Vietnam J. Agri. Sci. 2017, Vol. 15, No. 9: 1205 - 1212 Tạp chí Khoa học Nông nghiệp Việt Nam 2017, 15(9): 1105 - 1212
www.vnua.edu.vn

SCREENING AND CHARACTERIZATION OF CELLULASES PRODUCED BY *Bacillus* spp.

Samley Mam, Nguyen Thi Thanh Thuy*

Faculty of Food Science and Technology, Vietnam National University of Agriculture

Email: nttthuycntp@vnua.edu.vn*

Received date: 04.08.2017 Accepted date: 18.09.2017

ABSTRACT

Cellulases are enzymes synthesized by diverse groups of microorganisms. These enzymes have demonstrated biotechnological potentials in various sectors, including food, animal feed, brewing and wine, and pulp and paper industries. In this study, 100 strains of *Bacillus* spp. were incubated in MT1 agar medium, and cellulolytic activity was qualitatively determined by measuring the diameters of the hydrolytic clear zones. The three most active strains (A1.2, A1.8, and B6.4) showing clear zone diameters above 24 mm were selected. Cellulase activity in a liquid medium was further quantitatively determined by the dinitrosalicylic acid (DNS) method. Cellulolytic bacteria were identified by 16S rRNA gene sequencing, and neighbor-joining phylogenetic analysis was conducted to determine the evolutionary relationships between the selected strains and reported strains from the GenBank database. The strains were identified as *Bacillus cereus* (A1.2 and A1.8), and *Bacillus pumilus* (B6.4). Cellulase produced by *B. pumillus* B6.4, a GRAS bacterium showing the highest cellulase production in a liquid medium, was partially purified and characterized. The enzyme was most active at 55°C and pH 6.5. Half-lives (conducted at pH 5.0) of the enzyme at 55, 65, 75, and 85°C were 180, 180, 30 and 20 min, respectively. Similarly, half-lives (conducted at 37°C) of the enzyme at pH 5.5, 6.5, 7.5, and 8.5 were 130, 135, 80 and 70 min, respectively. The broad range of working temperatures and the stability under mild acidic conditions suggest that the cellulase of *B. pumilus* B6.4 could be a good candidate for application in the lignocellulosic bioethanol industry.

Keywords: *Bacillus* pumilus B6.4, cellulase, enzyme characterization.

Sàng lọc vi khuẩn *Bacillus* sp. sinh cellulase và xác định đặc tính của enzyme

TÓM TẮT

Cellulase là enzyme được sinh ra từ nhiều loại vi sinh vật khác nhau. Tiềm năng sinh học của những enzyme này thể hiện bởi sự ứng dụng đa dạng của nó trong các ngành công nghiệp khác nhau như thực phẩm, thức ăn chăn nuôi, bia và rượu vang, bột giấy và giấy. Tong nghiên cứu này, 100 chủng *Bacillus* sp. được ủ trong môi trường MT1 để xác định khả năng thủy phân cellulose thông qua đo đường kính vòng phân giải. Ba chủng (A1.2, A1.8 và B6.4) có đường kính vòng phân giải lớn nhất 24 mm được lựa chọn cho nghiên cứu tiếp theo. Hoạt độ cellulase được xác định gián tiếp thông qua định lượng đường khử bằng phương pháp acid dinitro-salicylic (DNS). Tên loài vi khuẩn sinh cellulase cao được xác định bằng cách giải trình tự gen 16S rRNA và sử dụng cây tiến hóa để hiển thị mối quan hệ giữa các chủng được chọn với các chủng khác trong cơ sở dữ liệu. Các chủng này được xác định là *Bacillus cereus* (A1.2 và A1.8) và *Bacillus pumilus* (B6.4). Cellulase tạo ra từ *Bacillus pumilus* B6.4, một vi khuẩn được xếp vào nhóm an toàn (GRAS), cho kết quả là cao nhất khi nuôi cấy trong môi trường lỏng sẽ bước đầu được tinh sạch và xác định đặc điểm. Enzyme cellulase hoạt động tốt nhất ở 55°C với pH 6,5. Thời gian bán rã (tiến hành ở pH 5,0) của enzyme ở nhiệt độ 55, 65, 75 và 85°C tương ứng là 180, 180, 30 và 20 phút. Tương tự, thời gian bán rã (tiến hành ở 37°C) của enzyme ở pH 5,5; 6,5; 7,5 và 8,5 lần lượt là là 130, 135, 80 và 70 phút. Phạm vi rộng của nhiệt độ tác động và sự ổn định trong điều kiện acid nhẹ cho thấy cellulase của *Bacillus pumilus* B6.4 có thể là ứng cử viên tốt sử dụng trong ngành công nghiệp nhiên liệu sinh học

