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Characterization of Xylanase Produced by *Bacillus pumilus* Strain PJ19

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Abstract *Bacillus pumilus* PJ19 isolated from *Pinus* leaves showed optimum xylanase production when grown in yeast tryptone broth at 37°C, pH 7.2, and shaken at 200 rpm after 48 h of incubation. Xylanase production was induced by xylan and xylose but repressed in the presence of glucose. Xylanase production by *B. pumilus* PJ19 was not growth-associated and the maximum enzyme production was found after 36 h of incubation.

Key words: Xylanase, characterization, *Bacillus pumilus* PJ19

Xylan is widely distributed in plant cell walls and forms a primary part of the hemicellulose portion. In some higher plants and agricultural wastes, the xylan constitutes 20–40% of the total dry weight. Xylan is a five carbon sugar consisting of a polymer of D-xylose linked by β-1,4-glycosidic linkages and is hydrolysed by xylanase. Many organisms including bacteria, fungi, yeast, and actinomycetes are capable of producing xylanase [8, 9].

Xylanase often occurs as more than one type of enzymes having similar specificities but differing in the amino acid or carbohydrate contents. This leads to differences in isoelectric point, relative stability, and pH optimum and stability [22]. Xylan and a variety of their catabolites [2, 21] induce the synthesis of xylanase by some xylanolytic microorganisms. In bacteria, the regulation of xylanase synthesis on the whole is inducible, although low level of activity resulting from constitutive synthesis may be required to generate low-molecular-mass inducers from native xylan [4]. There are, however, a few examples of wholly constitutive production of xylan-degrading enzymes [5]. In both cases, xylanase synthesis is repressed by readily metabolizable carbon sources like glucose or xylose or by one of the end products of xylan hydrolysis [7].

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It has been well documented that the production of xylan-degrading enzymes by xylanolytic organisms cultured with xylan as the carbon source is an inducible process. Since xylan is a large polymer which cannot be transported directly across the cell wall and membrane of microbial cells, it must be degraded extracellularly before the organisms could use its components. The signal for xylanase biosynthesis involved the low molecular weight catabolites such as xylobiose and xylotriose, formed by xylan hydrolysis by the actions of tiny amounts of constitutively produced extracellular or cell-surface xylanase. The low level of xylanase then reacts with xylan to produce a soluble molecule, which enters the cells and affects induction. The main products of xylan degradation are xylooligosaccharides and also D-xylose, which can be assayed by the DNS method to determine the enzyme activity [4].

This paper presents results on the characterization of xylanase produced by *B. pumilus* strain PJ19. The parameters studied were pH, temperature, agitation, and substrate requirements.

Materials and Methods

Isolation of Bacteria

Bacteria was isolated from *Pinus* sp. leaves, found within the campus of Universiti Putra Malaysia. Samples were plated on yeast extract agar containing (w/v) 0.5% tryptone, 1.0% yeast extract, 0.5% NaCl, 1.5% agar, and 0.2% of Remazol brilliant blue (RBB-xylan), where the RBB-xylan was prepared according to Biely *et al.* [4]. For primary isolation, 0.01% filter sterilized nystatin was incorporated into the agar to inhibit fungal growth. The pH of the media was maintained at 7.2. Plates were incubated at 37°C for 24–48 h. Bacterial colonies with zone of clearance were selected.

Characterization and Identification of Bacteria

The microbiological properties of the bacteria were identified according to the method described in Bergey's

Manual of Determinative Bacteriology [10, 11]. Identification of the genus and species was done using commercial kits API 50CHB and API 20E. The isolated bacteria was maintained on nutrient agar slant overlaid with sterile paraffin oil.

Preparation of Standard Bacterial Inoculum

The bacterial isolate taken from stock culture was grown in 10 ml yeast tryptone broth (YTB), pH 7.2, supplemented with 0.2% (w/v) oat spelt xylan (Fluka) in a 100-ml conical flask. The culture was incubated at 37°C in an orbital shaker (200 rpm) for 18 h. The cells were pelleted by centrifuging at 2000×g, 4°C for 10 min, washed once with sterile normal saline, and resuspended in the same solution to obtain an optical density reading of 0.5 at 550 nm.

