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Effect of pH, Temperature and Medium Composition on Xylanase Production by *Bacillus* sp. AQ-1 and Partial Characterization of the Crude Enzyme

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Bacillus sp. AQ-1 was isolated from household aquarium sediment. The isolate produced extracellular xylanolytic enzymes on xylan containing agar medium. Based on morphological, and physiological analysis, the isolate was identified as *Bacillus* sp. AQ1. The effect of temperature and pH on isolate growth and xylanase production were observed. The best condition observed for the enzyme production in Luria Broth supplemented with 0.5% oat spelt xylan medium was at 40 °C pH 7. The maximum enzyme production was 0.23 U mL^{-1} after 20 h of fermentation. Two different medium compositions (A and B) were examined for xylanase production. The maximum growth of the isolate and the xylanase production was better in A medium. Replacing oat spelt xylan in medium A with fruitless oil palm bunch in the medium caused the growth slightly slower than that of in the original formula. However, the xylanase production was 3 times higher in fruitless oil palm bunch medium. Optimum activity of the crude enzyme was observed at 60 °C and pH 7. Each ml of the crude enzyme contained 55.21 U xylanase, 8.12 U amylase and 0.50 U carboxymethylcellulase.

Key words: xylanase, *Bacillus* sp. AQ1, fruitless oil palm bunch

Xylan is a complex heteropolymer with a homopolymeric backbone chain of 1,4-linked β-D-xylopyranose units. The backbone consists of O-acetyl, α-L-arabinofuranosyl, α-1,2-linked glucuronic or 4-o-methylglucuronic substituents (Kulkarni *et al.* 1999). Several hydrolytic enzymes are needed to degrade the complex structure of xylan. Among these, endo 1,4-β-D-xylanase (EC 3.2.1.8), b-D-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55), α-D-glucuronidase and acetyl xylan esterase (EC3.1.1.6) are the most important enzymes (Rani and Nand 2001). Enzymatic hydrolysis of xylan produces xylooligosaccharides, xylobiose, and xylose (Kulkarni *et al.* 1999). Some *Bacillus* spp. have been reported as xylanolytic enzymes producers (Kulkarni and Rao 1996; Sunna *et al.* 1997a; Archana and Satyanarayana 1997; Samain *et al.* 1997; Dhillon *et al.* 2000a; and Bocchini *et al.* 2002). Another bacterial genus reported to produce xylanolytic enzymes was *Clostridium* spp. (Lee *et al.* 1987; Lee and Forsberg 1987; Sakka *et al.* 1999; Rani and Nand 2001).

Since the last decade xylanolytic enzymes have been studied concerning their properties and utilization in some commodities such as in pulp and paper, feed as well as, foods and beverage industries (Beg *et al.* 2001). In pulp and paper industry, xylanase was used as pretreatment prior to bleaching of pulp and paper to reduce application of chlorine (Garg *et al.* 1996; Kulkarni and Rao 1996; Chen *et al.* 1997; Zheng *et al.* 2000; Pala *et al.* 2006). In the food and beverage industry xylanolytic enzymes have been used for improving bread quality, clarification of fruit juices, wine and beer (Beg *et al.* 2001; Primo-Martin *et al.* 2005).

For industrial purposes, seeking a cheap, abundantly available substrate such as wheat straw, sugarcane baggase and rice straw for producing xylanase has been done by some researchers (Jain 1995; Dhillon *et al.* 2000a). The aims of this experiment were to study the effect of pH and temperature on isolate AQ-1 growth and xylanase production, to find a better medium composition formula and to attempt using fruitless oil palm brunch as a main carbon source for xylanase production.

MATERIALS AND METHODS

Microorganism and Growth Condition. Isolate AQ1 used in this experiment was isolated from fresh water aquarium sediment and cultivated in Luria Bertani (LB) medium. Morphological, physiological and biochemical properties of the isolate Q-1 were observed according to Case and Johnson (1984).

