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Xylanase Production by *Bacillus* sp. A-6 Isolated from Rice Bran

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Abstract A *Bacillus* sp. A-6 strain that produced xylanase was isolated from rice bran. The optimal temperature and pH for xylanase activity of the culture supernatant of *Bacillus* sp. A-6 were 40°C and pH 7, respectively. The optimal temperature and pH for xylanase production in the xylan medium were 30°C and pH 9, respectively. The optimal concentrations of oat spelt xylan and peptone for xylanase production were 0.5% and 1.5%, respectively. The best nitrogen sources for xylanase production was beef extract, but xylanase production was also supported comparably by tryptone and peptone. The bacterial growth in the optimal xylan medium reached stationary growth phase after 12 h of incubation. The xylanase production in the culture supernatant increased dramatically during the initial 12 h exponential growth phase and then remained constant at 23.8–24.5 unit/ml during the stationary growth phase. The pH of the culture medium decreased from 8.8 to 6.7 during the exponential growth phase and subsequently increased to 8.1 during the stationary growth phase. Rice bran, sorghum bran, and wheat bran as well as oat spelt xylan induced xylanase production. The xylanase production was repressed when glucose was added to the xylan-containing medium.

Key words: Xylanase, *Bacillus* sp., rice bran, xylan

Xylan, the main component of hemicellulose in the cell wall of plant, is widely distributed along with cellulose in the plant kingdom and is a polysaccharide that consists of xylose linked with β-1,4 glucosidic bonds, which form the main backbone structure. The xylose residues were linked with substituent sugars, such as D-glucose, L-arabinose, and D-glucuronic acid [3]. Thus, xylanase(endo-1,4-xylanase), β-xylosidase, α-glucuronidase, and α-L-arabinofuranosidase, and acetyl xylan esterase should act cooperatively to hydrolyze xylan completely. Xylanase takes the most important role among them in degradation of xylan by disintegrating the main backbone structure of xylan into xylose and/or xylooligosaccharides [7, 32].

Xylanase is widely utilized for pulp biobleaching, fruit beverage clarification, bakery production, and livestock feed supplementation [7, 21]. Microbial xylanases have been applied particularly to feed supplementation in the livestock industry [12, 31]. The grain by-products frequently used as raw materials for livestock feed contain non-starch polysaccharides (NSPs), such as xylan and β-glucan, which are indigestible for monogastric animals including pigs and chickens and young ruminant animals with immature digestive organs. The NSPs absorb water and form adhesive mucous materials in the intestine to prevent digestive enzymes from accessing nutrients and to slow down the proceeding of digesta in the intestine, which causes decrease in feed uptake and intestinal disorder. In order to ameliorate the digestion problem, livestock feed can be supplemented with the NSP-digesting enzymes, such as xylanase and β-glucanase, so the viscosity of digesta in the intestine is lowered and nutrient utilization efficiency is improved [1]. Furthermore, low manure output and decreased nitrogen excretion may reduce environmental pollution [15].

Isolation of xylanase-producing microorganisms and biochemical characterization of xylanase have been extensively carried out [7, 9, 21, 29]. Xylanases are produced by fungi, bacteria, yeast, marine algae, protozoans, *etc.*, but the principal commercial source is filamentous fungi [26]. Driven by industrial demands for enzymes that can operate under process conditions, a number of extremophilic xylanases have been isolated, in particular those from thermophiles, alkaliphiles, and acidophiles, whereas little attention has been paid to cold-adapted xylanases [11].

Alkaliphilic and thermophilic xylanase-producing *Bacillus* have been studied extensively for application in detergents and pulp biobleaching [11, 23]. Mesotrophic *Bacillus* spores are available commercially as probiotics for human use as dietary supplements and animal feed to prevent gastrointestinal infection [16]. The mechanisms for which *Bacillus* probiotics can inhibit the infection include

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immunomodulation and synthesis of antimicrobials. *Bacillus* may also contribute to the digestive function of animals by producing xylanase after germination in the intestine, and to prevention of environmental pollution by enhancing degradation of livestock manure.

