

Identification of Endoglucanase-producing/xylanase-Producing Strains Cultivated in Beancurd (soybean) Wastewater Medium

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To screen and identify both endoglucanase- and xylanase-producing microbes cultivated in bean curd wastewater as the sole medium for potential use in the feed industry, using traditional isolation methods such as hole observation, hydrolysis spot diameter measurement of CMC-Na and Xylan from soils samples collected from farmland. three strains, EM5, SB13, and BL14, showed maximum endoglucanase and xylanase activity. Morphological, physiological characteristics and 16S rDNA gene sequence analyses identified the isolated bacteria as *Bacillus megaterium* EM5, *Bacillus subtilis* SB13, and *Bacillus licheniformis* BL14. the endoglucanase activity of EM5, SB13 and BL14 cultured in bean curd wastewater medium, reached 20.35 ± 2.28 , 102.39 ± 8.76 and 64.73 ± 7.45 (U/ml), showing increases of 8.43, 1.37, and 2.29 times, respectively, compared with cultivation in basic medium for three days, and the endoglucanase activity was maintained for 10 days when cultured in beancurd wastewater medium. In addition, xylanase activity in bacteria cultured in beancurd wastewater medium, respectively reached 577.03 ± 21.28 , 611.98 ± 18.12 , 487.13 ± 11.06 (U/ml), showing increases of 0.37, 0.75, and 0.14 times, respectively, compared with bacteria cultured in basic medium for three days. These studies showed that beancurd wastewater is an adequate medium for the growth of strains EM5, SB13 and BL14 to produce both endoglucanase and xylanase.

Key words: Beancurd; Wastewater; Endoglucanase; Xylanase; *Bacillus subtilis*; *Bacillus licheniformis*.

To make full use of resources and save costs, materials such as wheat middling flour, cottonseed meal, straw powder and pasture, which have a high content of cellulose and hemicelluloses, are typically used to replace expensive materials, such as corn, soybean and wheat, to feed livestock. However, a high proportion of crude fiber is not only difficult for livestock to absorb, but many problems, such as diarrhea and slow growth, also occur. To improve feed quality, agriculturalists have realized the importance of adding digestive enzymes, particularly for the cellulose and hemicellulose, which cannot be synthesized in livestock animals.

The main ingredients of the plant cell wall are cellulose, hemicellulose and lignin. Cellulose and hemicellulose are coupled. After cellulose hydrolyzes plant cellulose, releasing free sugar, these two molecules are uncoupled, and the exposed hemicelluloses are hydrolyzed into xylose and other matter¹. Because endoglucanase and xylanase are the main hydrolytic enzymes of degraded celluloses, the dual hydrolytic action of endoglucanases and xylanases can be used to degrade high-content celluloses and hemicelluloses in feed into soluble micromolecular carbohydrates that can easily be absorbed and utilized, thereby increasing the palatability of the feed and improving the rate of absorption and utilization to greatly improve the health and growth of livestock.

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For the feed-processing industry, the addition of a single enzyme is insufficient for the complete degradation of plant cell wall celluloses and hemicelluloses, and the addition of various enzymes increases production costs. Thus, from the perspective of application effect and production cost, complex enzyme preparations are gaining popularity in the feed-processing industry. Many animal experiments have also shown that the addition of complex enzyme preparations can greatly improve the utilization efficiency of feed²⁻⁴. To this end, the microbes currently cultured and widely applied include *Trichoderma*, *Penicillium*, *Aspergillus niger* and other eumycetes. However, because the cellulose production cycle is long, potentially yielding many toxic substances, and crude metabolic enzymes from metabolism cannot be directly added to the feed, technical processes, such as membrane filtration, are used to remove toxic substances in the production of industrial cellulose. Apart from the many enzymes used in this process, the long cellulose production cycle increases production costs. Thus, the identification of strains that reduce the cellulose production cycle is desired. Moreover, safe metabolic products that degrade cellulose and hemicelluloses can be produced through environmental and cheap culture medium.