To assess the effect of temperature and pH on the cell growth and the enzyme production, the cultures were incubated at different temperatures and pHs in the above medium without the addition of agar. The temperature and pH observed were 30, 40, 50 °C and pH 7.0, 8.0 and 9.0, respectively. The growth was observed by counting the cell number in a Neubauer counting chamber. Bacterial starter was prepared in LB medium as follows: 1 loop bacterial culture taken from slant agar and inoculated into 25 mL of medium in 125 mL Erlenmeyer flask. The mixture was incubated in shaking incubator at 150 rpm for 6 h until the cell number reached about 10^9 cell mL^{-1}. A sum of 5 mL of inoculums was added into 45 mL of LB + xylan medium. Cell growth, xylanase activity and protein in cell free enzyme solution were observed every 4 h for 36 h incubation. Cell-free enzyme solution was obtained by centrifuging the fermented broth at 4 °C, 1100 xg for 15 min.

Effect of Medium Composition on Cell Growth and Xylanase Production. After obtaining optimum temperature

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and pH of xylanase production in LB medium, the isolate was cultivated in media suggested by Nakamura *et al.* (1994) and Dhillon *et al.* (2000b). Each 100 mL of Nakamura medium (medium A) contained 0.5 g polypeptone, 0.5 g yeast extract, 0.1 g K_2HPO_4, 0.02 g $MgSO_4 \cdot 7H_2O$ and 0.5 g oatspelt xylan. The pH was adjusted by adding 1% Na_2CO_3. Each 100 mL of Dhillon medium (medium B) contained 0.1 g KH_2PO_4, 0.1 g K_2HPO_4, 0.05 g $MgSO_4$, 0.1 g NH_4Cl, 0.3 g oat spelt xylan, 1 mL of stock vitamin solution (composition per 100 mL: biotin ± 2 mg, folic acid ± 2 mg, pyridoxine hydrochloride ± 10 mg, thiamine hydrochloride ± 5 mg, calcium D-pantothenate ± 5 mg, vitamin B12 ± 0.1 mg, *p*-aminobenzoic acid ± 5 mg, lipoic acid ± 5 mg) and 10 mL of trace elements (composition per 100 mL of stock solution: $C_6H_9NO_6$ (nitrilotriacetic acid) ± 1.5 g, $MnSO_4 \cdot 2H_2O$ ± 1.0 g, $FeSO_4 \cdot 7H_2O$ ± 0.2 g, $CoCl_2$ ± 0.2 g, $CaCl_2 \cdot 2H_2O$ ± 0.2 g, $ZnSO_4$ ± 0.2 g, $CuSO_4 \cdot 5H_2O$ ± 0.02 g, $Al(SO_4)_2$ ± 0.2 g, H_3BO_3 ± 0.02 g, $Na_2MO_4 \cdot 2H_2O$± 0.02 g, 2 mL nitrilotriacetic acid stock solution and 1 mL of the other stocks were mixed and the volume was made to 100 mL).

In the following step, the medium that showed the best bacterial growth was selected and modified. The oat spelt xylan was substituted with 0.35 g mL^{-1} of fruitless oil palm bunch (FPB). The FPB was prepared by chopping and screening through 35 mesh screen. The growth of bacterial cells and xylanase production was observed as previously done.

Assay of Xylanase Activity and Protein Content. The enzyme activity was measured according to Bernfeld (1955) with modification. As much as 150 µL cell-free enzyme solution was added into 150 µL of oatspelt xylan suspension (1 g in 100 mL 0.05M phosphate buffer (pH 7 and 8) and 0.05 M Tris-HCl buffer (pH 9)). The mixture was incubated for 15 min at the same temperature as that of the cultivation (30, 40 or 50 °C). The mixture was centrifuged at 4°C, 1100 xg for 5 min. The reaction was stopped by adding 200 µL 3,5-dinitro salicylic acid (DNS) and incubated at 100 °C for 5 min. The mixture was cooled and added with 2 mL distilled water. The absorbance was measured at l540 nm against blank reagent. One unit of xylanase was defined as the amount of enzyme that released 1 µmol reducing sugar equivalent to xylose per minute at assay condition. Protein content of the enzyme solution was measured according to a method suggested by Bradford (1976).

Effect of pH and Temperature on Xylanase Activity. Xylanase activity was measured at pH ranging from 4 to 10. Observation at pH 4, 5 and 6 were done using 0.05 M Citrate-phosphate buffer, pH 7 and 8 using 0.05 M phosphate buffer, pH 9 using 0.05 M Tris-HCl and pH 10 using 0.05 M glysine-NaOH. The enzymatic reaction was carried out at 50 °C for 15 min. The effect of temperature on xylanase activity was observed at temperature ranging from 30 to 90 °C with interval of 10 °C at optimum pH obtained from previous experiment.

