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To cite this article: N Y Hasnol Azahari *et al* 2020 *IOP Conf. Ser.: Mater. Sci. Eng.* **932** 012035

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# Isolation of alkali-thermotolerant cellulolytic bacteria from spent mushroom substrate

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**Abstract.** In this study, the isolation of new alkali-thermophilic bacteria with enhanced cellulase producing ability from the spent mushroom substrate was conducted. Repeated streaking was performed on the Microcrystalline-cellulose (MCC) to obtain a pure culture. Two thermophiles bacteria isolates were used for subsequent experiment. Morphological characteristic by gram staining and endospore staining also genetic identification of 16S rDNA was performed and the growth profile had been generated using Polymath Software. Filter paper assay (determination of enzyme activity), Bradford protein concentration were used in plotting the growth associated product curve. Based on the 16S rDNA sequencing results, the bacterium strains of *Anoxybacillus geothermalis*, incubated at 50°C, pH 7 and *Aeribacillus pallidus*, incubated at 60°C, pH 8 was successfully isolated. Both strains are gram-positive and endospore-forming bacteria. Based on the growth curve, the doubling time for *Anoxybacillus geothermalis* was calculated to be 49.5 minutes. Whereas, doubling time of *Aeribacillus pallidus* was 56.7 minutes. Both strains represent the growth associated product curve in which, when product formation is growth associated the specific rate of the product formation increases with specific growth rate and vice versa.

## 1. Introduction

Cellulosic or lignocellulosic compound is the organic matters that can be found abundantly in plants. Microorganisms that producing cellulose enzyme are capable of hydrolyzing the cellulosic materials [1]. Cellulose is the components that makes of plants' cell wall. It is presumably the most plenteous organic compound on the earth since it is made by all plants [2]. Cellulose, (C<sub>6</sub>H<sub>10</sub>O<sub>5</sub>)<sub>n</sub> is a polysaccharide comprising a linear chain of several hundred to many thousands of β(1→4) linked D-glucose units [3]. It has numerous uses over being the primary building material for plants by its nature to make paper, explosives, film, and plastic depending on how it is treated [3].

Cellulose is a water-insoluble compound and does not deliquesce effectively in water. It is chiral in structure and is biodegradable. It can be separated chemically into glucose units by treating it with concentrated mineral acids at high temperature or can be hydrolyzed by the enzyme, cellulase [4]. Those microorganisms that have cellulase will degrade the cellulose into glucose. Cellulase is being generated by cellulolytic microorganisms which include yeast, bacteria, fungi, and actinomycetes.

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Primitively, fungi are the precedence once due to its capability to produce copious amounts of cellulases, which are less complex, can be easily extracted and purified than bacterial glycoside hydrolases. However, due to certain benefits such as higher affinity and feasibility towards genetic engineering, a higher growth rate of bacteria, and its synergy of their complex enzyme system, bacteria are preferred over fungi [5].

A spent mushroom substrate (SMS) is the co-product that was produced in almost unconfined amount after done harvesting the mushrooms, either in the production of white button mushroom (*Agarius bisporus*), shiitake (*Lentinula edodes*) or oyster mushrooms (*Pleurotus spp.*). The mushroom cultivating process had mass-produced the compost of organic medium, spent mushroom substrate. The term "spent mushroom substrate (SMS)" or in other words, it's also being called "spent mushroom compost (SMC)" portraying the agro-residues and fungal mycelium left after harvesting of mushrooms [6]. Spent mushroom substrate typically rich in cellulosic compound [7]. Thus, spent mushroom substrate is a good source for cellulose exploration.

The present study is focused on the isolation of alkali-thermotolerant cellulolytic bacteria from spent mushroom substrate. Alkaliphile means the ability of some groups of the bacteria that are capable of survival in alkaline environment, roughly at pH 8.5 to 11. These extraordinary adaptation mechanisms are exhibited by certain types of bacteria only. Thermotolerant is the ability of the microorganism to grow at the higher array of temperature. Thermophiles have cell membranes that are drenched with fatty acids. Thus it provides a hydrophobic atmosphere for the cell to maintain the cell rigidity at high temperatures [8]. From the biotechnological judgment, the dual properties of alkaliphile and thermotolerant capability is attractive for applications such as household laundry detergents where enzymes and additives need to be active in callous washing conditions for improving fabrics softness and brightness [9].

