 50% reduction after exposure to a temperature of 75°C for the same period [29].

The strain reported in this study also produced the xylanse, which exhibited high activity and substrate stability under alkaline conditions and at 50 to 60°C. These characteristics exhibited by the xylanase indicated that it could be used in the Kraft pulp treatment, where using the pH adjustments and cooling are needed and use of this enzyme will avoid the pH adjustment and cooling costs.

Conclusions

The selected bacterial strain, *Bacillus pumilus,* can produce xylanase at pH 9.0 and above, and at temperature above 40°C. It gave highest xylanase enzyme activity in alkaline pH and at high temperatures and this activity was obtained in a short time of fermentation compared to all the other strains. Xylanase obtained from *Bacillus pumilus* showed zero order kinetics for 10 minutes. The pH optimum was 9.0 and temperature was 60°C. The Michaelis constant for soluble xylan was 6.7 gL^{-1}. Xylanase showed better stability at alkaline pH and higher stability at 50°C. The application of xylanase for the purpose of pulp bleaching demands good activity and stability under

alkaline conditions. Therefore the xylanase produced by the isolated strain, *Bacillus pumilus* has potential application in the paper industry.

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