PRODUCTION AND CHARACTERISATION OF CELLULASE BY *BACILLUS PUMILUS* EB3

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ABSTRACT

Cellulase production from bacteria can be an advantage as the enzyme production rate is normally higher due to the higher bacterial growth rate as compared to fungi. Screening of bacteria, optimisation of fermentation conditions and selection of substrates are important for the successful production of cellulase. This study is conducted to produce cellulase by our local isolate Bacillus pumilus EB3, using carboxymethyl cellulose (CMC) as substrate. Following that, cellulase produced from B. pumilus EB3 was purified using ion exchange chromatography with anion exchanger (HiTrap QXL) for characterisation of the cellulase. Cellulase was successfully produced in 2L stirred tank reactor (STR) with the productivity of 0.53, 3.08 and 1.78 U/L.h and the maximum enzyme activity of 0.011, 0.079 and 0.038 U/mL for FPase, CMCase and β-glucosidase respectively. Purification of cellulase from B. pumilus EB3 using ion exchange chromatography showed that 98.7% of total CMCase was recovered. Protein separation was however based on subtractive separation where the contaminants were bound to the column instead of CMCase. Characterisation of the enzyme found that CMCase by B. pumilus EB3 has a molecular weight range from 30-65 kDa and was optimally active at pH 6.0 and temperature 60°C. The CMCase also retained its activity over a wide pH range (pH 5.0–9.0) and temperature range (30-70°C).

Key words: *cellulase, Bacillus pumilus EB3, enzyme purification, enzyme characterisation*

INTRODUCTION

Cellulase is one of the most useful enzymes in industry. Cellulase can be produced by fungi, bacteria or actinomycetes, but the most common producer is fungi. High cost of cellulase is mainly due to the substrates used in production, and also the slow growth rate of fungi. Bacteria, which has high growth rate as compared to fungi has good potential to be used in cellulase production. However, the application of bacteria in producing cellulase is not widely used. Bacterial cellulase usually lacks one of the three cellulase activities, that is FPase. However, cellulases produced by bacteria are often more effective catalysts. They may also be less inhibited by the presence of material that has already been hydrolyzed (feedback inhibition). The greatest potential importance is the ease with which bacteria can be genetically engineered. This is needed especially in order to enhance cellulase production.

Product recovery is an important step after fermentation. In enzyme production, recovery process includes separation of particulates, i.e. biomass and solid particles, extraction of enzyme, enzyme concentration and purification. Purification is important to study the function and expression of the enzyme and to remove any contaminants (other proteins or completely different molecules) that are present in the mixture. Normally, purification of enzymes is done by chromatography. There are few types of chromatography, namely ion-exchange, gel filtration and affinity chromatography. The most common method for cellulase purification is by ion-exchange chromatography. Ion-exchangers have been the most widely used chromatographic technique for the direct recovery of proteins and other charged molecules as the technique is known to have high resolving power, high capacity, simple to operate, highly robust, generic and economical [1]. Studies and researches on cellulase purification have been widely studied, and most of the work adopted ion-exchange chromatography as the method of purification [2-5].

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The objective of this study is to screen and identify a locally isolated cellulase-producing bacterium for the production of cellulase using CMC as the substrate. Purification of the cellulase is also conducted to characterise the cellulase.

MATERIALS AND METHODS

Screening of Cellulase Producer

A total of 9 strains of bacteria and 2 strains of actinomycetes were obtained from MARDI (Malaysian Agricultural Research and Development Institute). The strains were isolated from oil palm empty fruit bunch (EFB) heap meant for composting. As they were isolated from rotten lignocellulose, there is high chance that the strains are cellulase producer. Therefore, a preliminary qualitative analysis for cellulolytic activity was conducted by using Congo red dye. The bacteria were grown on CMC agar containing (g/L) KH_2PO_4 1.0, $MgSO_4.7H_2O$ 0.5, NaCl 0.5, $FeSO_4.7H_2O$ 0.01, $MnSO_4.H_2O$ 0.01, NH_4NO_3 0.3, CMC 10.0, Agar 12.0. The pH was adjusted to 7.0 with 1M NaOH. The CMC agar plates were incubated at 37°C for 5 days to allow for the secretion of cellulase. At the end of the incubation, the agar medium was flooded with an aqueous solution of Congo red (1% w/v) for 15 minutes. The Congo red solution was then poured off, and the plates were further treated by flooding with 1M NaCl for 15 minutes. The formation of a clear zone of hydrolysis indicated cellulose degradation. The ratio of the clear zone diameter to colony diameter was measured in order to select for the highest cellulase activity producer. The largest ratio was assumed to contain the highest activity.