Bean curd wastewater is a leachate generated during bean curd production. The output of bean curd wastewater is 3-5 times the dry weight of soybean and the water-solid content of bean curd is 1%. Bean curd wastewater comprises soluble proteins, oligosaccharides, vitamins, lipids and microelements. China is the major producer and consumer of bean curd, and many highly concentrated organic wastewater substances are generated, resulting in the serious deterioration of nearby water quality, which threatens the living environment of humans. Studies have demonstrated the use of bean curd wastewater for the cultivation of *Bacillus cereus* and *Ochrobactrum anthropi* to address Cr (VI) and Ni (II) electroplating wastewater⁵. However, there is little research concerning the use of bean curd wastewater as the only nutrient substance to cultivate organisms that produce metabolic enzymes, particularly endoglucanase and xylanase. The aim of the present study was to identify and develop economic and environment-friendly

enzymes for the degradation of cellulose and hemicellulose. Moreover, we explored the use of safe, high-yielding endoglucanases and xylanases strains in bean curd wastewater as a culture medium to reduce the production cycle and provide a foundation for developing complex enzyme preparations of economic environment-friendly cellulose and hemicellulose.

MATERIALS AND METHODS

Samples and media

The soil samples screened for endoglucanase- and xylanase-producing bacteria were collected from the Farmland in Nanping City, Fujian Province of China (26.590° N, 118.255° E). The S-E medium used for the enrichment and subcultivation of endoglucanase- and xylanase-producing bacteria comprised the following ingredients (per liter): 150.0 g Straw powder, 5.0 g Okara powder, 2.0 g (NH₄)₂SO₄, 3.0 g KH₂PO₄, 3.0 g MgSO₄, 3.0 g CaCl₂, 0.2 g MnSO₄·H₂O, 0.2 g ZnSO₄·7H₂O and 0.2 g CoCl₂. S-I medium for isolation was as follows (per liter): 10.0 g carboxymethyl cellulose sodium (CMC- Na), 10.0 g beechwood xylan, 1.5 g (NH₄)₂SO₄, 2.0 g KH₂PO₄, 0.3 g MgSO₄, 0.3 g CaCl₂, 0.1 g FeSO₄, 0.1 g ZnSO₄. S-S medium for Screen was as follows (per liter): 10.0 g CMC-Na, 10.0 g beechwood Xylan, 2.0 g (NH₄)₂SO₄, 3.0 g MgSO₄·7H₂O, 1.0 g KH₂PO₄, 0.5 g NaCl, 20.0 g Ager and 0.2 g Congo red. Basic medium for producing endoglucanases and xylanases comprised the following ingredients (per liter): 10.0 g tryptone, 5.0 g beef extract and 5.0 g NaCl. Bean curd wastewater was obtained from a bean curd factory (the ratio of soybeans: water was 1:10) in Fuzhou, Fujian Province, China (26.222° N, 119.315° E). All media were adjusted to pH 7.4, dispensed into Erlenmeyer flasks, sealed with butyl rubber stoppers, and autoclaved at 121 °C for 25 min.

Enrichment and screening of endoglucanase/xylanase-producing strains

A 5.0-g soil sample was dried at room temperature for 7 days and subsequently transferred into an Erlenmeyer flask containing 150 ml of S-E medium and incubated in a shaker at 30 °C, 150 rpm for 5 days. The microbial cultures were serially diluted (10⁻⁵, 10⁻⁶ and 10⁻⁷) with physiological saline and plated onto solid S-S

plates. The plates were incubated at 30 °C for 3 days. Colonies that showed different phenotypic characters were selected and sub-inoculated three times. The isolated strains were inoculated into 100 ml of S-I medium in an Erlenmeyer flask and incubated in a shaker at 30 °C, 150 rpm for 1 day. The bacterial suspensions were used to evaluate enzymatic activity. CMCase and xylan activity were assayed using a Congo red overlay method with carboxymethylcellulose sodium and beechwood xylan as substrates⁶. The colonies with both endoglucanase- and xylanase-producing ability were purified and stored in 20% glycerol at -80 °C.