## 2. Materials and methods

### 2.1 Sample collection

Approximately 50 g of spent mushroom substrate was collected using a sterile microcentrifuge tube from Kaki Bukit, Perlis. The collected sample was sealed, labelled in right manner and stored at 4°C for further use.

### 2.2 Media preparation

Microcrystalline Cellulose (MCC) agar plate was prepared as the following composition: [agar powder, 15.0 g/L; KH<sub>2</sub>PO<sub>4</sub>, 1.5 g/L; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.0 g/L; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g/L; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g/L; NaCl, 2.0 g/L; yeast extract, 1.0 g/L; ZnSO<sub>4</sub>, 0.01 g/L; MnSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g/L; CoCl<sub>2</sub>, 0.01 g/L; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.5 g/L; MCC, 10 g/L]. The composition was added to 1000 mL of distilled water. The agar plates of different pH (7-13) were prepared. The medium was sterilized at 121°C, 15 psi for 15 minutes. MCC broth was prepared by dissolving all the components mentioned above except the agar powder. For Carboxymethyl Cellulose (CMC) broth, its preparation is same as MCC broth and just substitute MCC with CMC.

### 2.3 Isolation of bacteria

Approximately 5 g of spent mushroom substrate was added in 150 mL of sterile deionized water. After that, 3 mL from the solution was transferred to 97 mL of MCC broth. The sample was incubated for 2 days at 2 different temperatures, 50°C and 60°C. Subsequently, 100 µL of the sample was pipetted and spread on MCC agar of different pH. One with original concentration and the other with 10x dilution. Then, both plates were incubated in the oven for 2 days at temperature 50°C and 60°C respectively. A single colony grown on spread plates was picked and streak repeatedly until obtaining the pure colonies. The plates of pure colonies were kept at 4°C for preservation as well as further identification and characterization of cellulose-degrading bacteria. The isolates from the plate B2(2)-temperature

50°C, pH 7 and plate A2-temperature 60°C, pH 8 was used for the subsequent experiments after the isolation step.

#### *2.4 Morphological and genetic identification*

There are three test were conducted under morphological which are colony appearance, gram staining, and endospore staining, then the isolates was sent for analysis of 16S rDNA identification [10]. Colony appearance was performed by observing the bacteria under microscope to study its elevation, opacity, edge, margin and color [11]. Gram staining was performed according to its distinctive principle of applying crystal violet, mordant, ethanol and lastly safranin [12]. While endospore staining also was performed accordingly by applying Malachite green then washed with water then safranin was applied [13].

#### *2.5 Reagent preparation*

Concentration of 0.05 M of citrate buffer was prepared by dissolving 5.25 g of citric acid monohydrate in 500 mL of distilled water. The pH was adjusted to pH 4.8. While, for preparation of DNS reagent, ten grams of sodium hydroxide (NaOH) was added to 1 L of volumetric flask. Subsequently, 10 g of dinitrosalicylic acid (DNS), 2 g of phenol, 0.5 g of sodium sulphide and 500 mL of deionized water were added and stirred until all of the compounds dissolved. Finally, 200 g of potassium sodium tartrate was added and the deionized water was topped up to 1 L. This preparation was done under dark condition and stored in an amber bottle.

#### *2.6 Growth profile construction*

This being done by using two types of broth in which nutrient broth and CMC broth. Three percent of inoculums, was pipetted into each of 97 mL nutrient and CMC broth in a 250 mL flask. The flask then was incubated at 50°C for pH 7 and 60°C for pH 8 at 150 rpm for 24 hr. Sampling was done for every 1 hour (for nutrient), 3 hours each (for CMC) and absorbance of 600 nm was determine using a UV-vis spectrophotometer. The supernatant in CMC broth was indicated as crude enzymes. These crude enzymes were used in cellulase activity and protein content quantification experiments.

#### *2.7. Quantification of cellulase activity*

Cellulase activity was assayed by the determination of reducing sugar released from Filter Paper. The substrate was prepared by cutting the filter paper to 1.0 cm X 6.0 cm. Then, 1 mL of 0.05 M sodium citrate buffer with pH 4.8. was added to a test tube. Supernatant was collected after centrifugation of the culture from previous experiment at 10,000 rpm for 10 minutes. The supernatant indicated as the crude enzyme. An aliquot of 0.5 mL of crude enzyme then was transfered to different test tube. The substrate then was added into the test tube and incubated at 50°C, 60 minutes. To stop the reaction, 3.0 mL of 3,5- dinitrosalicylic acid (DNS) was added. The test tube then was placed in a water bath at 95°C for 5 minutes and was transferred into the cold water bath for 2 minutes. To measure the absorbance, 0.2 mL of the mixture was pipetted out to another new 14 mm of test tube and, 2.5 mL of distilled water was added. An aliquot of 1.0 mL of the diluted mixture was transferred to a cuvette and its absorbance was measured at 540 nm [14].