Mesotrophic xylanase-producing *Bacillus* strains were isolated from rice bran, sorghum bran, soil, and commercial feed supplement in this study. The production of xylanase by *Bacillus* strain A-6 isolated from rice bran was characterized.

MATERIALS AND METHODS

Isolation of *Bacillus* Strains Producing Xylanase

Rice bran, sorghum bran, soil, and commercial feed supplement were suspended in 0.5% peptone water and diluted appropriately to spread on standard count agar, which was then incubated at 30°C for 48 h. The colonies were randomly selected and transferred to a xylan agar [23], which consisted of oat spelt xylan 1 g (Sigma-Aldrich Co.), peptone 1 g, Tween 80 1 g, $(NH_4)_2SO_4$ 1.4 g, KH_sPO_4 2 g, urea 0.3 g, $CaCl_2$ 0.3 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $FeSO_4 \cdot 7H_2O$ 5.0 mg, $MnSO_4 \cdot H_2O$ 1.6 mg, $ZnSO_4 \cdot 7H_2O$ 1.4 mg, $CoCl_2$ 2.0 mg, and agar 15 g in 1 l distilled water, which was adjusted to pH 7.0. After incubation at 30°C for 24 h, the agar was flooded with 0.2% Congo red and washed with 1 M NaCl. The clear zone surrounding a colony indicated xylanase production.

Identification of *Bacillus* Strain

The selected strains that were grown on tryptic soy agar were observed microscopically after Gram staining and spore staining [5]. Scanning electron microscopic observation was provided by the Korean Basic Science Institute. Biochemical characteristics were examined using the API 20E and API 50CHB kit (bioMerieux Vitek, Inc).

Determination of Xylanase Activity

Xylanase activity in the culture supernatant was assayed using oat spelt xylan as substrate. The reducing sugar released from the xylan was determined by the 3,5-dinitrosalicylic acid method [23]. The reaction solution consisted of 0.5% oat spelt xylan in 50 mM sodium phosphate, pH 7.0. The culture supernatant, obtained after centrifuging at 10,000 ×*g* for 5 min and diluting 20 times with the phosphate buffer, was added to the reaction solution as enzyme solution. The reaction solution was then incubated at 40°C for 30 min and then 3,5-dinitrosalicylic acid reagent was added to the reaction solution, which was then heated at 100°C for 15 min. Absorbance at 550 nm was measured. D-Xylose (0–10 mM) was used as the standard solution. One unit of xylanase acitivy was defined as the amount of the enzyme to release reducing sugar equivalent to 1 μmol D-xylose per min.

Optimization of Culture Condition for Xylanase Production

The composition of the xylan medium (1 l) used initially to determine the optimal culture condition was oat spelt xylan 10 g, peptone 1 g, Tween 80 1 g, $(NH_4)_2SO_4$ 1.4 g, KH_2PO_4 2 g, urea 0.3 g, $CaCl_2$ 0.3 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $FeSO_4 \cdot 7H_2O$ 5.0 mg, $MnSO_4 \cdot H_2O$ 1.6 mg, $ZnSO_4 \cdot 7H_2O$ 1.4 mg, and $CoCl_2$ 2.0 mg at pH 7.0. The inoculum (1%) obtained after culturing sequentially twice in the xylan medium at 30°C for 24 h was transferred into the experimental culture. The culture was incubated at 30°C for 24 h in a shaking incubator at 100 rpm. The initial pH of culture, culturing temperature, carbon sources, and nitrogen sources were evaluated to optimize the culture condition for xylanase production.