Identification of endoglucanase- and xylanase-producing bacteria

Physiological and biochemical characteristics

The colony morphologies of the isolates were monitored on basic medium plates after incubation at 33 °C for 2 days. Gram staining was performed, and the bacteria was examined using a YS100 type microscope (Nikon, Japan). The cell morphology was examined using a JSM-6380/LV scanning electron microscope (Electron Optics Laboratory Co., Ltd. Japan). Biochemical and biophysical analyses were performed according to Bergey's Manual of Determinative Bacteriology (8th edition)⁷. Characteristics were analyzed using the appropriate tests, such as Voges-Proskauer (V. P.) test, MethylRed (M. R.) test, and catalase activity, hydrolysis of starch, gelatin liquefaction, indole production, and the utilization of sole carbon/nitrogen sources were also tested.

16S rDNA gene sequencing and phylogenetic analysis

Aliquots of the 24-h basic medium culture were used for DNA extraction. A total of 1.0 ml of the culture was centrifuged (12,000×g) to obtain the cell pellet for DNA extraction using the TIANamp Bacteria DNA Kit (Tiangen, Beijing, China). Polymerase chain reaction (PCR) was performed to amplify the V6-V8 regions of 16S rDNA using universal primers F968: 5'-AAC GCG AAG CTT AC-3' as the forward primer and R1401: 5'-CGG TGT GTA CAA GAC CC-3' as the reverse primer. The PCR reaction mixture comprised 2.5 µL of 10X NH₄ buffer, 2.5 µL of each 2 mM dNTP (dATP, dCTP, dGTP, and dTTP), 1 µL of 50 mM MgCl₂, 2 µL of each primer (5 pmol·mL⁻¹), 0.5 µL of BIO Taq DNA polymerase (2.5 U·mL⁻¹), 1 µL of template, and distilled ddH₂O water to a final volume of 25 µL.

The PCR program included 35 amplification cycles (1 min at 94 °C for denaturation, 1 min at 55 °C for annealing, and 1 min at 72 °C for extension), followed by a final extension cycle at 72 °C for 10 min. The sizes of the resulting PCR products were analyzed on 1.0% agarose gel to confirm 0.4 kb 16S rRNA. This 16S rRNA band was cut and purified using a DNA purification kit (Tiangen, Beijing, China). The purified DNA sample was sequenced at Sangon Biotech (Shanghai, China). The 16S rDNA sequences were confirmed and compared through a nucleotide BLAST search provided by the National Center for Biotechnology Information (NCBI) GenBank (U.S. National Library of Medicine, Bethesda, Maryland, USA). The phylogenetic analysis of the 16S rRNA gene sequences was performed using the ClustalW program within MEGA4 software version 14.0.0.162⁸. The phylogenetic tree was constructed using the neighbor-joining method. The data set was bootstrapped 1000 times to estimate the internal stability of the tree nodes.

Enzyme assay

The collected strains with both endoglucanase- and xylanase-producing ability were transferred into a Erlenmeyer flask containing 100 ml of basic medium and incubated in a shaker at 33 °C, 150 rpm for 1 day. A 1.0-ml aliquot of the culture broth was sub-inoculated into a Erlenmeyer flask containing 150 ml of beancurd wastewater medium. An Erlenmeyer flask containing 150 ml of basic medium was generated for comparison. Both flasks were incubated in a shaker at 33 °C, 150 rpm for periods ranging from 1 to 20 days. The enzymes produced for different amount of time in different media were identified from 50-ml samples obtained from the culture flasks for each replicate. The supernatant containing endoglucanases and xylanases was separated from the cellular fraction after centrifugation (5,000 ×g, 15 min, 4 °C) and filtration (0.2 µm) and subsequently assessed for both endoglucanase and xylanase activities. The endoglucanase (xylanase) activity was determined in 50 mM phosphate buffer, pH 5.0, containing 1% (w/v) carboxymethyl cellulose (CMC) (1% beechwood xylan). The enzymatic reactions containing 1.7 ml (10 ml) of substrate, 0.3 ml (1.0 ml) of phosphate buffer and 0.5 ml of enzyme solution were performed at 50 °C for 10 min. The concentration of reducing sugars was determined

using dinitrosalicylic acid reagent⁹. Glucose (xylose) was used for the standard curve. One unit (U) of activity was defined as the amount of enzyme releasing 1 µg of glucose (xylose) equivalents per minute under assay conditions.