Từ khóa: *Bacillus pumilus* B6.4, cellulase, đặc tính enzyme

1. INTRODUCTION

Cellulases contribute to 8% of the worldwide industrial enzyme load and demand is expected to increase drastically in the near future (Costa *et al.*, 2008). Cellulase is significant due to its key roles in biotechnology and industrial applications (Bhat, 2000). It has been widely utilized for bioremediation (Zahangir *et al.*, 2005), food processing (Chandara *et al.*, 2005), paper and pulp industry, supplementation in the animal feed industry (Chandara *et al.*, 2005), textile industry (Ali and Saad, 2008), alcoholic beverage, malting, and brewing (Sreeja *et al.*, 2013), formulation of washing powders, extraction of fruit and vegetable juices, and starch processing (Camassola and Dillon, 2007).

Although both fungi and bacteria have been exploited for their abilities to produce a wide variety of cellulases and hemicellulases, the isolation and characterization of novel cellulases from bacteria have become increasingly intensive. There are several reasons for these movements: i) bacteria often have a higher growth rate than fungi allowing for higher recombinant production of enzymes, ii) bacterial cellulases are often more complex and are in multi-enzyme complexes providing increased function and synergy, and iii) bacteria inhabit a wide variety of environmental and industrial niches like thermophilic or psychrophilic, alkaliphilic or acidophilic, and halophilic strains, which produce cellulolytic strains that are extremely resistant to environmental stresses (Sangrila and Tushar, 2013).

The aim of this study was to find novel cellulases of application potential for the cellulosic bioethanol industry from *Bacillus* sp. strains isolated in Vietnam.

2. MATERIALS AND METHODS

2.1. Materials

Bacteria strains: One hundred strains of *Bacillus* spp. were supplied by the Faculty of Food Science and Technology, Vietnam National University of Agriculture. These strains were collected from two different sources, namely from chili sauce (Muong Khuong, Lao Cai, Vietnam), and from cow rumen (Bavi, Hanoi, Vietnam).

2.2. Methods

The experimental flow diagram for the screening and characterization of cellulases produced by *Bacillus* spp. is indicated in Figure 1.

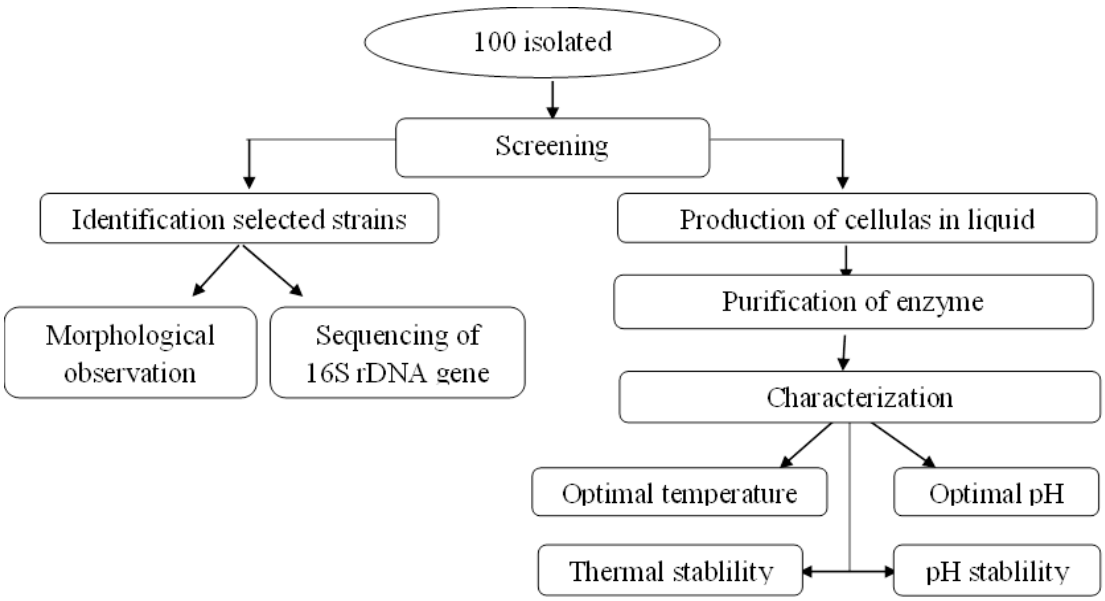

Fig. 1. Screening and characterization of cellulases produced by *Bacillus* spp.