Preparation of a Crude Enzyme Solution

Ten percent of bacterial inoculum was inoculated into 20 ml YTB in a 250-ml conical flask and was incubated at 37°C in an orbital shaker (200 rpm) for 24 h and/or 48 h. At the end of the incubation period, 1.0 ml of culture was removed and spun in a microcentrifuge for 5 min (13,000 rpm) at 4°C. The supernatant was appropriately diluted before being assayed for xylanase activity by the dinitrosalicylic acid (DNS) method with xylose as the standard [7]. One international unit (IU) is defined as the release of 1 µmol of reducing sugar as xylose per min under the specified assay conditions.

Effect of pH

The bacteria was grown in 10 ml YTB adjusted with acid (0.1 N HCl) or alkaline (0.1 M NaOH) to give pH ranges between pH 4 to 10. The culture was incubated for 24 h. The experiment was repeated for a narrower pH range from pH 6.5 to 8.0 and the incubation period was prolonged to 48 h.

Effect of Temperature

The bacteria was grown in YTB at its optimum pH (pH 7.2) for 48 h at different temperatures of 30, 37, 45, and 50°C.

Effect of Agitation

The bacteria was inoculated in YTB at optimum pH and incubated for 48 h at 37°C in an orbital shaker. The speed was varied from 0 rpm (static) to 100, 200, or 300 rpm.

Effect of Carbon Sources on Xylanase Production

In this study, the bacteria were grown in Dubois minimal media containing (g/l): $(NH_4)_2SO_4$, 0.2; $MgSO_4$, 0.5; $CaCl_2{\cdot}2H_2O$, 0.25; KH_2PO_4, 3.0; and yeast extract, 2.0 with pH of 7.2. Known amounts of xylan, cellulose, and carboxymethylcellulose (CMC) as the substrates were added to the Dubois minimal media and then sterilized at 121°C, 15 psi for 15 min. The carbon sources such as glucose, xylose, fructose, cellobiose, maltose, and sucrose were filter-sterilized using a 0.45 µm filter membrane. Known amounts of the sterilized carbon sources were added to the presterilized Dubois media.

Ten percent of the bacterial inoculum was inoculated into 20 ml Dubois media in a 250-ml conical flask and incubated at 37°C in an orbital shaker (200 rpm) for 24 or 48 h. The culture was then centrifuged at 13,000 rpm at 4°C for 5 min in a microcentrifuge (MicroCentaur MSE, U.K.). The supernatant was assayed for xylanase activity by the DNS method [7]. Controls were set up as above except without the addition of any carbon sources.

Growth Curve of Bacteria Producing Xylanase

Ten percent of the bacterial inoculum was inoculated into four 1-litre conical flasks, each containing 200 ml of YTB with 1% (w/v) xylan, pH 7.2. The culture was incubated at 37°C in an orbital shaker (200 rpm) for 48 h. In the first 24 h, 4.0 ml aliquots of the cultures were withdrawn at two-hourly intervals and in the next 24 h aliquots were withdrawn every 4 h. Samples were taken out 5 times from each flask, and the following analyses were carried out: pH, total bacterial count, and xylanase activity.

RESULTS AND DISCUSSION

The isolate was identified as *B. pumilus* PJ19 [15]. The isolate was different from *B. pumilus* IPO [20] proven by nucleotides sequence of *B. pumilus* PJ19 [14].

Effect of pH

The production of xylanase during growth on YTB containing xylan was shown to be influenced by the culture pH in the range of pH 4.0 to 10.0 with the optimum pH of 7.2. The enzyme was not secreted at a very acidic

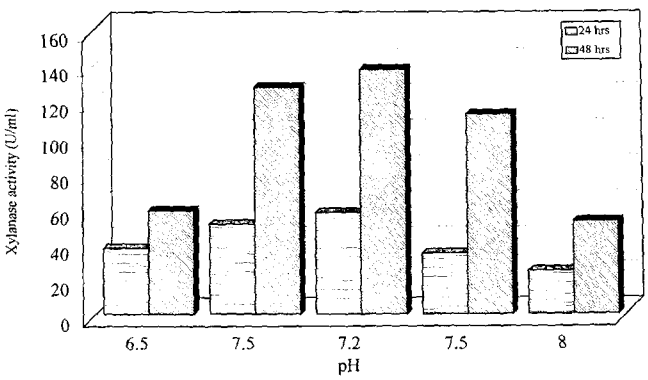

Fig. 1. The effect of culture pH on xylanase production of *B. pumilus* PJ19 after 24 h and 48 h of incubation.
The points represent the averages of four samples of which the standard error of means (SEM) are less than 5%.