RESULTS

Screening and isolation of endoglucanase / xylanase-producing bacteria

To screen and isolate endoglucanase/xylanase-producing bacteria, soils samples were collected from farmland. In the preliminary screening, 18 isolates showing endoglucanase and xylanase activities with digestion circles on solid S-S plate were transferred to S-I medium. Among these 18 isolates, 7 isolates did not appear in the digestion circles using the Congo red overlay method, indicating that these bacteria did not possess endoglucanase and xylanase activity. However, 11 colonies appeared in the digestion circles, and the largest digestion circle indicated the highest endoglucanase and xylanase activity. Among the 11 colonies, three strains, EM5, SB13, and BL14, showing maximum endoglucanase and xylanase activity, were selected for further study.

Identification of endoglucanase /xylanase-producing bacteria

The morphological and physiological characteristics of the three endoglucanase and xylanase bacterial strains are summarized in Table 1. Scanning electron microphotographs revealed that all strains were rods (Fig. 1). Strain EM5 was the largest, with a cell size of (2.0–5.0)×(1.2–1.5) µm. All three strains were opaque, white or brown, Gram-positive bacteria, possessing endospore-forming ability. However, the colonies differed in configuration and surface: BM5 and SB13 formed round colonies and BL14 produced trilobed colonies; BM5 and BL14 showed wrinkled surfaces and SB13 showed smooth colonies. Physiological analyses showed that all three strains were positive for V-P test, catalase activity, and starch hydrolysis, and negative for M-R test and gelatin liquefaction activity. Furthermore, all strains assimilated glucose, D-fructose, and sucrose, and weakly assimilated mannitol and xylose. Only BM5 could assimilate maltose, and α- lactose, and no strains could assimilate melezitose.

To characterize the three endoglucanase

and xylanase bacteria at the species level, 16S rDNA sequencing was performed. The sequences of strains SB13 and BL14 showed 100% homology with *Bacillus subtilis* and *Bacillus licheniformis*, respectively. Strain EM5 exhibited 16S rDNA sequence greater than 98% similarity to *Bacillus megaterium*.

Based on these results, the isolated bacteria were identified as *Bacillus megaterium* (EM5) *Bacillus subtilis* (SB13), and *Bacillus licheniformis* (BL14). The phylogenetic relationships among EM5, SB13 and BL14 are shown in Fig. 2. The 16S rDNA sequence (385, 375 and 397bp, respectively) for strains EM5, SB13 and BL14 have been deposited in the GenBank database under the accession numbers of KJ627223, KJ627222 and KJ627224, respectively. Also, strains EM5, SB13 and BL14 have been collected in China General Microbiological Culture Collection Center (CGMCC) under the numbers of 8866, 8867 and 8865, respectively.

Endoglucanase/xylanase activity and glucose/xylose contents

The endoglucanase activities in the crude protein samples from different medium are presented in Fig. 3. When strains BM5, SB13 and BL14 were grown in basic medium the maximum fermentation activity at 20.35±2.28, 102.39±8.76, 64.73±7.45 (U/ml), respectively, was observed after 3 days. In bean curd wastewater medium, the maximum activities of strains EM5, SB13 and BL14 were observed on the second day and third day: 192.95±10.96, 242.08±21.00, 213.80±9.74 (U/ml), respectively. After fermenting for three days, the activities of strains in the two mediums declined, and the endoglucanase activity was not detected for 6 days in basic medium and 13 days in bean curd wastewater medium. In addition, the glucose contents showed a downward trend throughout the fermentation process in basic medium. Conversely, in bean curd wastewater medium, the glucose content increased after one day of cultivation, with concentrations ranging from 1283.06 ± 31.54 (µg/ml) up to 2869.56 ± 34.10, 2766.04 ± 38.88, and 3204.68 ± 39.61 (µg/ml). After 1–2 days of fermentation, the glucose content gradually declined.

The xylanase activities of the crude protein samples and xylose contents from different medium are presented in Fig. 4. Both the xylanase

activity and xlyose content in bean curd wastewater medium were higher than those in basic medium, but similar trends were detected for strains grown in two different media throughout the fermentation process. The maximum activity appeared on the 3th day of fermentation. Similarly, the xlyose contents obviously increased after one day of cultivation, and gradually decline thereafter. Both the xylanase activity and xlyose content could be detected after 20 days of cultivation.