- Screening of cellulase producing bacteria

Microorganisms were activated in NB medium (per liter: 10 g peptones, 5 g NaCl, 5 g meat extract, pH 7), and then 100 µl of culture broth was sporred on an agar plate containing MT2 medium (per liter: 20 g CMC; 0.25 g yeast extract; 3.5 g KH_2PO_4; 5 g $MgSO_4$; 0.625 g KNO_3; 20 g agar; pH 7) (Thi and Quyen, 2014) for primary detection of cellulase. The formation of a clear zone of hydrolysis indicated cellulose degradation. The difference (in mm) of the clear zone and colony diameters qualitatively reflecting enzyme activity was recorded. Enzyme activity was also determined by using the 3,5-dinitrosalicylic acid (DNS) method (Miller *et al.*, 1959). The strains showing the highest cellulase activity were selected for further study.

- Cellulase enzyme assay

Briefly, the cellulase activity (U/ml) was measured by estimation of reducing sugars liberated from CMC. A 1% CMC solution was prepared in 50 mM sodium acetate buffer (pH 5.0). The enzyme assay was performed by incubating 0.1 ml enzyme with 0.9 ml of 1% CMC solution at 37°C for 30 min. After incubation, the reaction was stopped by the addition of 1.2 ml of DNS reagent, and boiled at 100°C in a water bath for 10 min. Liberated sugars were determined by measuring the absorbance at 540 nm. Cellulase production was estimated by using a glucose calibration curve. One unit (U) of enzyme activity was expressed as the quantity of enzyme required to release 1 µmol of reducing sugar per minute under testing conditions (37°C, pH 5.0) (Singh *et al.,* 2013).

- Identification of selected strains

The strains showing the highest cellulase activities were identified based on morpho-physiological characteristics (Gram staining, colony and cell morphology, mobility) (Apun *et al.*, 2000) and 16S rRNA gene sequencing.

- Analysis of 16S rRNA gene sequence

Genomic DNA was extracted and purified using CTAB (Current Protocol in Molecular Biology, 2009), and DNA purity was spectrophotometrically assessed by the A260/A280 ratio. The fragment of the 16S rRNA gene was amplified using the universal primers 8F (5 -AGAGTTTGATCCTGGCTCAG-3') and 1492R (5′-GGTTACCTTGTTACGACTT-3'). The PCR conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 90 sec; 72°C for 5 min. A positive control (*E. coli* genomic DNA) and a negative control (without DNA template) were also used in the PCR reactions. DNA products (about 1.5 kb) were gel purified using a QIAquick Gel Extraction Kit (Qiagen) as recommended by the manufacturer and sent out for sequencing (First BASE Laboratories, Selangor, Malaysia). DNA sequences were then compared to published sequences in GenBank using the BLAST engine hosted at NCBI (Bethesda, USA).

- Characterization of cellulase

For the crude enzyme preparation, a fresh colony of a selected strain was inoculated in 2.5 ml of NB medium at 37°C on a rotary shaker at 150 rpm for 24 h. After that, 2 ml of culture broth was added into 250 ml of MT1 medium (per liter: 10 g CMC; 1g D-glucose; 2 g yeast extract; 0.5 g KH_2PO_4; 0.2 g $MgSO_4.7H_2O$; 0.04 g $CaCl_2$; 0.02 g $FeSO_4.7H_2O$; 0.75 g KNO_3; pH 7) (Lisdiyanti *et al.,* 2012) in a 500 ml conical flask and incubated at 37°C, 150 rpm for 24 h. The broth was then centrifuged at 6000 rpm for 15 min at 4°C and the supernatant was used as crude enzyme for further studies.

Partial purification of cellulase: Cellulase was precipitated by adding 4 volumes of 96% ethanol. The slurry was centrifuged at 6000 rpm, 4°C for 30 min, and the supernatant was discarded. The pellet was washed three times with 50 mM phosphate buffer, pH 7.0, and then re-dissolved in 20 ml of sodium acetate buffer, pH 5.0. The partially purified enzyme was used for further characterization.