Identification of Cellulase Producer

Strain EB3 which was the highest cellulase activity producer was picked for identification and further investigation. Preliminary identification by morphological analysis was conducted by using light microscope and SEM (Scanning Electron Microscopy, JEOL-6400). The genotype of the strain was also identified. The 16S rDNA sequences of the strain were determined. Extraction of DNA was carried out by using GF_1 DNA Extraction Kit (Vivantis (M) Sdn Bhd). PCR-mediated amplification of the 16S rDNA gene and sequence determination were performed by Vivantis (M) Sdn Bhd by following the manufacturer's instruction. Comparisons of the sequence between different species suggest the degree to which they are related to each other. This was done by constructing phylogenetic tree using neighbour-joining (N-J) method.

Cellulase Production in 2L Stirred Tank Reactor

Medium for cellulase production contained (g/L) KH_2PO_4 1.0, K_2HPO_4 1.145, $MgSO_4.7H_2O$ 0.4, $(NH_4)_2SO_4$ 5.0 $CaCl_2.2H_2O$ 0.05 and $FeSO_4.7H_2O$ 0.00125. 10 g/L of CMC was used as carbon source. The pH was adjusted to initial pH 7.0 by 1M NaOH. Fermentation was done in a 2L STR at the temperature of 37°C and constant impeller speed of 450 rpm. The air was supplied continuously at 0.5 vvm. Five milliliter of samples were withdrawn at intervals and centrifuged at 5000 rpm for 15 minutes at 4°C. The culture filtrates were analysed for FPase, CMCase and β-glucosidase activities, as well as protein content, cells growth and reducing sugars content.

Protein Purification by Ion Exchange Chromatography

Ion-exchange chromatography was used for purification of cellulase. Cellulase purification was conducted using AKTA FPLC (Fast Protein Liquid Chromatography) by Amersham Pharmacia Biotech. Running buffer used was 50 mM phosphate buffer (pH 7.0) and elution buffer was 1M NaCl in 50mM phosphate buffer pH 7.0.

Characterisation of CMCase (Endoglucanase)

Purified cellulase was characterised for its molecular weight, pH and temperature optimum. For molecular weight determination, SDS-PAGE was conducted [6] and was performed on a BIO-RAD Mini Protean 3 Cell (BIO-RAD, U.S.) with 12% acrylamide gel using Fermentas protein molecular weight marker. For optimum pH activity, the enzyme was tested in the pH 4.0 – 12.0 range: pH 4-6, 50mM acetate buffer; pH 7-8, 50mM phosphate buffer; pH 9-10, 50mM Glycine/NaOH; pH 11-12, 50mM KCl/NaOH. The enzyme was also tested within the temperature range of 30-80°C to determine the optimum temperature.

Analytical Methods

Carboxymethyl-cellulase (CMCase) activity: CMCase activity was assayed using a modified method described by Wood and Bhat [7] with some modifications. 0.2 mL of culture filtrate was added to 1.8 mL of 1% CMC prepared in 0.05M sodium citrate buffer (pH 4.8) in a test tube and incubated at 40°C for 30 minutes. The reaction was terminated by adding 3.0 mL of dinitrosalicylic acid (DNS) reagent and by subsequently placing the reagent tubes in water bath at 100°C for 15 min. One milliliter of Rochelle salt solution (40 g Rochelle salt in distilled water to make the volume 100 mL) was then added to stabilise the colour. The absorbance was recorded at 575 nm against the blank (of 0.05M sodium citrate buffer). One unit of CMCase activity was expressed as 1 μmole of glucose liberated per mL enzyme per minute.