DISCUSSION

The purpose of the present study was to identify microorganisms for the degradation of



Fig. 1. Scanning electron microphotographs of strains BM50SB13 and BL14. All strains were grown at 33 °C for 1 day on basic medium.

cellulose and hemicellulose through screening *Trichoderma*, white rot fungi, *penicillium*, and other fungi, and bacteria, including *Clostridium*, *Cellulomonas*, *Bacillus*, *Thermomonospora*, *Acetovibrio*, *Microbispora*, *Clostridium*, *Thermocellum*, *Bacteroides cellulosolvens* and *Streptomyces*¹⁰. The high-yield xylanase-producing microbes included fungi comprising *Aspergillus niger*¹¹⁻¹², *Trichoderma reesei*¹³, *Penicillium*¹⁴ and bacteria, such as *Bacillus subtilis*¹⁵, *Paenibacillus*¹⁶, *Ammonia nitrate coli*¹⁷. Complex enzymes produced from *Penicillium oxalicum* have also been reported¹⁸. However, the aim of the present study was to use bean curd wastewater as a nutrient medium to screen endoglucanase- and xylanase-producing bacteria. Herein, these strains were obtained through the conditional filtering of special soil samples containing microorganisms enriched after adding straw and bean dregs powders to the medium. The strains were identified as *Bacillus megaterium* EM5, *Bacillus subtilis* SB13, and *Bacillus*

Table 1. Morphological and Physiological characteristics of strains BM5, SB13 and BL14

Tests	Strain BM5	Strain SB13	Strain BL14
Morphological characteristics			
Configuration	Round	Round	Trilobed
Surface	Wrinkled	Smooth	Wrinkled
Color	White	White, Brown	White
Opacity	Opaque	Opaque	Opaque
Cell shape	Rod shaped	Rod shaped	Rod shaped
Cell size	(2.0–5.0)×(1.2-1.5) ìm	(1.5–2.8)×(0.6-0.7) ìm	(0.5–3.0)×(0.6-0.8) ìm
Endospore	+	+	+
Gram stain	+	+	+
Physiological characteristics			
V-P test	+	+	+
M-R test	-	-	-
Catalase	+	+	+
Starch hydrolysis	+	+	+
Gelatin Liquefaction	-	-	-
Maltose	+	w	w
á- lactose	+	-	-
Glucose	+	+	+
Cellobiose	-	w	w
D-fructose	+	+	+
Sucrose	+	+	+
Mannitol	w	w	w
Xylose	w	w	w
Melezitose	-	-	-

+ positive; - negative; w, weak positive

licheniformis BL14, all belonging to *Bacillus* and showing positive results in growth and short fermentation periods. The strain with highest enzyme production was SB13, and such *Bacillus subtilis* strains have been widely used as probiotic bacteria in China due to the characteristics of no pollution, no residue, and no resistance¹⁹. Other studies have shown the degradation of cellulose or cellulose derivatives through endoglucanase hydrolysis mediated through *Bacillus subtilis*, generating cellodextrins, cellobiose, cellotriose and other substances, which can improve the rate of absorption and utilization of high-fiber fodder in animal feeds. Thus, such strains can be used to produce various enzymes for bacteria without separate programs for the addition of enzyme and other metabolites. The use of these *Bacillus* strains will simplify production processes and reduce production and fodder costs.

Studies have shown that the nutrient conditions of growth can significantly affect the output of cellulose and hemicellulose, and carbohydrates and their derivatives can be used as secretion-inducing substances to promote the

secretion of most cellulases²⁰. In the present study, endoglucanase- and xylanase-production were identified in three strains, compared and cultivated using beancurd wastewater and basic media. The results showed that endoglucanase activity could obviously be improved when beancurd wastewater is used as a medium. EM5 showed an 8.43-fold improvement in endoglucanase activity times, whereas SB13 and BL14 strains showed 1.37- and 2.29-fold increase in endoglucanase activity. The xylanase activity in EM5, SB13 and BL14 strains was also increased 0.37, 0.75, and 0.14-fold,