Optimum temperature and pH: For determination of the optimum temperature, reaction mixtures containing the enzyme preparation and 1% CMC in 50 mM sodium acetate buffer, pH 5.0 were incubated at different temperatures, ranging from 40°C to 80°C for 30

min. Relative cellulolytic activity was determined by measuring the amounts of reducing sugar released. Similarly, optimum pH was determined by incubating the reaction mixtures at different pHs, ranging from 5.0 to 8.0 at 37°C using 50 mM sodium acetate buffers.

Temperature and pH stability: Thermal stability was investigated by pre-incubating the partially purified enzyme at various temperatures, ranging from 45°C to 85°C. At different time points, samples were taken and residual cellulase activity was determined by a reducing sugar assay using the DNS method. The relative activity at different time points was calculated as a percentage of the maximum activity observed for each given temperature. Similarly, pH stability was studied by pre-incubating the enzyme at 37°C in 50 mM sodium acetate buffer with the pH ranging from 5.5 to 8.5. The residual cellulase activity was determined at different time points as described above.

3. RESULTS AND DISCUSSION

3.1. Screening of cellulase producing bacteria

All 100 *Bacillus* spp. strains were cultured on CMC agar plates for screening of cellulase activity. The diameters of clear zones produced by the tested strains varied from 3 to 24 mm. The results are summarized in Table 1. Among the tested stains, we eliminated 32 strains since they were morphologically identical and produced clear zones with diameters less than 10 mm. There were 9 isolates that showed large clear zones (21-25 mm).

Although it indicates hydrolytic activity, the plate-screening method is not quantitative and there is a poor correlation between enzyme activity and the size of the clear zone (Maki *et al.,* 2009). Thus, the three strains (A1.2, A1.8, B6.4) that showed largest clear zones (≥ 24 mm) were selected for further screening on the basis of CMCase production in a liquid medium.

The strains were cultivated in MT1 medium containing 1% CMC at 37°C for 36 h for cellulase production. CMCase activities of the strains are presented in Table 2.

Table 2. Cellulase activity of 3 selected strains

Strain	Cellulase activity (IU/ml)
A1.2	1.14 ± 0.01
A1.8	1.01 ± 0.01
B6.4	3.01 ± 0.02

The most active cellulase producer was B6.4, which showed CMCase activity of 3.01 U/ml. It was previously reported that the maximum cellullase activity of *Bacillus velesensis* was 0.02 U/ml (Ancharida *et al.,* 2014), and *B. safensis* was 0.23 U/ml (Khianngam *et al.,* 2014). Thus, in comparison with published data, the cellulase activity of B6.4 was significantly higher.

3.2. Identification of selected strains

All 3 isolates (A1.2, A1.8, and B6.4) were Gram positive, endospore forming, and mobile. The 16S rRNA gene sequences obtained for the three selected strains were compared with the GenBank nucleotide database using the BLAST tool. Strains A1.8 and A1.2 were most closely related to *Bacillus cereus* and shared 99% and 97% homology, respectively, to the latter. Strain B6.4 was most closely related to *Bacillus pumillus* YQQ24 (97% of homology). The phylogenetic tree generated using the 16S rRNA gene sequences for *B. pumilus* B6.4 is presented in Figure 2. According to the FDA (2015), *B. pumilus* is regarded as GRAS. *B. pumilus* B6.4 was therefore chosen for further studies.

3.3. Characterization of cellulase produced by B. pumilus B6.4

- Optimum temperature: Temperature greatly affects enzyme activity. The effect of temperature on *B. pumilus* B6.4 cellulase was studied in the range from 40°C to 80°C with 5°C intervals. The results revealed that cellulase activity of *B. pumilus* B6.4 increased when the temperature increased from 40°C to 50°C, and reached a maximum at 55°C (5.157 U/ml), then activity gradually decreased as the temperature increased to 80°C (Figure 3).

