

# Immobilization of Cellulase from *Bacillus subtilis* UniMAP-KB01 on Multi-walled Carbon Nanotubes for Biofuel Production

Sandrasedkaran Naresh<sup>1</sup>, Siew Hoong Shuit<sup>1,\*</sup>, Balakrishnan Kunasundari<sup>1</sup>, Peng Yong Hoo<sup>1</sup>, Qi Hwa Ng<sup>1</sup> and Yi Peng Teoh<sup>1</sup>

<sup>1</sup>Department of Chemical Engineering Technology, Faculty of Engineering Technology, Universiti Malaysia Perlis (UniMAP), P.O Box 77, D/A Pejabat Pos Besar Kangar, Perlis, 01000, Malaysia.

E-mail: shshuit@unimap.edu.my

**Abstract.** *Bacillus subtilis* UniMAP-KB01, a cellulase producer was isolated from Malaysian mangrove soil. Through morphological identification it was observed that the *B. subtilis* appears to be in rod shaped and identified as a gram positive bacterium. Growth profile of isolated *B. subtilis* was established by measuring optical density (OD) at 600 nm for every 1 hour intervals. Polymath software was employed to plot the growth profile and the non-linear plot established gave the precision value of linear regression, R<sup>2</sup> of 0.9602, root mean square deviation (RMSD) of 0.0176 and variance of 0.0025. The hydrolysis capacity testing revealed the cellulolytic index of 2.83 ± 0.46 after stained with Gram's Iodine. The harvested crude enzyme after 24 hours incubation in carboxymethylcellulose (CMC) broth at 45°C and 100 RPM, was tested for enzyme activity. Through Filter Paper Assay (FPA), the cellulase activity was calculated to be 0.05 U/mL. The hydrolysis capacity testing and FPA shown an acceptable value for thermophilic bacterial enzyme activity. Thus, this isolated strain reasoned to be potential for producing thermostable cellulase which will be immobilized onto multi-walled carbon nanotubes and the cellulolytic activity will be characterized for biofuel production.

## 1. Introduction

Cellulase is an enzyme widely being utilized to breakdown cellulose. The abundance of the readily available lignocellulosic biomass such as agricultural waste drives the exploitation of the cellulase production in order to sustain the bioconversion of waste to the useful products [1]. Cellulase can be produced by microorganisms and plants. Nonetheless, microbial cellulase is much preferred as it easily available as extracellular enzyme in most of the system [2].

Currently, the commercial cellulase are being produced using the well-known *Trichoderma reesei* strain. *T. reesei* which has been referred as the best cellulase producer is a mesophilic fungal strain. Numerous studies have been carried out to enhance the cellulase production focusing on the optimization of fermentation conditions and recombinant technology to alter the gene of the mentioned fungal species [3]. Even so, bacterial enzyme was discovered to have some advantages compared to fungal enzyme as available in multi-enzyme complexes and the complexity is much higher than the latter [4]. This factors drive the interest of current research to take advantage of the bacterial enzymes.



The major bottleneck of using cellulase in bioconversion is the percentage enzyme recovery. Enzyme recovery is the process involving separation of enzymes from fermentation broth at the end of experiment for reusing it for subsequent batches. In order to do this, the enzyme immobilization technique has been introduced. Sodium alginate is the commonly used an entrapment technique to capture the enzymes thus enabling the recovery process [5]. Apart from this, there are a few other methods to immobilize the enzyme such as using the multi-walled carbon nanotube (MWCNT). Enzymes can be bounded on the MWCNTs using covalent and noncovalent approaches. The noncovalent approach has the advantage as less enzymes were denatured during the process compared to the covalent approach. Recently, the functionalized MWCNTs have gained wide interest among the researchers to serve as the base of immobilization for enzymes because of their intrinsic properties, such as high surface area and well-developed surface morphology and porosity. MWCNTs can be functionalized using organic, biological or polymeric molecules which enables the controlled immobilization and precision bounding of the enzymes on MWCNTs [6]. These properties made the recovery of enzyme immobilized onto the MWCNTs become possible.

*B. subtilis* is an aerobic, gram-positive, mesophilic bacterium commonly found in soil [7]. The *B. subtilis* UniMAP-KB01 was isolated from tropical mangrove soil at Taman Paya Bakau, Lumut, Perak, Malaysia. The strain is not fully tested for its capability for production of cellulase. Thus, this study will function as the preliminary data for better understanding of the isolated *B. subtilis* UniMAP-KB01 strain in production of cellulase which will be immobilized onto MWCNTs. The product from the enzymatic activity on cellulose (glucose) can be used as the main substrate of fermentation for future biofuel productions.