RESULTS AND DISCUSSION

The bacterial isolates from rice bran, sorghum bran, soil, and commercial feed supplement were screened to select mesotrophic bacteria that produced xylanase after incubating on xylan agar at 30°C for 48 h and thus showed a clear zone around the colony on the xylan agar after staining with Congo red. Nine isolates of xylanase-producing bacteria were obtained from rice bran, sorghum bran, and commercial feed supplement. The two isolates from rice bran and commercial feed supplement showed stronger xylanase activities than other isolates. They were Gram-positive rods and formed spores. The biochemical characteristics of a *Bacillus* strain designated A-6 isolated from rice bran were further characterized by using API 20E and API 50 CHB. The biochemical reactions of the A-6 strain were positive in β-galactosidase, catalase, Voges Proskauer, gelatinase, and sugar fermentation of L-arabionose, D-ribose, D-xylose, methyl-β-D-xylopyranoside, D-galactose, D-glucose, D-

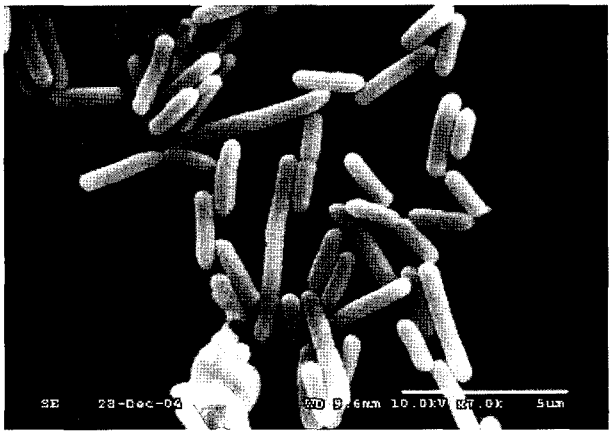

Fig. 1. Scanning electron microscopic photograph of *Bacillus* A-6. The bar at low left indicates 5 μm.

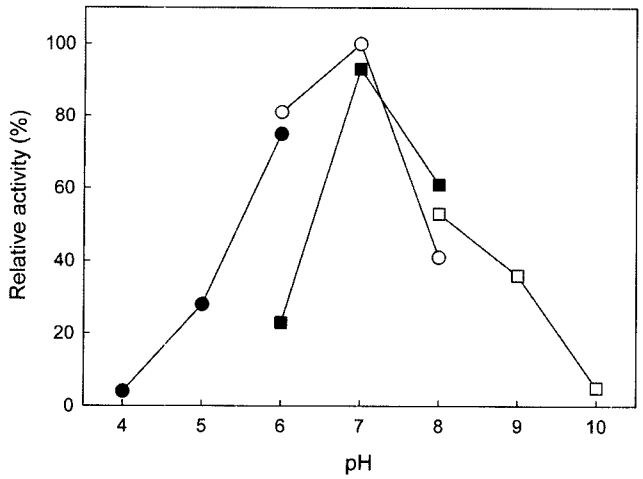

Fig. 2. Effects of pH on the xylanase activity of the culture supernatant of *Bacillus* A-6.
The enzyme reaction was carried out at 40°C for 30 min in 0.05 M acetate buffer (●; pH 4.0–6.0), 0.05 M phosphate buffer (○; pH 6.0–8.0), 0.05 M MOPS buffer (■; pH 6.0–8.0) and 0.05 M Tris-HCl buffer (□; pH 8.0–10.0).