Table 1. Clear zones produced by 100 tested *Bacillus* spp. strains on CMC agar

Diameter of clear zone (mm)	Number of strains	Source of isolation	
		Chili sauce (A)	Cow rumen (B)
0-5	5	2	3
6-10	27	7	20
11-15	34	11	23
16-20	25	6	19
21-25	9	2 (A1.2; A1.8)	7 (B2.6, B2.7, B4.6, B4.9, B5.5, B6.4, B8.1)

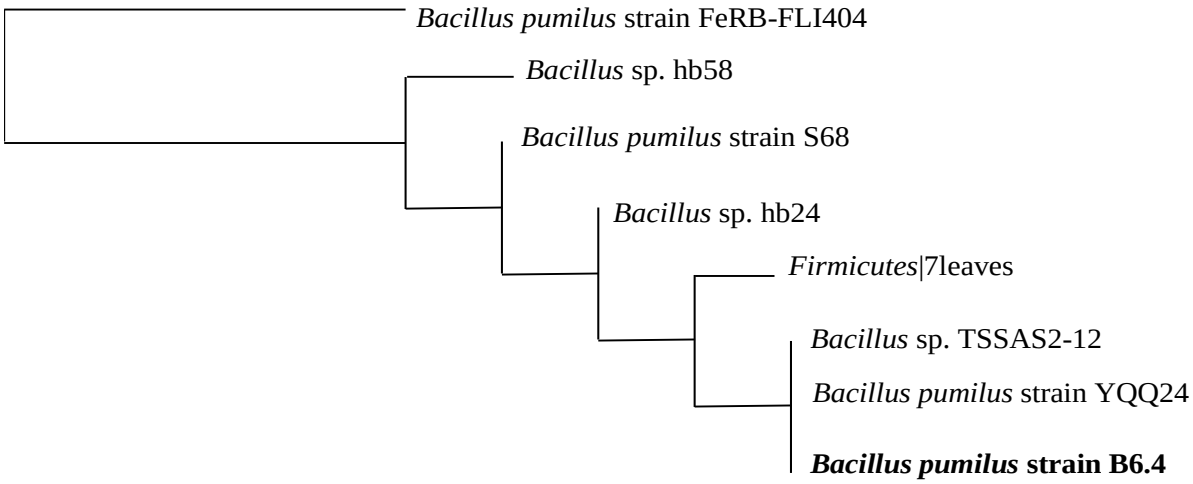

Fig. 2. Phylogenetic tree based on the 16S rDNA sequences of *B. pumilus* B6.4

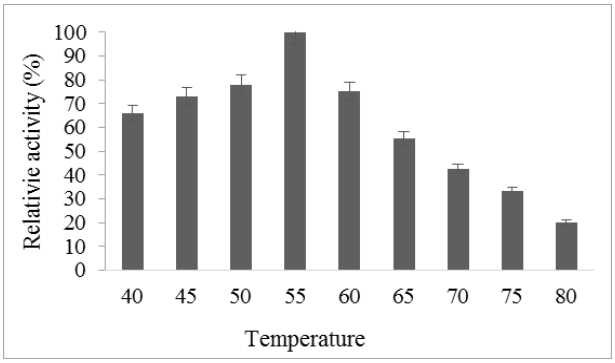

Fig. 3. The effect of temperature on *B. pumilus* B6.4 cellulase activity

The optimum temperature (55°C) of *B. pumilus* B6.4 cellulase was slightly different from that previously reported for *B. pumilus*. *B. pumilus* S124A cellulase functioned optimally at 50°C (Natesan and Nelson, 2014), and 60°C was the optimum temperature for *B. pumilus* EB3 cellulase (Ariffin *et al.,* 2006). Other *Bacillus* cellulases also share a similar range of optimum temperatures, for example, cellulases of *B. mycoides* S122C and *Bacillus subtilis* YJ1 have optimum temperatures of 50°C (Balansubramanian *et al.*, 2012), and 60°C (Li *et al.,* 2010), respectively.

- pH optimum: *B. pumilus* B6.4 cellulase showed the highest activity at pH 6.5, but it also demonstrated rather high activity in light

acidic (pH 5.5 - 6.0) and mild alkaline (pH 7.5) conditions (Fig. 4). For instance, at pH 5.5 and pH 7.5, the enzyme retained 79% and 80% of its maximum activity, respectively. However, a steep reduction in cellulase activity was noticed in alkaline conditions. At pH 8, the relative activity was only 36%.