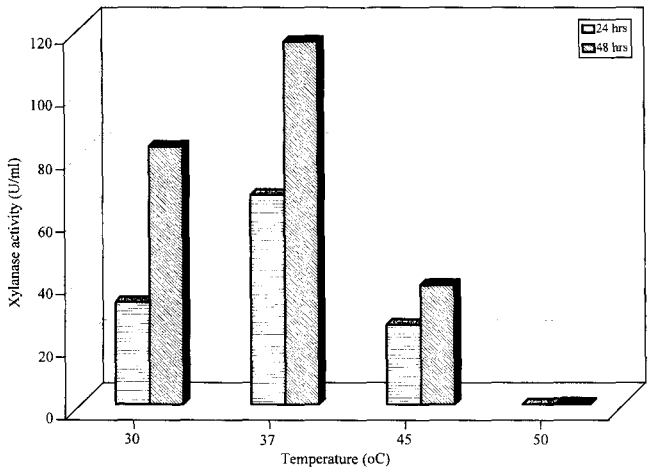

Fig. 2. Effect of temperature on xylanase production of *B. pumilus* PJ19 after 24 and 48 h incubation.
The points represent the averages of four samples where SEM < 5%.

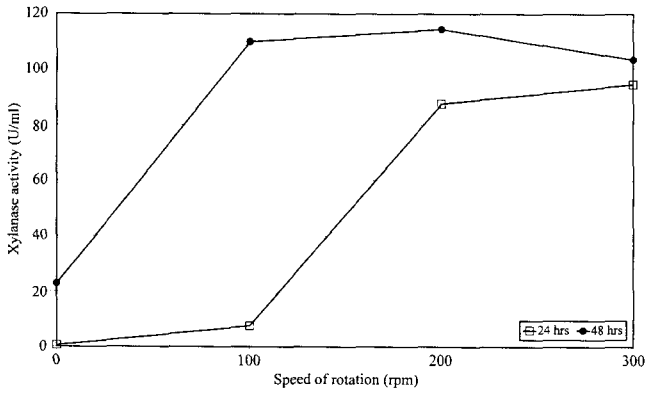

Fig. 3. The effect of speed rotation on xylanase production of *B. pumilus* PJ19.
This was done at optimum temperature and pH.

pH (<4.0). The production of xylanase was increased after 48 h incubation compared to 24 h (Fig. 1). The growth pH for *B. pumilus* IPO [12] and *B. circulans* WL12 [6] was at pH 6.5. Based on the literature, it was noted that most of *Bacillus* sp. producing xylanase were alkalophilic in nature, produced xylanase at an optimum pH of 8.0-12.0 [13].

Effect of Temperature

The *B. pumilus* strain PJ19 showed maximum xylanase activity at 37°C, and no xylanase was produced at 50°C even though growth was observed (Fig. 2). It has been found that all these mesophilic strains of *B. pumilus* produced the highest xylanase activity when incubated at 37°C. Other mesophilic strains of bacteria also exhibited the highest xylanase production at 37°C [16].

Effect of Agitation

The xylanase production was also affected by agitation where higher xylanase activity was found when the cultures were shaken at 200 rpm, but declined at 300 rpm (Fig. 3). One-way analysis of variance on the effect of speed rotation showed a significant difference at $p<0.05$ between the speeds tested. Adequate aeration during the growth phase is important for the cell growth and synthesis of enzyme in aerobic organisms. However, at a very vigorous rotation and longer incubation time, the shear forces gathered through agitation may damage the cells and disrupt the enzyme synthesis [12, 23]. The result obtained on the effect of agitation is in good agreement with other xylanase producing *Bacillus* sp. where the optimum rotational speed was between 150 rpm to 250 rpm [12].

Effect of Carbon Sources

In order to determine the effect of various carbon sources on the production of extracellular xylanase the cells were grown in Dubois minimal media with a little amount of yeast extract (0.2%, w/v) to initiate cell growth. Sugars such as xylan, xylose, cellulose, cellobiose, glucose, fructose, maltose, and sucrose were added to the media in appropriate concentrations. The highest xylanase production was observed in medium containing xylan. At lower sugar concentration, xylan was the best inducer for xylanase followed by xylose; however, at an elevated concentration there was a reduction in xylanase activity (Table 1). These two carbon sources were further tested to find the optimum concentration needed to enhance xylanase production. There was a decreasing trend in xylanase activity as higher xylan concentrations were used (Fig. 4). The highest level of xylanase activity occurred at 10 mg/ml of xylan supplied. Xylose at 5 mg/ml showed the highest activity after 24 h incubation and exhibited a decrease in activity after 48 h.