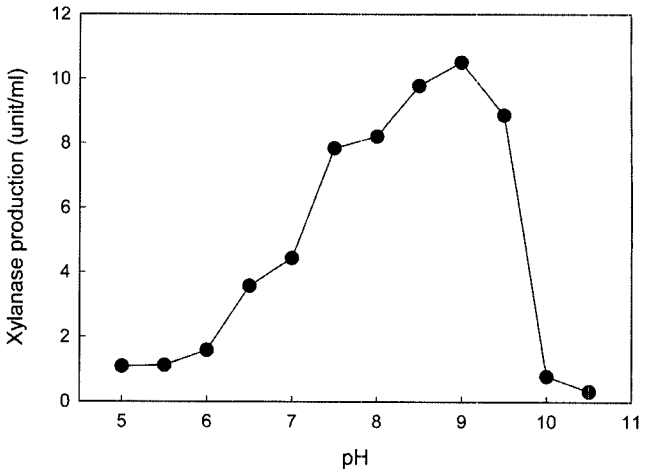

Fig. 4. Effects of initial pH of the xylan medium on the xylanase production by *Bacillus* A-6.

fructose, D-mannose, inositol, D-mannitol, amygdalin, arbutin, esculin, salicin, cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-melezitose, D-raffinose, amidon, glycogen, gentiobiose, and D-turanose. The rest of the reactions in the API 20E and API 50CHB were negative. The scanning electron microscopic photograph (Fig. 1) showed that the cells were 0.6 μm in width and 1.7–3.0 μm in length. The spores were ellipsoidal at the center or terminal of the vegetative cell. The A-6 strain was presumptively identified as *Bacillus circulans*.

The effects of pH and temperature on the xylanase activity of the culture supernatant of *Bacillus* sp. A-6 are shown in Fig. 2 and Fig. 3, respectively. Acetate buffer (0.05 M) at pH 4, pH 5, and pH 6, 0.05 M phosphate buffer and 0.05 MOPS buffer at pH 6, pH 7, and pH 8, and 0.05 M Tris-HCl buffer at pH 8, pH 9, and pH 10 were used, as shown in Fig. 2. The xylanase was active in the range from pH 6 and pH 8 and the optimal pH was 7. The xylanase activities at pHs below 6 and above 9 were below 30% of its maximal activity at pH 7. The optimal temperature for xylanase activity was 50°C. The relative activity of xylanase at 20°C was 27%, as shown in Fig. 3.

The optimal pH for xylanase production in the xylan medium at 30°C was pH 9, as shown in Fig. 4. The xylanase production at pH 7, pH 8, and pH 9 was 4.1, 9.0, and 10.3 unit/ml culture, respectively and decreased sharply above pH 9.5. The optimal temperature for xylanase production at pH 9 was 30°C as shown in Fig. 5. However, xylanase production at 20°C was 6.8 unit/ml, which was

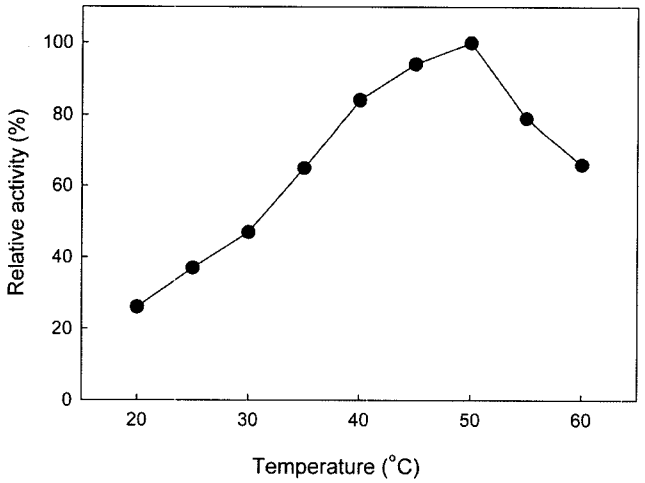

Fig. 3. Effects of incubation temperature on the xylanase activity of the culture supernatant of *Bacillus* A-6.