Optimum pH values of 4.5 to 8.0 have been reported for different microbial cellulases (Immanuel *et al.,* 2007; Dutta *et al.,* 2008). Each enzyme has its own optimum pH and if the pH increases or decreases beyond the optimum, the ionization groups at the active site may change by slowing or preventing the formation of an enzyme substrate complex (Eijsink *et al.,* 2005). The pH range suitable for cellulase from *B. circulans* was found to be 4.5 to 7.0 (Kim, 1995). For other *Bacillus* strains, pH optima were 5.0 to 6.5 (Mawadza *et al.,* 2000) and 6.0 to 6.5 in *B. subtilis* YJ1 (Li *et al.,* 2010).

- Thermal stability

Thermal stability of cellulase from *B. pumilus* B6.4 was determined by measuring the relative cellulolytic activity at various temperatures, ranging from 45°C to 85°C, and at different time points, from 30 to 240 min at pH 5.0 (Fig. 5). More than 58% of cellulase activity was maintained at temperatures ranging from 55 to 65°C after 150 min incubation at pH 5.0 and it remained more than 40% after 240 min. Interestingly, the enzyme was less stable at 45°C. At 45°C, about 38% of the activity was maintained after 150 min and then dramatically decreased to 25% after 240 min. Cellulase degradation at 45°C could be explained by the presence of associated proteases, although this might require further verification. At 75°C and 85°C, less than 15% of the activity was observed after 150 min, and 1% after 240 min.

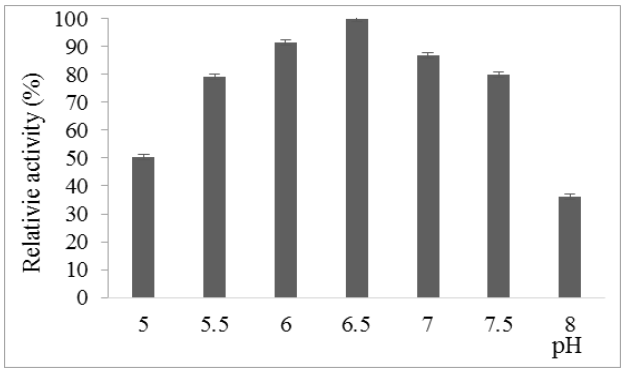

Fig. 4. The effect of pH on *B. pumilus* B6.4 cellulase activity

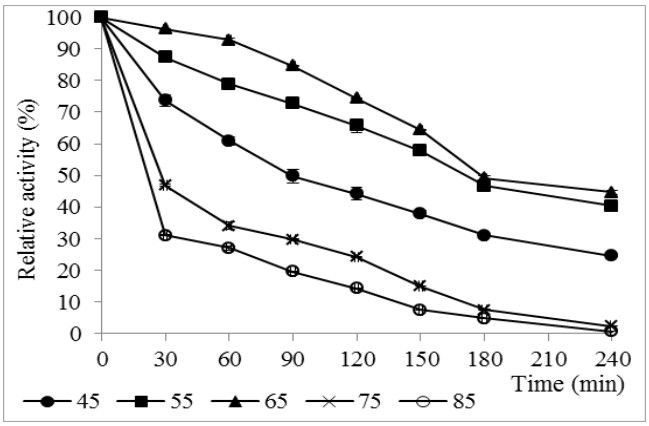

Fig. 5. Thermal stability of *B. pumilus* B6.4 cellulase

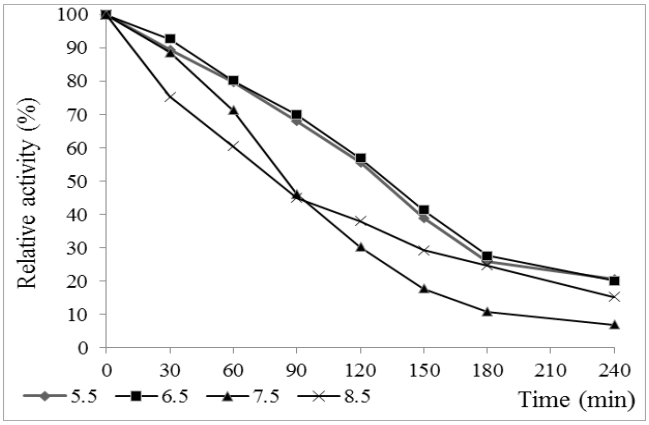

Fig. 6. pH stability of *B. pumilus* B6.4 cellulase

Natesan and Nelson (2014) reported that *B. pumilus* S124A cellulase was stable at 60°C to 70°C. Cellulase from *B. amyoliquefaciens* DL-3 was stable at temperatures ranging from 50°C to 70°C (Lee *et al.,* 2008), and purified cellulase produced by *B. subtilis* was stable at 40°C to 60°C (Rekha and Lakshmi, 2012). Cellulases produced by *Bacillus* sp., *Pseudomonas* sp., and *Serratia* sp. isolates were found to be stable up to 55°C (Prabesh *et al.*, 2016).