Filter-paperase (FPase) activity: The activity of FPase was assayed according to the method explained by Wood and Bhat [7] with some modifications. Briefly, the methods are similar to the CMCase assay method, but the substrate used was Whatman no. 1 filter paper strip (1 x 3 cm) soaked in 1.8 mL 0.05M sodium citrate buffer (pH 4.8). The samples were incubated at 40°C for 60 minutes. The reaction was terminated by adding 3.0 mL of dinitrosalicylic acid (DNS) reagent and by subsequently placing the reagent tubes in water bath at 100°C for 15 min. One milliliter of Rochelle salt solution (40 g Rochelle salt in distilled water to make the volume 100 mL) was then added. The absorbance was recorded at 575 nm against the blank (of 0.05M sodium citrate buffer). One unit of FPase activity was determined as 1 μmole of glucose liberated per mL enzyme per minute.

β-Glucosidase activity: 0.2 mL of culture filtrate was added to 2.0 mL of 0.15g/L pNPG (p-nitrophenyl-β-D-glucopyranoside) prepared in 0.05M sodium citrate buffer (pH 4.8) and incubated at 40°C for 30 minutes. Two milliliter of 1M Na_2CO_3 was added to terminate the reaction. The method was described by Wood and Bhat [7] with some modifications. The absorbance was recorded at 400 nm. One unit of β-glucosidase activity was determined as 1 μmole of p-nitrophenol liberated per mL enzyme per minute.

Protein content: Protein content was determined by Bio-Rad Protein Assay (dye reagent) obtained from Bio-Rad Singapore. The assay method is based on the method of Bradford [6]. In this assay, five milliliter of diluted dye reagent was pipetted into 100 μL of sample solution. The mixture was then incubated at room temperature for at least 5 minutes, but not more than 1 hour. The absorbance was measured at 595 nm against the blank of deionised water.

Reducing sugars content: Reducing sugars analysis was conducted based on the method as described by Wang [8]. In this method, 2 mL of diluted sample was added to 3 mL of DNS and boiled for 15 minutes. After boiling, 1 mL of Rochelle salt was added. The absorbance was recorded at 575 nm using spectrophotometer against the blank of distilled water.

RESULTS AND DISCUSSION

Screening and Identification of Cellulase Producer

Screening of bacteria was conducted by using the Congo red test as a preliminary study for identifying cellulase producers. After 5 days of incubation, all 9 strains of bacteria (EB1 – EB9) and 2 strains of actinomycetes (Act 1 and 2) showed signs of growth on CMC agar and demonstrated positive results in the Congo red test. Since the sole carbon source in CMC agar was CMC (cellulose), therefore the result of the test was a strong evident that cellulase was produced in order to degrade cellulose.

EB3 gave the highest ratio of clear zone diameter to colony diameter. This indicated more cellulose degradation in CMC agar plate cultured with EB3 as compared to plates cultured with the other strains. Preliminary study on morphology of the bacterium suggested that the bacterium is from *Bacillus* species. The bacterium has rod shape and had a positive gram stain reaction. SEM (Scanning Electron Microscopy) analysis was conducted and based on the SEM micrograph (Figure 1), it was evident that the bacterium has rod shape and the size of the bacterium was about 2 -3 μm. In order to characterise the strain, the nucleotide sequences of the 16S rDNA of the strain was determined. Phylogenetic tree was constructed by the neighbour-joining (N-J) method based on the 16S rDNA sequences. The results revealed that strain EB3 exhibited high level of 16S rDNA similarity (99%) with *B. pumilus*.