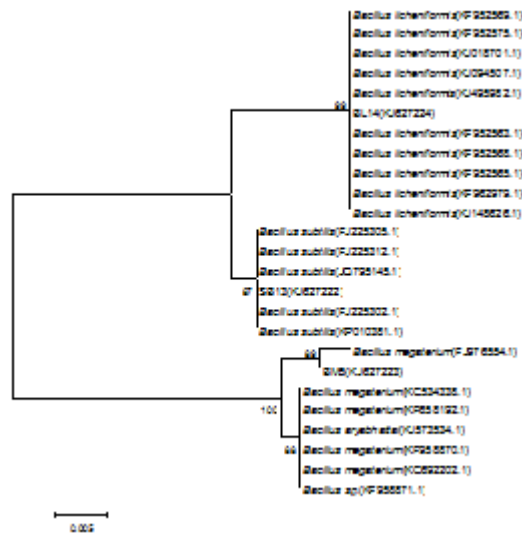


Fig. 2. A neighbor-joining phylogenetic tree based on 16S rDNA gene sequences showing the phylogenetic relationship among EM5, SB13, BL14 and other species. The sequences were aligned using the Clustal W program and MEGA4 software. The phylogenetic tree was generated using the neighbor-joining method. Bootstrap values, as percentages of 1,000 replications, are given at branch points.

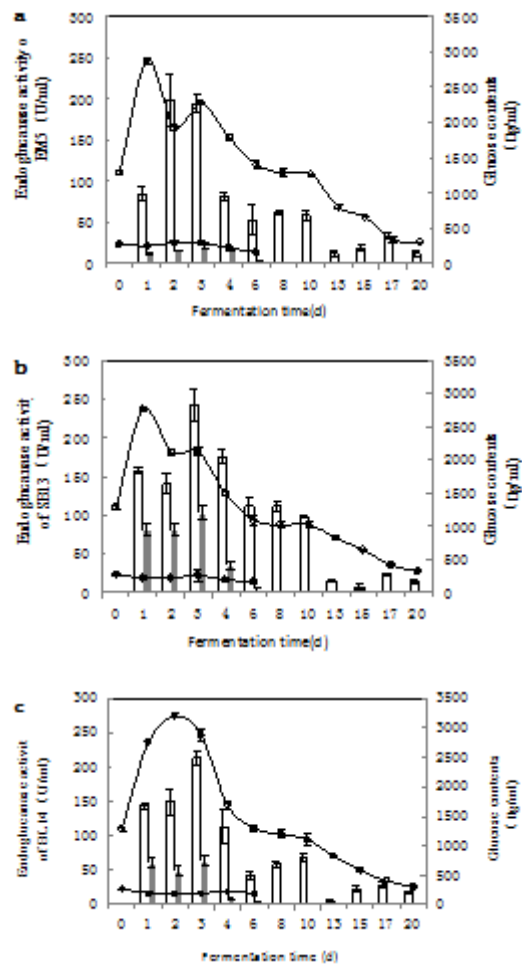


Fig. 3. Endoglucanase activity(white bar in beancurd wastewater media,gray bar in basic media) and glucose content (white circle in beancurd wastewater media,black circle in basic media) measured in the supernatant of strains BM5(a), SB13(b) and BL14(c) grown in two different media.

respectively. Presumably, the oligomeric polysaccharides, lipids, other carbohydrates and microelements contained in beancurd wastewater play important roles in the secretion of cellulose. The endoglucanase activity of different strains was obviously different in both media, the positive effect of cellulose through the enzymatic ion might vary with the composition of different cellulose systems from various sources²¹. Furthermore, the activity of endoglucanase in basic medium was no longer detected after 4 days of fermentation, suggesting that these strains enter decline and death phases after fermentation in basic medium for 4 days, which could not generate endoglucanase, and the enzymes generated through fermentation are rapidly inactivated. In

beancurd wastewater medium, the endoglucanase activity of these strains could be significantly detected on the tenth day of fermentation, indicating that beancurd wastewater not only contains inducing substances that promote endoglucanase secretion but also contain oligosaccharides, lipids and other substances that could protect enzymatic activity.