Tabel 1. Effect of different carbon sources on xylanase production by *B. pumilus* PH19. The xylanase activity from xylan was taken as reference (100%).

Carbon sources	Concentrations (mg/ml)	
	1.0	100
Xylan	100.00	100.00
Xylose	95.30	12.80
Cellulose	1.11	6.53
Carboxymethylcellulose	4.76	7.30
Cellobiose	0.85	1.23
Glucose	0.05	0.09
Fructose	0.15	0.11
Maltose	0.28	0.51
Sucrose	0.10	0.04
Control	0.65	0.65

Since xylose have a negative effect on xylanase production, the effects of xylan and xylose and their combination were carried out. The results are shown in Table 2. When the bacteria was grown on Dubois minimal

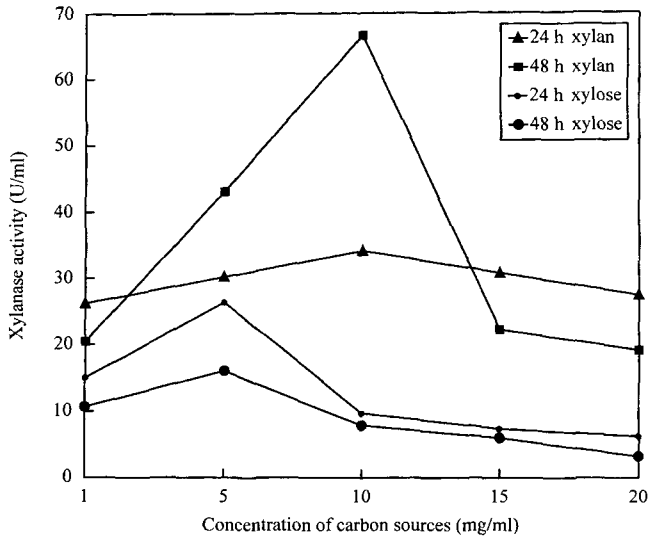

Fig. 4. The effect of different concentration of carbon sources on xylanase production of *B. pumilus* PJ19.
The points represent the average of four samples where SEM < 5%.

Table 2. The effect of the combination of xylan and xylose on xylanase procuction by *B. pumilus* strain PJ19.

Treatment	Xylanase activity (U/ml)±SD (p<0.05)			
	PH19 (24 h)		PH19 (48 h)	
Yeast Tryptone Broth	22.5±0.22	1[a]	55.33±2.57	1[a]
Xylan (10 mg/ml)	15.06±0.75	2[a]	20.20±0.38	2[a]
Xylose (5 mg/ml)	12.5±0.87	3[a]	7.02±0.85	3[a]
Xylan (10 mg/ml) +Xylose (5 mg/ml)	18.21±1.81	4[a]	17.61±0.92	4[a]
Glucose (10 mg/ml)	0.14±0.25	5[a]	0	5[a]
Glucose (10 mg/ml) +Xylan (10 mg/ml)	1.07±0.33	5[a]	0	5[a]
Control	0.0032±0.007	5[a]	0.19±0.15	5[a]

[a]Significant; treatments which are not significantly different are followed by the same number (p<0.05).

media containing xylan (10 mg/ml) or xylose (5 mg/ml) or a combination of xylan+xylose, (10 mg/ml+5 mg/ml), xylanase activity was significantly higher (p<0.05) after 24 h of incubation. However, after 48 h of incubation, the activity was significantly higher in medium containing only xylan. This result showed that although xylose produced an inductive effect on xylanase production, the presence of xylose in xylan medium at prolonged incubation time did not increase enzyme production. This could be due to the accumulation of products of hydrolysis which result in catabolite repression [16].

In order to confirm the glucose effect on xylanase production, the bacteria were grown in media containing only glucose (10 mg/ml) or in a mixture of glucose (10 mg/ml)+xylan (10 mg/ml). The production of xylanase was very minimum in both media and was insignificant after 24 h incubation, and totally inhibited after 48 h. In the presence of glucose, the bacteria was not able to produce xylanase even in the presence of xylan since glucose may have caused feedback inhibition to the enzyme system [2, 21].