Activity of Crude Enzyme on Various Carbohydrate Substrates. The presence of other carbohydrase was analyzed using soluble starch, carboxymethylcellulose, oatspelt and birchwood xylan. Amylase, celulase and xylanase activity were measured according to Bernfeld (1955), Miller (1959) and Bailey (1992), respectively. The enzymatic reactions were done at pH 7, 60 °C for 15 min.

RESULTS

Identification of Isolate AQ1. The isolate showed xylanolytic activity on LB medium containing 2 g agar and 0.5 g oatspelt xylan per 100 mL medium. Observation on morphological, physiological and biochemical properties of isolate Q-1 are shown in Table 1. The data showed that the isolate was a rod shaped, aerobic, Gram positive, endospore-forming microorganism. The endospores were oval-shaped and located in the center of the cells. The cell diameter was less than 0.1 µm. According to Bergey's manual, these characteristics indicate that the isolate belongs to *Bacillus* genus (Claus and Berkely 1986).

Effect of Temperature and pH on Bacterial Growth and Xylanase Production. Fig 1 shows bacterial growths at 30, 40 and 50 °C at various medium pH (7, 8 and 9). Fig 2 shows xylanase and protein content in the fermented broth at temperature and pH observed as mentioned above.

Using LB medium supplemented with oatspelt xylan as xylanase inducer, the maximum cell count was observed in the culture incubated at 50 °C, pH 7 for 12 h with cell density of 1.78 x 10^9 (Fig 1c), however, the optimum condition for xylanase production was observed at 40 °C and pH 7 for 24 h fermentation with enzyme activity of 0.24 U mL^{-1} and 0.26 mg mL^{-1} protein (Fig 2b).

Effect of Medium Composition on Cell Growth and Xylanase Production. Cell growth in Nakamura medium was about the same as that of in Dhillon medium (Fig 3). The optimum growth was reached after 12 h with cell density of 4.79 x 10^8cfu mL^{-1} and 6.31 x 10^8 cfu mL^{-1} in Dhillon and Nakamura medium, respectively. In Nakamura medium with substitution of carbon source from oatspelt xylan to fruitless oil palm bunch (FPB), the cell growth was slower compared to in original Nakamura medium, where the number of cells reached at 7.94 x 10^8 cfu mL^{-1} after 18 h fermentation.

Protein content in the fermented broth using Dhillon medium was higher (0.48 mg mL^{-1}) and reached faster than that of in the other medium formulae observed (Fig 4). The

Table 1 Morphological, physiological and biochemical properties of isolate AQ1

Test	AQ1
Xylanolytic index	0.18
Colony color	white
Colony shape	spread
Elevation	flat
Gram staining	+
Endospore	central
Growth at 50 °C	+
Growth at 7% NaCl containing medium	+
Growth in anaerobic agar	+
Cell shape	rod
Hydrolysis of indole	-
Methyl Red	-
Voges-Proskauer	+
Citrate	+
Catalase	+
Nitrate reduction	+
Carbohydrate fermentation	+
D-glucose	+
Acid formation	+
Gas formation	-
D-xylose	+
L-arabinose	+
D-mannitol	+