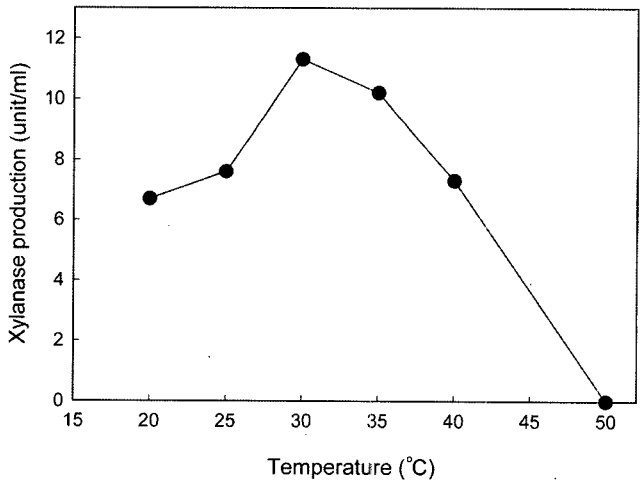

Fig. 5. Effects of culture temperature on the xylanase production by *Bacillus* A-6.

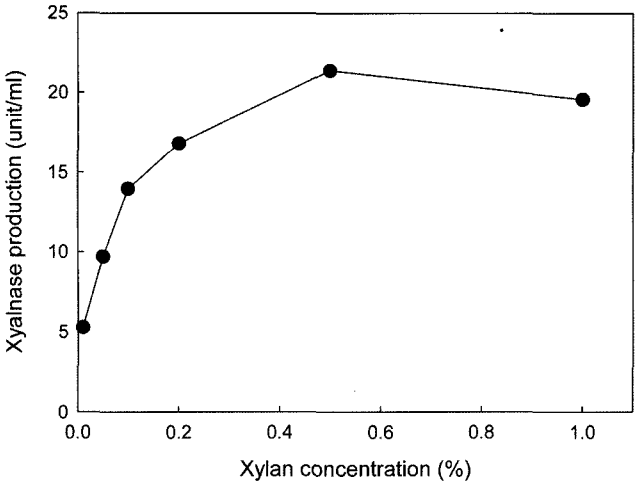

Fig. 6. Effects of xylan concentration on the xylanase production by *Bacillus* A-6 in the xylans medium containing 1.5% peptone.

still significantly high compared with 11.5 unit/ml at 30°C. However, xylanase production at 50°C was very low. These results suggested that *Bacillus* sp. A-6 should produce xylanase at ambient temperature and alkaline pH.

As the concentration of oat spelt xylan increased in the xylan medium containing 1.5% peptone at pH 9, the xylanase production increased up to 21.4 unit/ml at 0.5% oat spelt xylan, as shown in Fig. 6. The xylanase production by *Bacillus* sp. A-6 was inducible by oat spelt xylan. The induction was linearly dependent on xylan concentrations at low range. It has been reported that xylanases produced by various bacteria and fungi are usually inducible enzymes in the media containing pure xylan or xylan-rich substances [4, 21]. However, constitutive production of xylanase has also been reported [18].

The xylanase production was maximal at 1.5% peptone, as shown in Fig. 7. When the effects of various nitrogen

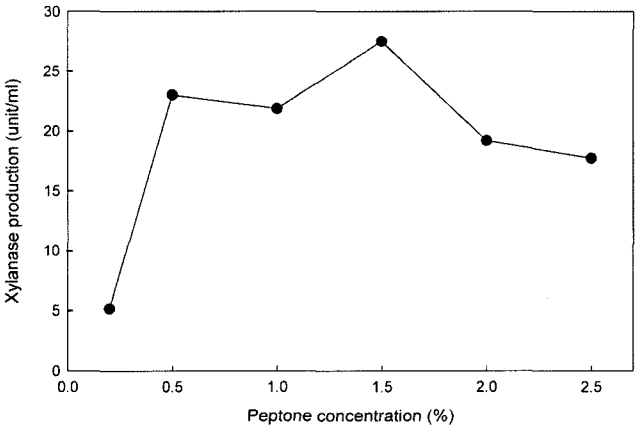

Fig. 7. Effects of peptone concentration on the xylanase production by *Bacillus* A-6 in the xylan medium containing 0.5% oat spelt xylan.