In bean curd wastewater medium, the glucose and xylose content were obviously increased after one day of cultivation, likely resulting from the degradation of polysaccharides and other macromolecule carbohydrates in bean curd wastewater through endoglucanase and xylanase produced by these strains. After 1-2 days of fermentation, the glucose and xylose in the bean curd wastewater culture solution begin to decline, and on the 20th day, glucose was nearly exhausted and xylose also showed a downward trend, indicating that the three strains metabolize glucose and xylose, consistent with the physiological and biochemical characteristics of the bacteria identified in this study (see Table 1). Similarly, in basic medium, the fermentation of glucose and xylose consistently showed a linear downward trend, as peptone and beef extract are used as nutrients in basic medium, no macromolecules can be degraded to monosaccharides, thus no increase in glucose and xylan was observed. In addition, because basic medium lacks carbohydrates, no positive effect on the induction of endoglucanase and xylanase gene was observed, and the activity of endoglucanase and xylanase in these three strains was significantly lower in basic medium than in beancurd wastewater.

Studies have shown that glucose participates in feedback inhibition on the secretion of cellulase from *Coptotermes formosanus* and filamentous fungi, and the normal expression of cellulase is observed after elimination of glucose²²⁻²³. In the present study, the glucose and xylan content was highest in the culture solution after the fermentation of various strains for one day, suggesting that inductive substances, such as oligomeric polysaccharides in beancurd wastewater, that promote endoglucanase and xylan secretion is decomposed and exhausted. However, the endoglucanase and xylanase activity in bacteria reaches the maximum value on the third day of cultivation, suggesting feedback inhibition

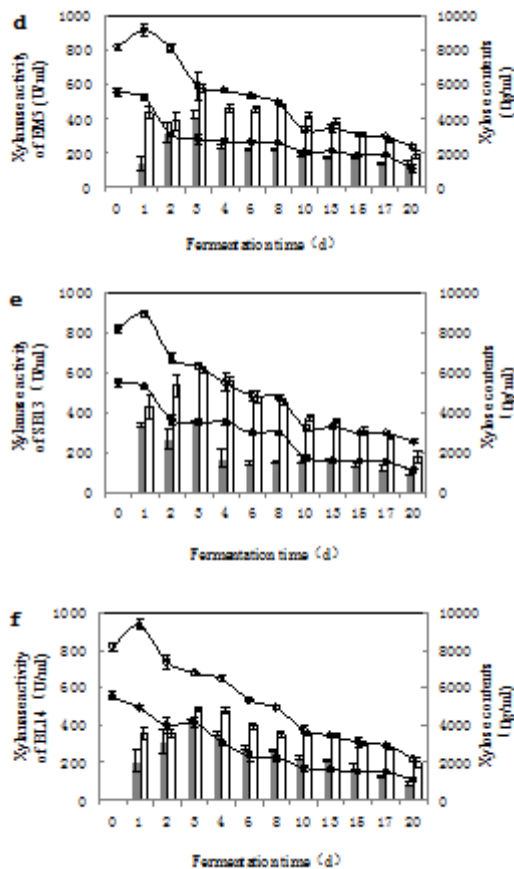


Fig. 4. Xylanase activity(white bar in beancurd wastewater media,gray bar in basic media) and xylose contents (white circle in beancurd wastewater media,black circle in basic media) measured in the supernatant of strains BM5(d), SB13(e) and BL14 (f)grown in two different media.

through the secretion of these enzymes at high concentrations of glucose and xylose in the culture solution. When the glucose in the culture medium is partly consumed as a cell nutrient, the enzyme expression returns to normal. Ultimately, the activity of these two enzymes is decreased, reflecting the decline of enzyme activity after the growth and metabolism of the microorganisms enters the decline phase.

CONCLUSIONS

3 endoglucanase- and xylanase-producing bacteria, cultured in beancurd wastewater as the sole medium, were screened and identified in the present study. Strain EM5 was identified as *Bacillus megaterium*, SB13 was identified as *Bacillus subtilis*, and BL14 was identified as *Bacillus licheniformis*. Compared with basic medium, all strains showed higher endoglucanase and xylanase activities in beancurd wastewater medium. The endoglucanase activity cultured in beancurd wastewater medium was maintained for a longer period than that in basic medium. These results show that beancurd wastewater is an adequate medium for strains EM5, SB13, and BL14 to produce both endoglucanase and xylanase. Among the three strains identified in the present study, SB13 showed the highest endoglucanase and xylanase activity, representing a good strain with broad development potential in the feed industry.

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