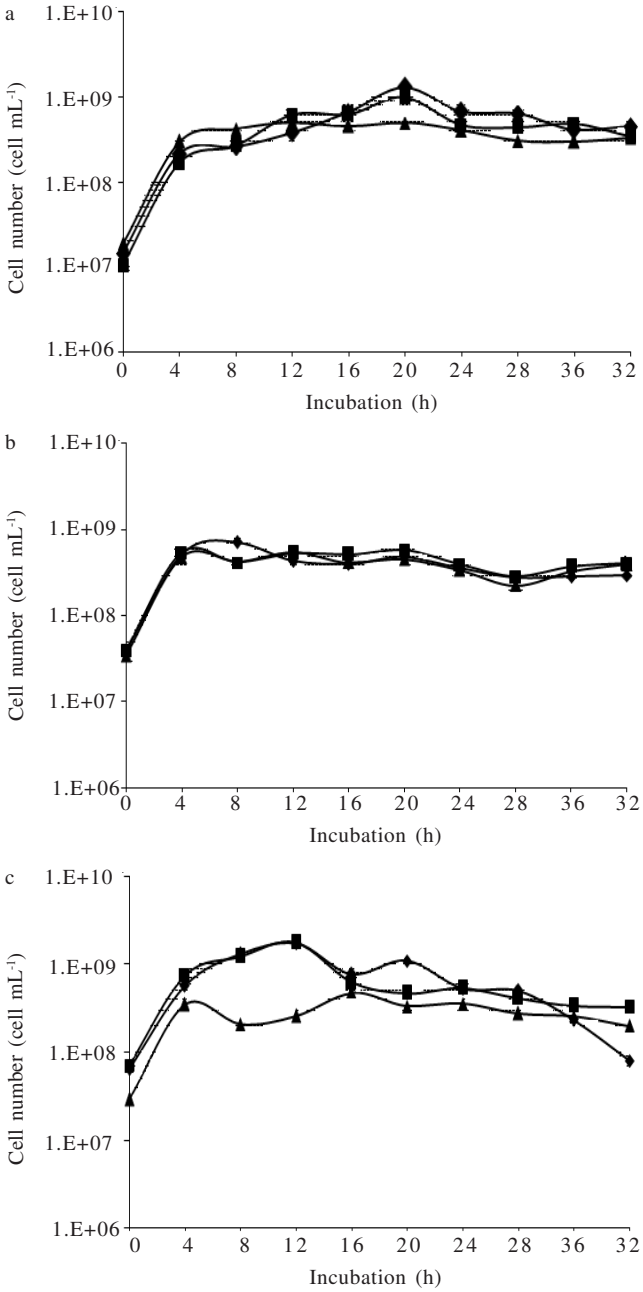

Fig 1 Effect of pH and temperature on isolate AQ1 growth. a, 30 °C; b, 40 °C; c, 50 °C; ◆ pH 7, ▲ pH 8, ■ pH 9.

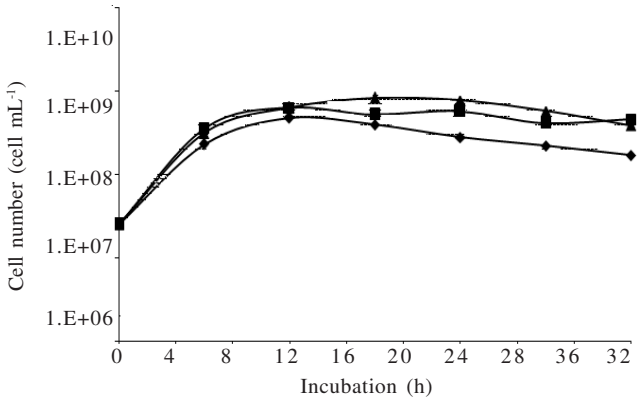

Fig 3 Effect of medium composition on isolate AQ1 growth at 40 °C, pH 7. ◆, oat spelt xylan (Dhillon); ▲, oat spelt xylan (Nakamura), ■, FPB (Nakamura).