#### *2.8 Quantification of protein content*

Protein Bradford test used BSA (Bovine Serum Albumin) as standard and 30 µl of crude enzyme was transferred into test tube . Then, 1.5 mL of Bradford Reagent was added into the solution. After 5 mins, 1 mL of each solution was pipetted into cuvette and the absorbance was read at 595nm.

#### *2.9 Growth profile construction using POLYMATH software*

The data from the nutrient broth from exponential phase region was used and was tabulated in the software for calculating of regression model used [15].

$$\text{Non-linear model equation: } xt = x_0 e^{mt} \quad (1)$$

$$\text{Doubling time equation: } t_d = \frac{\ln 2}{\mu} \quad (2)$$

### 3. Results and discussion

#### 3.1 Isolation and screening of alkali-thermotolerant cellulolytic bacteria

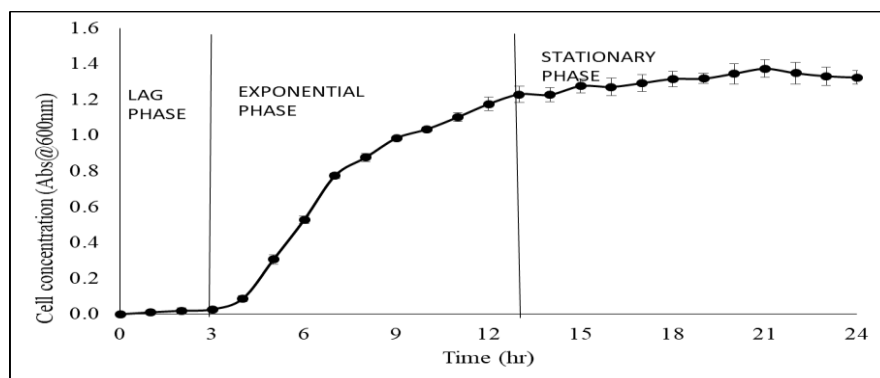
From the isolation steps of spread and streak plate procedures, different colonies were identified and further streaked to obtain a single colony. After morphological identification, there were five plates of single colony obtained in which incubated at their respective condition of 50°C, pH 7 (B2-2, A1), pH 7, 60°C (A1-2, B) and 60°C, pH 8 (A2). MCC agar plate was used from the beginning as a selective medium in which only cellulase producers can grow. The screening results based on filter paper assay revealed that sample 1 (B2-2) has liberated the high glucose content, (0.26 mg/0.5 mL at 12hrs sampling compared to other samples. Only one isolate has been obtained from conditions at 60°C, pH 8. Based on the cellulase activity results, strain B2-B and A2 have been selected for the subsequent experiments. Table 1 shows the data of the morphological characteristic for both strain. While, according to the 16S rDNA the strain is identified as *Anoxybacillus geothermalis* (50°C, pH 7) and *Aeribacillus pallidus* (60°C, pH 8) and both with 99 % similarity. Based on previous study, the exact same result as shown in table 1 of this present study was determined [16,17,18].

**Table 1.** Morphological Characteristic for isolated bacteria of both strains of *Anoxybacillus geothermalis* and *Aeribacillus pallidus*