Table 1. Effects of nitrogen sources on the xylanase production by *Bacillus* A-6*.

Nitrogen sources	Xylanase production (unit/ml)	Relative production (%)
Beef extract	36.9±2.4	100.0
Tryptone	30.1±2.0	81.5
Peptone	23.1±1.4	62.5
Yeast extract	16.2±1.5	43.9
Soytone	5.1±1.4	13.8
Casamino acid	2.6±1.3	7.1

*Basal carbon source: 0.5%(w/v) oat spelt xylan.

sources on xylanase production were evaluated, beef extract was shown to be the best nitrogen source (Table 1). Tryptone and peptone were comparably good nitrogen sources, but the relative xylan productivities of soytone and casamino acid were 13.8% and 7.1%, respectively.

From these results, it was concluded that the optimal composition of the xylan medium (1 l) for xylanase production was oat spelt xylan 5.0 g, tryptone 10.0 g, beef extract 5.0 g, tween 80 1.0 g, $(NH_4)_2SO_4$ 1.0 g, KH_5PO_4 1.4 g, urea 0.3 g, $CaCl_2$ 0.3 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, $FeSO_4 \cdot 7H_2O$ 5.0 mg, $MnSO_4 \cdot H_2O$ 1.6 mg, $ZnSO_4 \cdot 7H_2O$ 1.4 mg, and $CoCl_2$ 2.0 mg, at pH 9.

The changes of xylanase production, cell number, and pH by *Bacillus* sp. A-6 in the optimal xylan medium at 30°C for 48 h are shown in Fig. 8. The cells seemed to reach the exponential growth phase just after inoculation. The cell growth slowed down in the late exponential growth phase after 6 h and then reached the stationary growth phase after 12 h. The xylanase production increased slowly during initial 6 h and then sharply in late exponential growth phase after 6 h until 12 h, and remained constant at 23.8–24.5 unit/ml during the stationary growth phase. The xylanase production did not look exactly proportional to cell growth during the exponential growth phase. There was

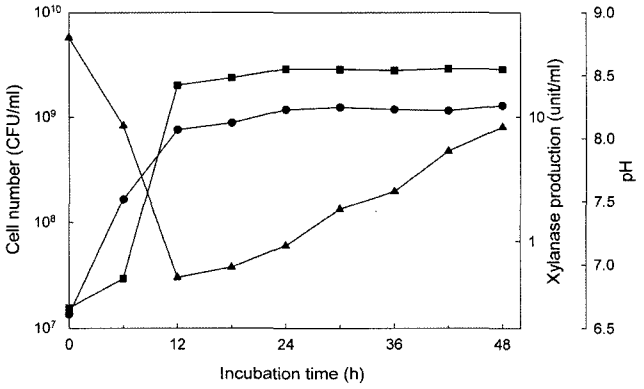

Fig. 8. Time course of cell growth, xylanase production, and pH change in the optimal xylan medium culture of *Bacillus* A-6.
●, Cell number; ■, xylanase production; ▲, pH.

not significant increase in the xylanase production during the stationary growth phase.

The pH of the culture decreased from 8.8 to 6.9 in the exponential growth phase and then increased gradually to 8.05 in the stationary growth phase. It seemed that the organic acids produced from xylose released from xylan acidified the culture medium during the exponential growth phase, and then the ammonia produced from tryptone and beef extract alkalinized the culture medium during the stationary growth phase. Acidification of the culture medium to neutral pH during the exponential growth phase presumably slowed down the bacterial growth, which led to the stationary growth phase. It was suggested that *Bacillus* sp. A-6 should be a moderately alkaliphilic bacteria.

Many alkaliphilic or alkalo-tolerant *Bacillus* that produced xylanase have been isolated [10, 13, 25, 27]. However, the optimal pHs for activities of most purified xylanases were near neutral pH [10, 13, 24]. One of the xylanases from alkaliphilic *Bacillus* sp. strain 41M-1 was most active at pH 9.0 [25].