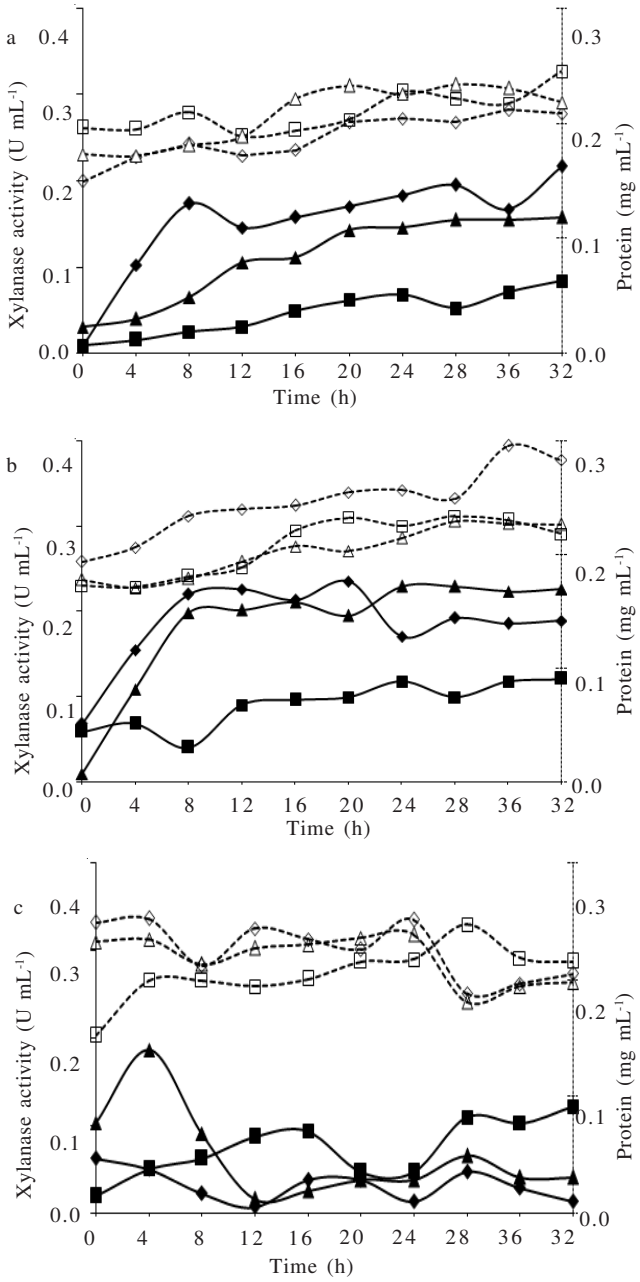

Fig 2 Effect of pH and temperature on xylanase production (◆, ▲, ■) and protein content (◇, △, □). a, 30 °C; b, 40 °C; c, 50 °C. ◆, pH 7; ▲, pH 8; ■, pH 9; ◇, pH 7; △, pH 8; □, pH 9.

maximum protein content in this medium was observed after 18 h fermentation but it did not increase significantly throughout 36 h observation, whereas the protein content in Nakamura medium after 18 h fermentation was only 0.23 mg mL^{-1} and did not increase significantly either during 36 h of fermentation. Protein in fermented broth in Nakamura medium with FPB substitution was not different with that of in Nakamura original formulae which was 0.24 mg mL^{-1} after 18 h fermentation.

Xylanase production in Dhillon medium was slightly higher compared to in Nakamura medium, which was 0.07 U mL^{-1} after 18 h and 0.09 U mL^{-1} after 30 h, respectively. However, the substitution of xylan source with FPB in Nakamura medium, xylanase production was much faster and

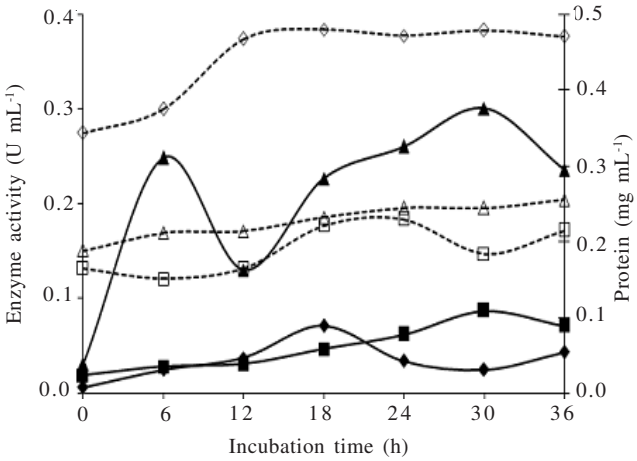

Fig 4 Effect of medium composition on xylanase production (◆, ■, ▲)and protein content (◇, □, △)at 40°C and pH 7. ◆, oatspelt xylan (Dhillon); ■, oatspelt xylan (Nakamura); ▲, FPB (Nakamura); ◇, oatspelt xylan (Dhillon); □, oatspelt xylan (Nakamura); △, FPB (Nakamura).

higher than that of in original Nakamura and Dhillon formula. The enzyme production was about three times higher compared to in original Nakamura medium which 0.30 U mL^{-1} was after 30 h.

Effect of pH and Temperature on Crude Xylanase Activity. The pH of reaction mixture significantly affected xylanase activity as shown in Fig 5, where the highest enzyme activity was observed at pH 7. The temperature higher than 60 °C decreased the enzyme activity significantly (Fig 6). The optimum activity was observed at 60 °C.