The production of extracellular xylanase in the presence of some mono-, di-, and polysaccharides was also studied in order to determine whether the enzyme was synthesized constitutively or produced only as a response to certain carbon sources. The results revealed that the enzyme was produced inductively by xylan or xylose (Table 1). The results also showed that xylan exhibited the highest induction power than the other carbon sources tested. We have demonstrated also that xylose had less repressor effect at low concentrations, but this effect was more pronounced at higher concentrations due to catabolite repression (Fig. 4).

It has been found that glucose is a repressor in xylanase production. The phenomenon of "glucose effect" had also been reported by Nakamura *et al.* [19] for *Bacillus* sp. Likewise, Biely *et al.* [3] reported that the low basal synthesis of xylanase was repressed to a certain level when bacteria is grown in the presence of well-utilized carbohydrates such as glucose or xylose. On the other hand, the enzyme production was not repressed in the presence of high concentration of cellulose or CMC. Therefore, it can be assumed that increased levels of utilizable sugars would result in significant catabolite repression. Although xylan supplemented with xylose resulted in enhanced xylanase production, at longer incubation times, xylanase activity was significantly reduced. Hence, xylanase production in our study was induced directly by the amount of substrate present in the medium and is dependent upon incubation time. Therefore, the two factors that control xylanase synthesis are induction and catabolite repression. This has also been reported in the case of cellulase biosynthesis [1] in which the cellulase synthesis was inhibited by the presence of glucose and induced by cellulose, cellobiose, sophorose, and xylan [2].

Growth Curve

The *B. pumilus* culture was grown at 37°C in YTB containing 0.2% (w/v) oat spelt xylan since this medium gave the highest xylanase production in previous experiments (Table 1). Following inoculation with a fresh culture, there was a very short lag period and after only 2 h the growth began a log phase. The cells grew logarithmically and reached the maximum growth in about 8 h, and then entered stationary phase after 24 h (Fig. 5).

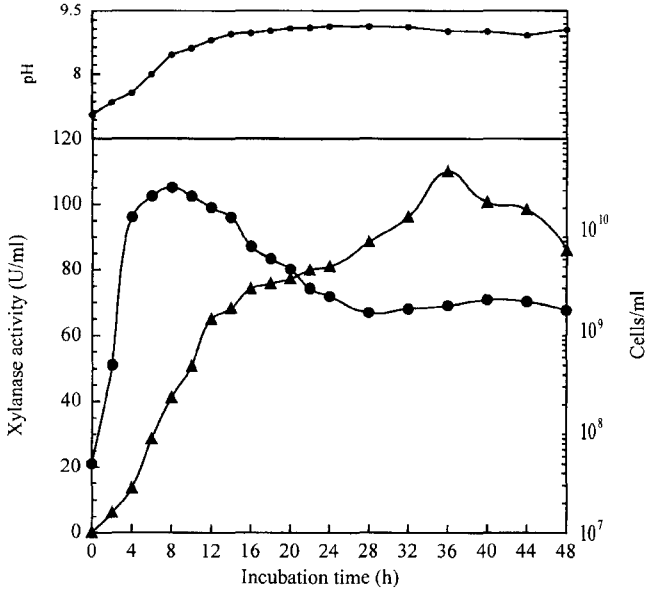

Fig. 5. Time course of xylanase production by *B. pumilus* PJ19. Xylanase activity (▲); Cells/ml (○); pH (●).

Enzyme production started as early as 2 h after incubation and it probably was not growth-associated since the enzyme was continuously secreted even after the growth had ceased. Maximum enzyme production was after 36 h of incubation. This phenomenon of non-growth association was also demonstrated in *Clostridium acetobutylicum* [17] and *B. stearothermophilus* [13]. The high xylanase production was maintained due to the induction effect from the xylan present in the medium even though the cellular growth was inhibited by some components in the medium [13]. Prolonged incubation may have reduced enzyme production because of the accumulation of toxic products in batch culture that inhibit the cells from growing. The pH of the culture was reduced from 7.2 to 6.5 at the beginning of incubation due to acid production during the lag phase. As the growth proceeded, the pH of the medium increased to a highly alkaline pH between 8.5 to 9.0 because of the accumulation of ammonium ions from the degradation of yeast extract by the bacteria. The pH was then stabilized after 20 h of incubation. The same pattern of pH variation during enzyme production was also demonstrated in the study made by Maheshwari & Kamalam [18] using *Melanocarpus albomyces*. The production of xylanase by bacteria has been associated with alkaline pH, whereas xylanase produced by fungus has always been related to acidic pH [1, 7, 12].

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