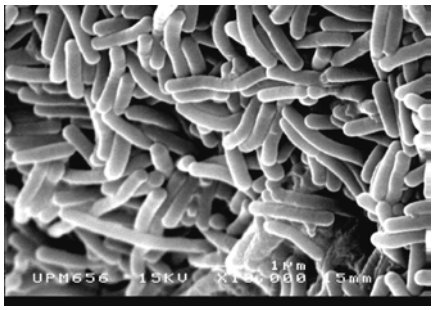

Figure 1: SEM micrograph of B. pumilus EB3 (x10,000)

Cellulase Production in 2L Stirred tank Reactor

Figure 2 illustrates cellulase activities and total protein content in the broth during fermentation for cellulase production in 2L STR. Overall, maximum cellulase activity was obtained after 24-hour of fermentation with the activities recorded were 0.011, 0.079 and 0.038 U/mL for FPase, CMCase and β-glucosidase respectively. Figure 3 shows the growth profile of the bacterium during fermentation. From the figure, it was seen that the cellulase was being produced during growth phase of the *B. pumilus* EB3. This is in agreement with the previous study which showed that cellulase is a growth-associated product [9].

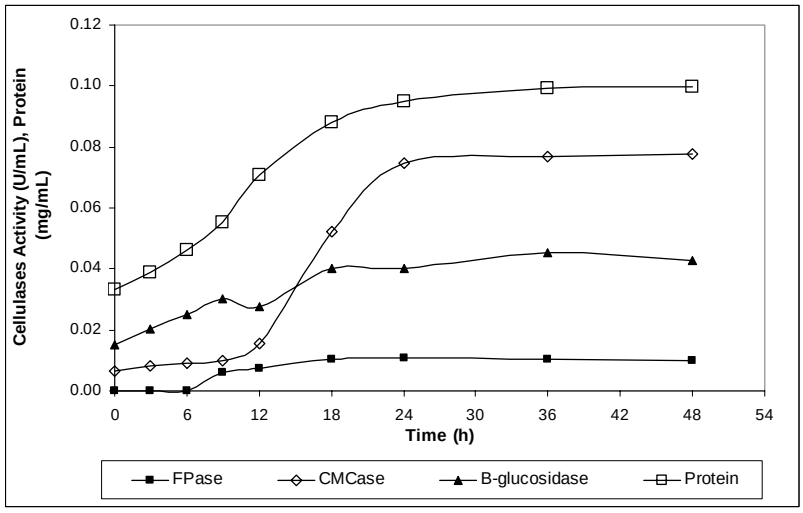

Figure 2: Cellulase activities and total protein content during fermentation in 2L STR

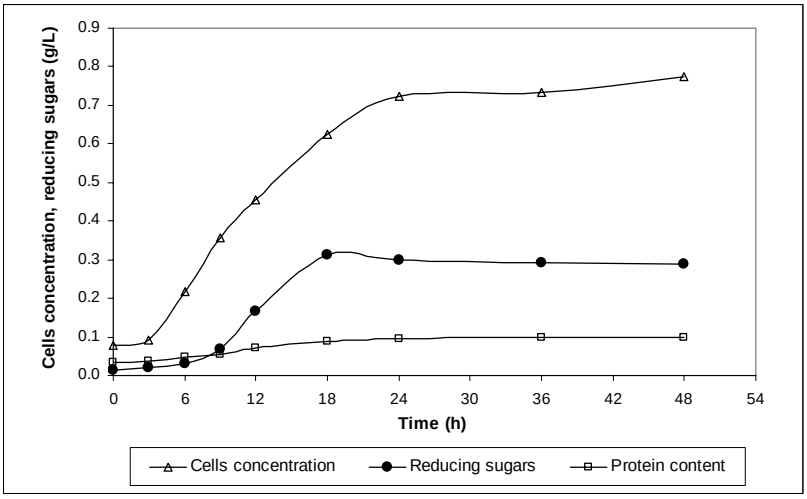

Figure 3: Growth profile of B. pumilus EB3 during fermentation for cellulase production in 2L STR

Purification of Cellulase

The enzyme was purified by ion-exchange chromatography. Table 1 shows the results for purification of endoglucanase using Q XL HiTrap column at pH 7. It was seen that 94.8% of CMCase was recovered from the flowthrough and washing fractions, and the overall CMCase yield was high at 98.7%. This indicated that the CMCase was unbound to the column. Based on the results obtained, the technique can be considered as subtractive-separation [10] as generally in chromatography, the protein of interest is bound while the contaminants are allowed to pass through the column. However, in this case the results are contrary to the general condition and hence, are called subtractive-separation.