Activity of the Crude Enzyme on Various Carbohydrate Substrates. Activity of the crude enzyme on some carbohydrate was showed at Fig 7. The crude enzyme mainly contained xylanase as indicated by the highest activity was on oat spelt xylan (55.21 U mL^{-1}) and birchwood xylan (43.03 U mL^{-1}). The crude enzyme also contained amylase (8.12 U mL^{-1}) but hardly cellulase (0.50 U mL^{-1}).

DISCUSSION

Based on morphological, physiological and biochemical analysis, isolate AQ-1 was identified as *Bacillus* sp. *Bacillus* spp. have been reported as xylanolytic enzymes

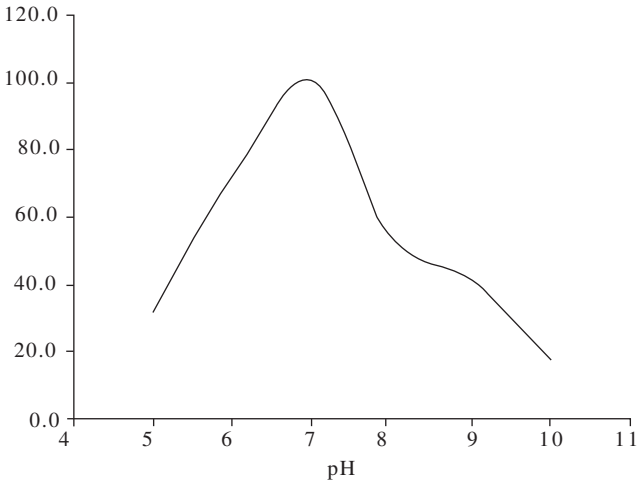

Fig 5 Effect of pH on xylanase activity at 50 ºC

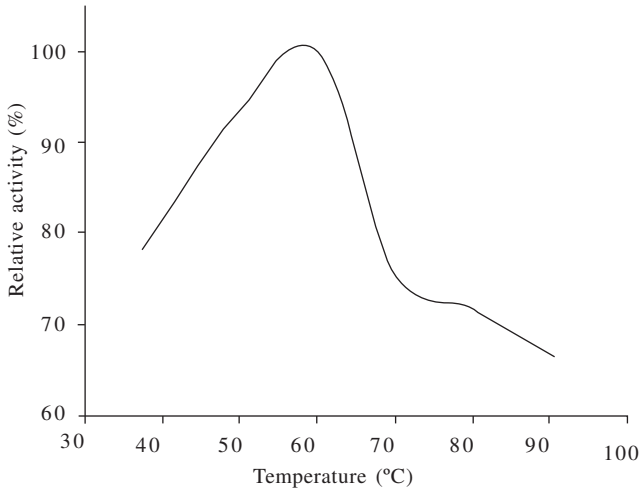

Fig 6 Effect oftemperature on xylanase activity at pH 7.0.

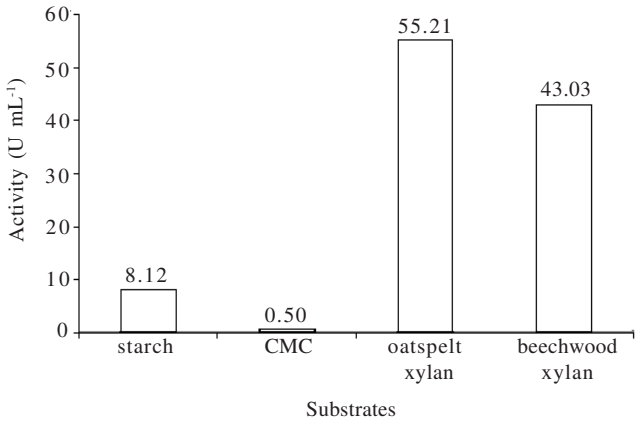

Fig 7 Activity of isolate AQ1 crude enzyme on various carbohydrate substrates at pH 7, 60 °C.