- pH stability

The stability of the enzyme when incubated at different pHs between 5.5 and 8.5 was determined (Fig. 6). The results showed that more than 56% of cellulase activity was maintained at a pH range of 5.5 to 6.5 after 120 min of incubation, and more than 21% after 240 min. About 30% of activity remained at pH 7.5 to 8.5 after 120 min and then dramatically decreased to 7% after 240 min.

Thus, cellulase obtained from *B. pumilus* B6.4 was rather stable at a pH of 5.5 to 6.5. Some previous studies have also reported that cellulases produced by several *Bacillus* sp. were stable over a wide pH range (Mawadza *et al.,* 2000; Lee *et al.,* 2008). *B. subtilis* BY-4 cellulase was found to be stable at a pH ranging from 4.5 to 6.0, and most stable at pH 5.0 (Lima *et al.,* 2015). *B. amyloliquefaciens* DL-3 cellulase was stable over a broad pH range, from 4.0 to 9.0 (Lee *et al.,* 2008), *B. halodurans* IND18 cellulase showed stability at a pH from 6.0 to pH 9.0, and was most stable at pH 9.0 (Gao *et al.,*2008).

4. CONCLUSIONS

One hundred strains of *Bacillus* spp. were qualitatively screened for cellulase activity using an agar plate assay and the three most active strains (A1.2, A1.8, and B6.4) were selected. Strains A1.2 and A1.8 were identified as *Bacillus cereus,* and B6.4 as *Bacillus pumillus* based on 16S rRNA gene sequencing. Cellulase produced by *B. pumillus* B6.4, a GRAS bacterium showing the highest cellulase production in a liquid medium, was partially purified. The cellulase was most active at 55°C and pH 6.5. The enzyme maintained more than 58% activity after treatment at 55°C or 65°C for 150 min. It also maintained more than 56% activity at pH 5.5 or 6.5 for 120 min. These properties suggest that *B. pumilus* B6.4 could be a good candidate for application in the cellulosic biofuel industry.

REFFERENCES

Ali U. F., and Saad E. H. S. (2008). Production and partial purification of cellulase complex by *Aspergillus niger* and *A. nidulans* grown on water hyacinth blend. Journal of Applied Sciences Research, 4: 875-891.

Ancharida S., Thanawan T., Jaruwan S., and Somboon T. (2014). Characterization of cellulase producing *Bacillus* and *Paenibbacillus* strains from Thai soils. Journal of Applied Pharmaceutical Science, 4(05): 6-11.

Bhat MK, Bhat S. (1997). Cellulose degrading enzymes and their potential industrial applications. Biotechnology Advances, 15: 583-620.

Bhat M. K. (2000). Cellulases and related enzymes in biotechnology. Biotechnology Advances, 18(5): 355-383.

Camassola M., and Dillon A. J. P. (2007). Production of cellulases and hemicellulases by *Penicilliumechinulatum* grown on pretreated sugarcane bagasse and wheat bran in solid-state fermentation. Journal of Applied Microbiology, 103: 2196-2204.

Chandara S. K. R., Snishamol C., and Prabhu N. G. (2005). Cellulase production by native bacteria using water hyacinth as substrate under solid state fermentation. Malaysian Journal of Microbiology, 1: 25-29.

Dutta T., Sahoo R., Sengupta R., Ray S. S., Bhattacharjee A., and Ghosh S. (2008). Novel cellulases from an extremophilic filamentous fungi *Penicilliumcitrinum*: production and characterization. Journal of Industrial Microbiology and Biotechnology, 35: 275-282

Eijsink V., Gåseidnes S., Borchert T., and Burg V. D. (2005). Directed evolution of enzyme stability. Biomolecular Engineering, 22(1-3): 21-30.