Table 1: Purification table for CMCase purified using HiTrap Q XL column at pH 7.0 and flow rate 0.5 mL/min

Purification Stage	Volume (mL)	Total protein (mg)	CMCase Activity (U)	CMCase Yield (%)	Specific Activity (U/mg)	Purification factor
Clarified feedstock	2	0.87	1.54	100	1.77	1
Flowthrough	2	0.19	0.38	24.67	2.02	1.14
Washing	5	0.56	1.08	70.13	1.91	1.08
Elution	10	0.11	0.06	3.89	0.57	0.32
Recovery		**0.86**	**1.52**	**98.69%**		

Characterisation of Cellulase from *B. pumilus* EB3

SDS-PAGE was conducted to determine the molecular weight of the cellulase produced by *B. pumilus* EB3. Figure 4 shows the SDS-PAGE result for crude and purified cellulase from *B. pumilus* EB3. Purified enzyme has a molecular weight range from 30 – 65 kDa. Some molecular weight endoglucanase (23 - 42kDa) from *Bacillus sp*. has been reported [3, 11]. The highest molecular weight of endoglucanase from *B. pumilus* EB3 that is 65 kDa is almost similar with the molecular weight of endoglucanase from *B. pumilus* as being studied previously [12]. A range of molecular weight suggested that the enzyme is an isoenzyme. Endoglucanase as isoenzyme has been found by a few researchers [13-14].

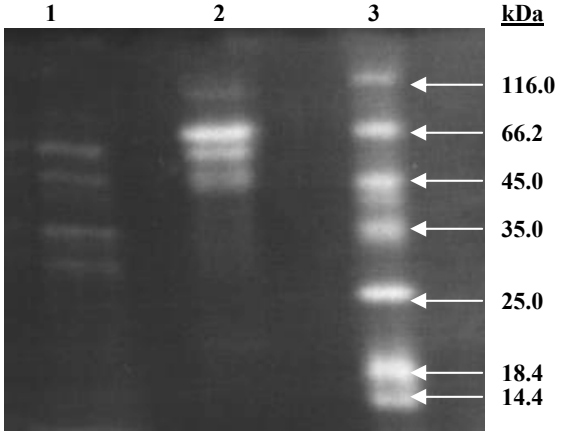

Figure 4: SDS-PAGE of purified cellulase
Lane 1 - purified cellulase, Lane 2 - commercial cellulase (from Trichoderma sp. – Celluclast 1.5L)
Lane 3 - molecular weight markers

B. pumilus EB3 cellulase exhibited the highest activity at pH 6 for temperatures 50 and 60°C (Figure 5). The enzyme was optimally active between pH 5 and 9. When incubated in pH 9 buffer at 60°C, the enzyme was able to retain 69% of its maximum activity. The result was almost similar with the previous research [15] where the endoglucanase from *B. pumilus* was shown to be active at a pH ranging from pH 5-8. Another researcher reported on endoglucanase from *B. pumilus* and the enzyme was active at an optimum pH of 6.5 [16]. As compared to fungal endoglucanase, bacterial cellulase or specifically endoglucanase from *B. pumilus* EB3 has advantage in term of pH optimum as buffering system is not needed in saccharification or hydrolysis of cellulose as the enzyme can optimally hydrolyse the cellulose in near neutral pH. Most of the fungal cellulase

has an optimum pH of 4.0–6.0 [13, 17-19]. Besides, the wide range of optimum pH (pH 5–9) is also an advantage as the enzyme can retain its activity over a wide pH range.

Incubation temperature also affects the activity of endoglucanase. To find the optimum temperature for endoglucanase activity from *B. pumilus* EB3, the enzyme was incubated at a temperature range of 30- 80°C with various pH (pH 4–12). This was done as pH also affects cellulase activity. The highest endoglucanase activity was obtained when the enzyme was incubated in pH 6 buffer at temperature 60°C (Figure 6). This is similar with two previous reports on endoglucanase by *B. pumilus* where both of the reports showed that endoglucanase from *B. pumilus* has an optimum temperature of 60°C [12, 15].