producers, such as *Bacillus* sp. NCIM 59 (Kulkarni and Rao 1996), *B. thermoleovorans* strain K-3d and *B. flavothermus* strain LB3A (Sunna *et al.* 1997a), *B. licheniformis* (Archana and Satyanarayana 1997), *Bacillus* sp XE (Samain *et al.* 1997) *B. circulans* AB16 (Dhillon *et al.* 2000b), *B. circulans* D1 (Bocchini *et al.* 2002), *B. subtilis* B230 (Oakley *et al.* 2003). Growth and enzyme production condition of most *B. subtilis* was reported at pH 6-8 and temperature of 37-70 °C. *Bacillus* sp. AQ1 could grow and produce enzyme at the pH (7-9) and temperature (30-50 °C) observed. The data showed that even though the fastest cell growth was at 50 °C, pH 7 and 8, the growth also declined faster than that of at 30 and 40 °C. The enzyme and protein content were better at pH 7, temperature of 30 and 40 °C than that at other pH and temperature observed, therefore it can be suggested that the enzyme production was better at pH 7 and temperature close to 40 °C. Previous studies reported that xylanase production by *B. subtilis* B230 was best at pH 8, 37 °C (Oakley *et al.* 2003) and *B. subtilis* 168 at pH 7, 37 °C (St John *et al.* 2006). The pH and temperature conditions were similar to that condition of enzyme production by AQ1 strain that was concluded as *B. subtilis* as well (unpublished).

Most xylanases were reported as inducible enzymes. Some studies showed the used of different kinds of commercial extracted xylan as well as xylan containing agricultural wastes for inducing xylanolytic enzyme production. Sunna *et al.*

(1997a) used beechwood xylan for inducing xylanase production by *B. thermoleovorans* K-3d and *B. flavothermus* LB3A. Sunna *et al.* (1997b) also reported that beechwood xylan was used to induce xylanolytic enzyme production by *Thermotoga maritima, T. neapolitana* and *T. thermarum*. Rani and Nand (2000; 2001) studied substrate specificity of *Clostridium absonum* CFR-702 using different kinds of xylans such as birchwood, larchwood, oatspelt xylan. Kulkarni and Rao (1996) used wheat bran as enzyme inducer for producing xylanolytic enzyme from *Bacillus* sp. NCIM 59 whereas Dhillon *et al.* (2000a) used rice bran and straw, wheat and maize bran, as well as sugarcane baggase to induce xylanase production by *B. circulans* AB16. Dhillon *et al.* (2000b) also used different kinds of substrates in another experiment including sugarcane bagasse, rice straw and wheat straw, as the carbon source in basal medium to produce xylanolytic enzymes by *B. circulans* AB 16. The experiment also reported that the present of simple sugar lowered xylanolytic enzyme production by *B. circulans* AB16. These studies showed that various agricultural wastes could be used as substrates for producing xylanolytic enzymes by various microorganisms. These studies also reported that an inducer for certain microorganism could be an inhibitor for others (Biely 1985; Paul and Varma 1990; Nakamura *et al.* 1995; Oakley *et al.* 2003). As a consequence, it is important to choose a proper inducer for certain microorganism.

In this experiment, cell growth and enzyme production in the medium containing oatspelt xylan and FPB as carbon source were observed. The results showed cell growth in medium composition according to Dhillon *et al.* (2000b) was slightly better than that of in medium of Nakamura *et al.* (1994), however, xylanolytic production was better in Nakamura medium than that of in Dhillon medium. Since in Dhillon medium xylanase production did not tend to increase, even decreased during fermentation time observed, therefore substitution of oatspelt xylan with FPB was done using Nakamura medium. In Nakamura FPB containing medium, xylanolytic enzyme production was better than that of using the original Nakamura medium composition. According to Irawadi (1991), FPB contained minerals as followed: 2.13% K, 0.18% Ca, 0.17% Mg, 0.05% Mn, 0.63% Na, 0.59% Fe, 0.14% P_2O_5. The experimental results showed that enzymes production by AQ-1 using FPB was three times higher than that of using oatspelt xylan therefore FPB might be advised to be used as a substrate for xylanolytic enzymes production.

Crude enzymes produced by *Bacillus sp* AQ1 using FPB, not only showed xylanolytic activity but also amylolytic and cellulolytic activity as well (Fig 7). The presence of cellulolytic enzyme in crude xylanase is common, especially fungal xylanase (Kulkarni *et al.* 1999). The isolate AQ1 only produced very little cellulolytic enzyme (0.5 U mL^{-1}) compared to that xylanolytic activity on oatspelt (55.21 U mL^{-1}) and on birchwood (43.03 U mL^{-1}). Based on the available data from this experiment, the difference in crude enzyme on the different xylan substrate could not be explained yet. It is still needed more complete studies to elaborate the type of xylanolytic activities present in the crude enzyme.

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