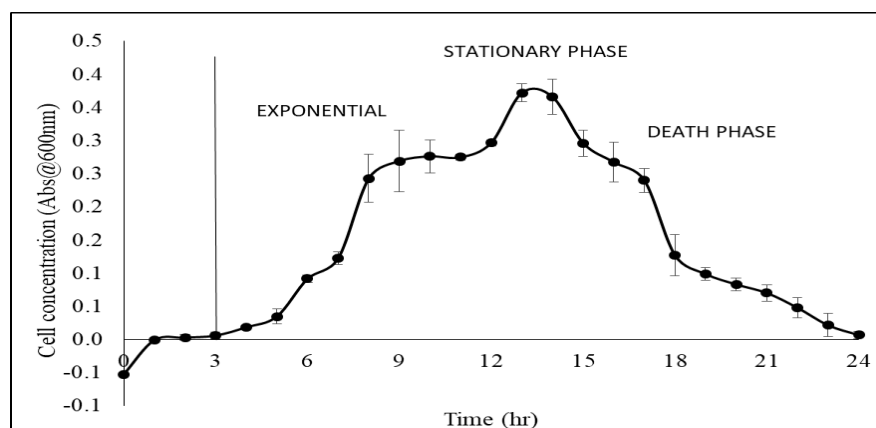
Test Analysis	Types	Features	Result
Morphology characteristic	Microscopic examination	Gram's staining	+
		Shape	Rods arranged in pairs
		Endospore formation	+
	Macroscopic appearance: colony morphology	Colour /pigmentation	White to off
		Elevation	Raised
		Margins	Entire
	Opacity	opaque	
	Form	Circular	

#### 3.2 Growth curve profile construction using nutrient broth

Figure 1 and Figure 2 shows the dynamic of bacterial growth by plotting the graph of cell concentration versus time of incubation (h). Generally, bacterial growth curve is in sigmoid curve, and *Anoxybacillus geothermalis* (Figure 1) present the exact sigmoid curve. However, the graph observed for *Aeribacillus pallidus* (Figure 2) is in unsymmetrical sigmoid shape due to some constrain such as nutrient in the medium, temperature and pH of the nutrient broth itself [19]. This growth curve had been constructed successfully using alkalo-thermotolerant cellulolytic bacterium as it is incubating at 50°C, pH 7 and 60°C, pH 8. The data from exponential phase was further analyzed using Polymath software in order to measure its growth accurately.



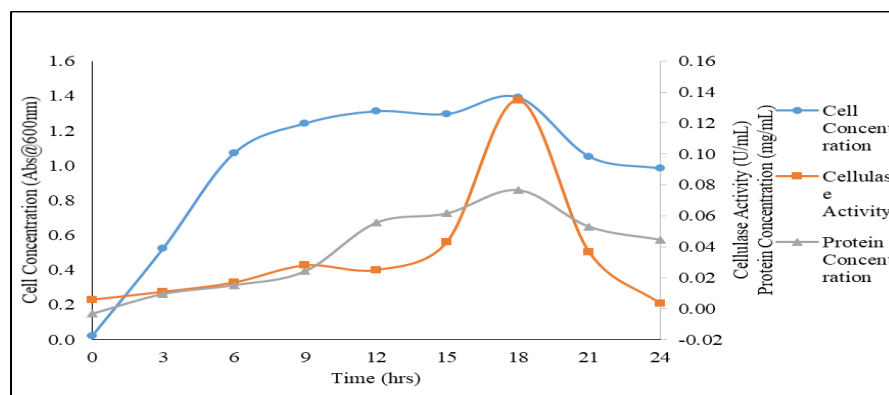
**Figure 1.** The graph of absorbance versus time of incubation of *Anoxybacillus geothermalis* at 50°C for pH 7 in nutrient broth medium.



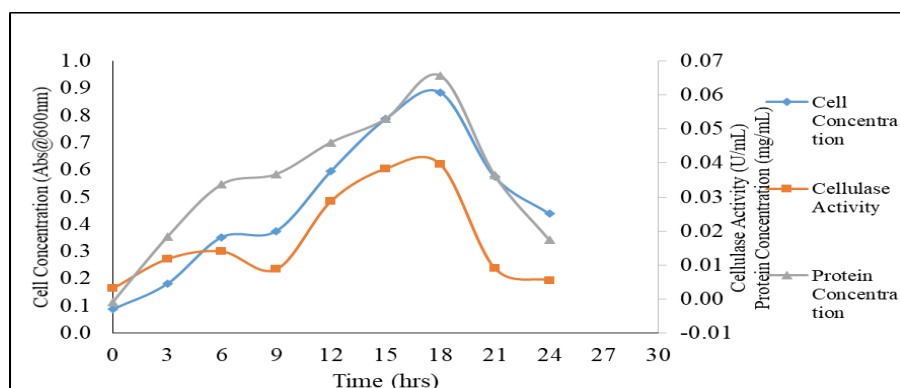
**Figure 2.** The graph of absorbance versus time of incubation of *Aeribacillus pallidus* at 60°C for pH 8 in nutrient broth medium.