Endoglucanase from *B. pumilus* EB3 was observed to be active over a wide temperature range, up to 70°C. It retained 66% of its optimum activity when incubated at 70°C. However, the activity was decreased abruptly with only 13% of the activity retained when the enzyme was incubated at 80°C. The high temperature for optimum endoglucanase activity is also an advantage over fungal cellulase as common cellulase from fungus, namely *Trichoderma* and *Aspergillus sp.* has an optimum temperature of 40 - 55°C [13,17-18].

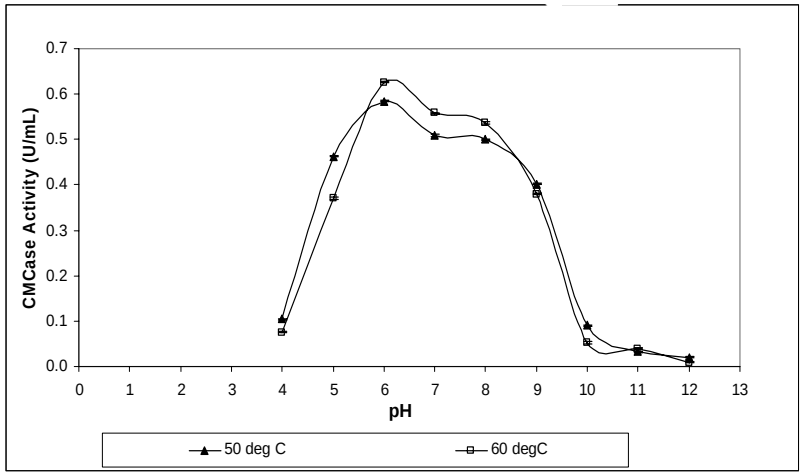

Figure 5: Effects of pH on endoglucanase activity incubated at temperature 50 and 60ºC. The vertical bars represent the standard deviation of the mean calculated for duplicates

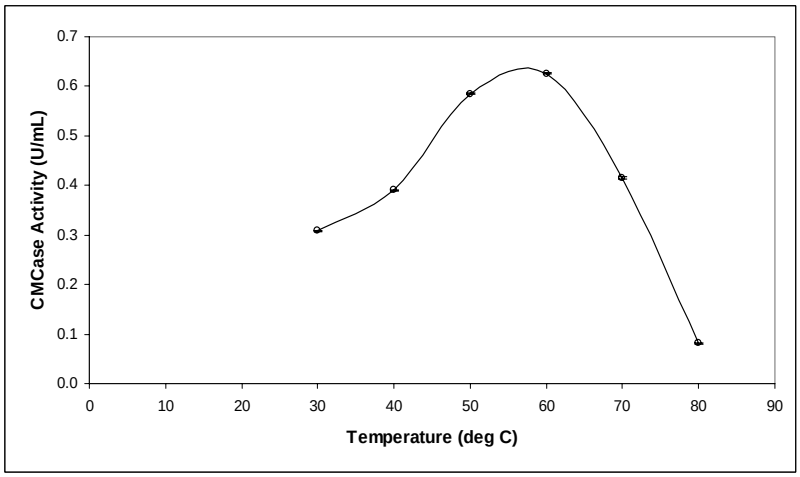

Figure 6: The effect of temperature on endoglucanase activity incubated at pH 6.0

CONCLUSION

A locally isolated bacterium was successfully screened for cellulase production and was identified as *B. pumilus* EB3. Cellulase from *B. pumilus* EB3 was successfully produced in 2L STR with the maximum activities recorded were 0.011, 0.079 and 0.038 U/mL respectively for FPase, CMCase and β-glucosidase. The cellulase

was purified using subtractive anion exchange chromatography and was later characterised. Purified CMCase has a molecular weight range of 30-65 kDa, optimum temperature of 60°C and pH 6.0. Comparison of the characteristics of CMCase from *B. pumilus* EB3 revealed that the CMCase has an advantage over other fungal cellulases. Higher optimum pH and temperature can lead to a higher reaction rate. Besides, CMCase from *B. pumilus* EB3 also retained its activity over a wide pH and temperature range. This allows the manipulation of process parameters when CMCase from *B. pumilus* EB3 is used.

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