Iflah W., Muhammad G., and Shahnaz C. (2014). Cellulase production from newly isolated bacterial strains from local habitat. International Journal of Recent Scientific Research, 5(8): 1454-1459.

Immanuel G., Dhanusha R., Prema P., and Palavesam A. (2006). Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effuents of estuarine environment. International Journal of Environmental Science and Technology, 3(1): 25-34.

Immanuel G., Bhagavath C. M., Raj I. P., Esakkiraj P., and Palavesam A. (2007). Production and partial purification of cellulase by *Aspergillusniger* and *A. Fumigatus* fermented in coir waste and sawdust. The Internet Journal of Microbiology, 3(1). doi:10.5580/49

Gao J., Weng H., Zhu D., Yuan M., Guan F., and Xi Y. (2008). Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover. Bioresource Technology, 99: 7623-7629.

Khianngam S., Yupa P., Taweesak T., and Somboon T. (2014). Screening and identification of cellulase producing bacteria isolated from oil palm meal. Available online at http://www.japsonline.com. DOI: 10.7324/JAPS.2014.40416. Journal of Applied Pharmaceutical Science, 4(04): 90-96.

Lee Y. J., Kim B. K., Lee B. H., Jo K. I., Lee N. K., Chung C. H., Lee Y.C., and Lee J. W. (2008). Purification and characterization of cellulase produced by *Bacillus amyoliquefaciens* DL-3 utilizing rice hull. i. 99: 378-386.

Lima A. O., Quecine M. C., Fungaro M. H., Andreote F. D., Maccheroni W. J., Araujo W. L., SilvaF. M. C., Pizzirani K. A. A., and Azevedo J. L. (2005). Molecular characterization of a beta-1,4-endoglucanase from an endophytic *Bacillus pumilus* strain. Applied Microbiology and Biotechnology, 68: 57-65.

Lisdiyanti P., Suyanto E., Gusmawati N. F., and Rahayu W. (2012). Isolation and characterization of cellulose produced by cellulolytic bacteria from peat soil of Ogan Komering Ilir, South Sumatera. International Journal of Environment and Bioenergy, 3: 145-153.

Maki M., Leung K. L., and Qin W. (2009). The prospects of cellulase producing bacteria for the bioconversion of lignocellulosic biomass. International Journal of Biological Sciences, 5: 500-516.

Mawadza C., Hatti K. R., Zvauya R., and Mattiasson B. (2000). Purification and characterization of cellulases produced by two *Bacillus* strains. Journal of Biotechnology, 83(3): 177-187.

Natesan B., and Nelson S. (2014). *Bacillus pumilus* S124A carboxymethyl cellulase; a thermos stable enzyme with a wide substrate spectrum utility. International Journal of Biological Macromolecules. 67: 132-139.

Prabesh K., Jahed A., Mehadi H. S., Kamrul I., and Abul K. A. (2016). Isolation, screening and characterization of cellulase producing bacterial isolates from municipal solid wastes and rice straw wastes. Journal of Bioprocessing and Biotechniques. 6, 280. doi:10.4172/2155-9821.1000280

Rawat R., and Tewari L. (2012). Purification and characterization of an acidothermophilic cellulase enyme produced by *Bacillus subtilis* strain LFS3. Extremophiles, 16(4): 637-644.

Rekha R., and Lakshmi T. (2012). Purification and characterization of an acidothermophilic cellulase enzyme produced by *Bacillus subtilis* strain LFS3. Extremophiles. 16, 637-644. DOI 10.1007/s00792-012-0463-y.

Sangrila S. and Tushar K. M. (2013). Cellulase production by Bacteria: A review. British Microbiology Research Journal, 3(3): 235-258.

Sreeja S. J., Jeba M. P.W., Sharmila J. F. R., Steffi T., Immanuel G., and Palavesam A. (2013). Optimization of cellulase production by *Bacillus altitudinis* APS MSU and *Bacillus licheniformis* APS2 MSU, gut isolates of fish *Etroplussuratensis*. International Journal of Advancements in Research and Technology, 2: 401-406.

Thi Q. D., and Quyen D. T. (2014). Enzyme supplement natural and recombination in feed. Natural Sciences and Technology Publisher.

Zahangir A. M., Nurdina M., and Erman M. M. (2005). Production of cellulase enzyme from oil palm biomass as substrate by solid state bioconversion. American Journal of Applied Sciences, 2: 569-572.