### 3.3 Quantification of cellulase activity and protein content

The Figure 3 and Figure 4 shows the graph of growth associated product curve for all of the analysis performed. The result obtained indicate that when product formation is growth associated the specific rate of the product formation increases with specific growth rate and vice versa.



**Figure 3.** The graph of the cell concentration, cellulase activity of filter paper assay, protein concentration versus time, h for *Anoxybacillus geothermalis* incubate at 50°C, pH 7.

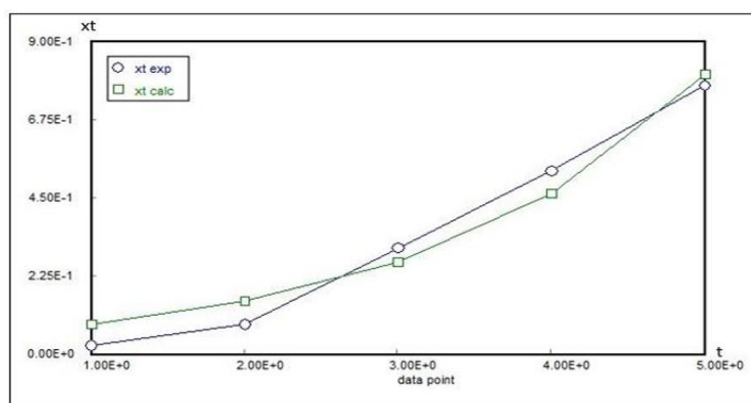


**Figure 4.** The graph of the cell concentration, cellulase activity of filter paper assay, protein concentration versus time, h for *Aeribacillus pallidus* incubate at 60°C, pH 8.

According to the graph (Figure 3) for *Anoxybacillus geothermalis* incubate at 50°C, pH 7, and (Figure 4) for *Aeribacillus pallidus* incubate at 60°C, pH 8, increasing in the number of cell concentration resulting in increasing of protein concentration and cellulase activity over time. The highest value of the three test for both strains was in 18hr of incubation time, with 0.0766 mg/mL and 0.1348 U/mL of *Anoxybacillus geothermalis* and for *Aeribacillus Pallidus*, 0.0675 mg/mL and 0.0397 U/mL for its protein concentration and cellulase activity respectively. The graph verified that, product formation is growth associated the specific rate of the product formation increase with increasing of specific growth rate and vice versa. However, *Anoxybacillus geothermalis* was more stable compare to *Aeribacillus pallidus* as it cell concentration plotted, or bacterial growth curve was in symmetrically sigmoid curve. Radchenkova, N *et al.*, (2013) had reported that the time for reaching maximum productions of exopolysaccharides (cellulase) for *Anoxybacillus sp.* is at 15hr and *Aeribacillus sp.* is at 20hr [16]. The less the time for maximum production, the more the product. The lag phase of the growth profile also shorter [20]. Thus, *Aeribacillus pallidus* isolated from spent mushroom substrate are more stable as it has shorter time to reach maximum activity. Meanwhile, *Anoxybacillus geothermalis*, its activity is a bit slower (less productive) and it may due to the different species of bacteria and also from previous study, it was isolated from hot spring in Bulgaria and the *Anoxybacillus geothermalis* was isolated from spent mushroom substrate at mild conditions.

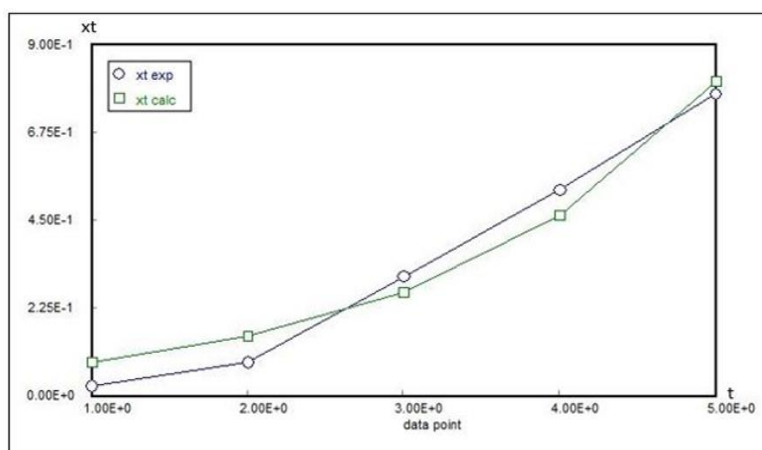
### 3.4 Growth profile studies of growth using Polymath software