The effects of various carbon sources including oat spelt xylan on xylanase production are compared in Table 2. Oat spelt xylan was the best carbon source of all to induce xylanase production. Rice bran, sorghum bran, and wheat bran also induced xylanase production significantly. The polysaccharides, such as starch and cellulose, the disaccharides, such as maltose, cellobiose, and lactose, and the monosaccharides, glucose, xylose, and arabinose, did not induce xylanase production significantly. These results suggested that the xylanase production by *Bacillus* sp. A-6 should be inducible by the xylan in rice bran, sorghum bran, and wheat bran, which provided inducers for xylanase synthesis.

Bernier *et al.* [8] reported that xylanase production by *Bacillus subtilis* in the medium containing xylan and cellobiose as nitrogen sources and urea as a nitrogen source was 0.85 unit/ml, which is lower than those shown in Table 1. Sá-Pereira *et al.* [28] reported that xylanase production by *Bacillus subtilis* in DXM medium containing soybean as a nitrogen source was 1.2–1.5 unit/ml. However, the xylanase production by *Bacillus* sp. strain 41M-1 in the medium containing polypeptone and yeast extract as nitrogen sources was 19.4 unit/ml [25], which was comparable to those of peptone and yeast extract (Table 1). *Bacillus* strains seemed to produce different amounts of xylanase, which were also affected by nitrogen sources containing amino acids, peptides, nucleic acids, and vitamins.

Table 2. Effects of carbon sources on the xylanase production by *Bacillus* A-6.

Carbon sources	Xylanase production (unit/ml)	Relative production (%)
Oat spelt xylan	21.4±0.7	100.0
Rice bran 1	12.7±1.3	59.3
Wheat hull	9.8±0.9	46.7
Sorghum bran	7.7±0.8	36.0
Carboxymethylcellulose	7.7±0.7	35.7
Rice bran 2	7.3±0.6	33.9
Maltose	5.1±0.6	23.6
Glucose	4.4±0.7	20.5
Soluble starch	4.1±0.7	19.0
Xylose	4.0±0.6	18.7
Lactose	2.8±0.8	12.9
Cellulose	2.0±0.7	9.3
Arabinose	1.9±0.7	8.7
Cellobiose	1.6±0.6	7.6

Table 3. Effects of glucose concentration added to the xylan medium on the xylanase production by *Bacillus* A-6.

Glucose concentarion (%)	Xylanase production (unit/ml)	Relative production (%)
0.0	21.8	100.0
0.1	11.5	52.8
0.2	0.72	3.3
0.3	1.77	8.1
0.4	1.95	8.9
0.5	2.85	13.1
0.6	1.05	4.8
0.7	0.53	2.4
0.8	0.36	1.7
0.9	0.35	1.6
1.0	0.34	1.5

The effects of glucose addition to the optimal xylan medium on xylanase production are shown in Table 3. As the concentration of glucose increased, the xylanase production generally decreased. The relative xylanase productivity at 1.0% glucose was 1.5%. This result indicated that xylanase production by *Bacillus* sp. A-6 was repressed by glucose.

Esteben *et al.* [14] reported that xylanase was undetected in glucose-grown cultures of *B. circulans* WL-12, but xylose, mannose, and cellobiose supported xylanase production. Bataillon *et al.* [6] reported that glucose was a suppressor of xylanase synthesis. Xylanase synthesis by *Bacillus* sp. A-6 seemed to be induced only by xylooligosaccharides released from xylan and grain by-products by residual xylanase in the culture medium. The relatively low synthesis of xylanase in the presence of grain by-products may be due to its low concentration of xylan and presence of other polysaccharides, such as starch. Solid state fermentation using a high concentration of grain by-products should be studied for production of xylanase and cell mass in future [2, 20].

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