In this research, the growth of bacteria was studied using Polymath software. The absorbance reading from growth profile were used in this software to generate a non-linear graph based on selected equation. From the generated graph which shown in Figure 5 and Figure 6, the value inserted near linear line and this indicated the accuracy of the data based on equation provided (Eq.1). The rectangular pattern in the graph represent the inserted equation for the kinetic growth of non-linear pattern. On the other hand, round pattern represents the value from the absorbance which has been inserted into polymath software. For *Anoxybacillus Geothermalis*, the initial concentration was 0.0273 and its specific growth rate was determined to be 0.84 hr<sup>-1</sup>. A regression plot (Figure 5), was established and its shows two lines.  $X_t$  calc represents the concentration of the biomass at the specific time calculated based on the equation of non-linear model (Equation 1). Meanwhile, the  $X_t$  exp indicates the experimental values obtained for the cell concentration. The plot was established by using the linear regression (R<sup>2</sup>) valued which was computed to be 0.9615 and its adjusted R<sup>2</sup> valued was determined to be 0.9832 which is also very near to experimental R<sup>2</sup> and the regression model is correct as the value close to 1. The value of m and variance were 0.8366 and 0.0050 respectively [15].



**Figure 5.** Non-linear graph of *Anoxybacillus geothermalis* incubate at 50°C, pH 7, generated from polymath software

Meanwhile, for *Aeribacillus Pallidus*, the initial concentration was 0.0062 and its specific growth rate was determined to be  $0.94 \text{ hr}^{-1}$ . The regression plot (Figure 6) was verified and ( $R^2$ ) valued which was computed to be 0.9865 and the adjusted  $R^2$  valued was 0.9487 which is very near to experimental  $R^2$ . The regression model is correct as the value obtained also close to 1. The value of  $m$  and variance were 0.7339 and 0.0001 respectively.



**Figure 6.** Non-linear graph of *Aeribacillus pallidus* incubate at 60°C, pH 8, generated from polymath software

RMSD is the square root of the variance, is always non-negative and a value of 0 is almost never achieved in practice and indicate a perfect fit to the data. Lower RMSD and variance value is better than a higher one, thus this RMSD value of the experiment for both *Anoxybacillus geothermalis* and *Aeribacillus pallidus* is acceptable and the accuracy of the growth profile is higher [15].

Naresh *et al.*,(2018) revealed that *Anoxybacillus sp* has doubling time of 32.3 minutes while the *Anoxybacillus geothermalis* had 49.5 minutes of doubling time which is longer and it may be due to some different condition such as temperature, pH used, carbon source and salts concentration. Instead of CMC agar, MCC agar was used in the present study. Its composition was slightly different and its carbon content also different thus it lead to slow doubling time of *Anoxybacillus geothermalis* [21]. On the other hand, theres no previous research that had discovered the doubling time for *Aeribacillus pallidus* and it had been determined to be 56.7 minutes.

#### 4. Conclusion

There were two isolates of alkali-thermotolerant cellulolytic-degrading bacteria were identified from this research which are *Anoxybacillus geothermalis* (50°C, pH 7) and *Aeribacillus pallidus* (60°C, pH 8). Both strains are gram positive and endospores-forming bacteria. Cellulolytic activity of *Anoxybacillus geothermalis* and *Aeribacillus pallidus* was analyzed and both are best at 18hr of incubation time. However, *Anoxybacillus geothermalis* showed the highest cellulolytic activity (0.1348 U/mL) compared to *Aeribacillus pallidus* (0.0397 U/mL); approximately three times higher. From the data that were used in POLYMATH software of regression model, the analysis of the growth profile revealed that the doubling time of *Anoxybacillus geothermalis* was 49.5 minutes with specific growth rate of 0.84 hr<sup>-1</sup>. Whereas, doubling time of *Aeribacillus pallidus* was 56.7 minutes and its specific growth rate is 0.94 hr<sup>-1</sup>.

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### **Acknowledgements**

The authors would like to express their gratitude to the Department of Chemical Engineering Technology, Faculty of Engineering Technology (FETech) of Universiti Malaysia Perlis.