## 2. Materials and Methods

*Bacillus subtilis* UniMAP-KB01 was used throughout this study. The isolated bacterium was morphologically identified to verify it is free of contaminations of other microorganisms followed by construction of growth profile. Hydrolysis capacity testing and cellulase activity determination based on colorimetric assay were carried out using carboxymethylcellulose (CMC) as sole carbon source.

### 2.1. Morphological Identification

The morphology identification of bacteria was examined by using Gram staining method. Using a cooled sterile loop, a drop of sterile water was placed on a slide cleaned using 70% Ethanol. Then, the loop was sterilized again and cooled before picked a single colony bacteria from the agar plate. The colony then was gently stirred in the water drop to create a smear. Heat fixing was carried out by passing the slide a few times over the flame. A drop of crystal violet was placed onto the smear which covered the whole smear area. After 1 minute, using the distilled water the dye was washed before staining it with Gram's Iodine and left it for 1 minute. Then the dye gently rinsed before a few drops of ethanol were added to decolourize for 15 to 30 seconds. The smear was flooded with safranin and left for 45 seconds after rinsed of the ethanol. The safranin was washed with distilled water and the smear was dried prior observed under light microscope [8].

### 2.2. Growth Profiling

*B. subtilis* was cultured and maintained on the CMC agar plate containing of 1.36 g of potassium hydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ); 0.2 g of magnesium sulphate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ); 2.0 g of sodium chloride (NaCl); 0.01 g of iron sulphate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ); 1.0 g of ammonium sulphate ( $(\text{NH}_4)_2\text{SO}_4$ ); 3.0 g of Carboxymethylcellulose (CMC); 1.0 g of yeast extract and 15.0 g of agar powder in 1 L of distilled water, pH 7.0 [9]. Inoculum was prepared by transferring two loopful of the bacterium into 30 mL of Nutrient Broth (NB) and incubated at 45°C, 100 RPM for 24 hours. Then, 3.0 mL of the 24 hours culture was pipetted transferred aseptically to 97.0 mL of NB in a 250 mL Erlenmeyer flask. Optical density at 600 nm was determined for every sample at 3 hours interval for 24 hours. The growth profile was established by plotting OD versus time for each sampled points [10].

### 2.3. Hydrolysis Capacity Testing

The CMC agar plate was divided into 4 quadrants before inoculating with a single colony of bacterium at the middle of each quadrants. The inoculated agar plate was incubated for 24 hours followed by staining with Gram's Iodine (2.0 g of potassium iodide and 1.0 g of iodine in 300.0 mL distilled water) for 5 minutes [11]. The cellulolytic index of *B. subtilis* was calculated by dividing clear zone diameter by colony diameter.

### 2.4. Cellulase Production

Cellulase production was carried out in a batch fermentation with 100 mL of working volume in 250 mL of Erlenmeyer flask. The inoculum was prepared as in section 2.2. Three milliliter of culture was transferred into flask containing 97.0 mL of CMC broth medium (section 2.2). The fermentation was carried out at 45°C, 100 RPM for 24 hours.

### 2.5. Determination of Cellulase Activity

Cellulase activity was measured using Filter Paper (FP) Assay. First, the fermentation broth was centrifuged at 4,000 RPM for 10 minutes. The supernatant was used as crude enzyme sample. An aliquot of 0.5 mL crude enzyme was incubated in a test tube containing filter paper (1 × 6 cm) and 1.0 mL of 0.05M Citrate buffer of pH 4.8 at 50°C for 60 minutes. Then, 3.0 mL of DNS reagent was added immediately to end the reaction and the test tubes were placed in the waterbath at 95°C for 5 minutes. The test tubes then were transferred to the ice bath and 1.5 mL of potassium sodium tartrate was added in each tubes. Then, 0.2 mL of the colour-developed mixtures were transferred to 2.5 mL of distilled water before measuring the Absorbance at 540 nm [5-6].

## 3. Results and Discussions

Figure 1 shows the *B. subtilis* UniMAP-KB01 cultured onto the CMC agar plate which was used throughout of the experiment.

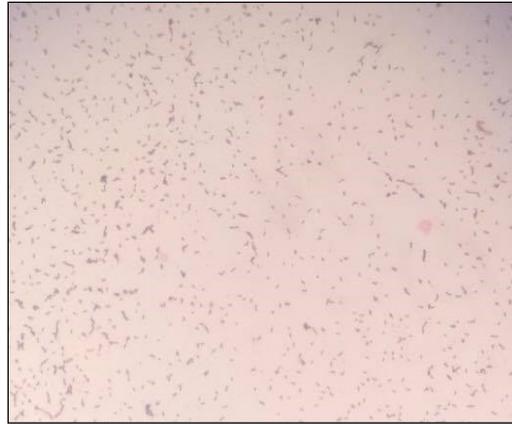


**Figure 1.** *Bacillus subtilis* UniMAP-KB01 cultured on CMC agar plate using streak plating technique.

### 3.1. Morphological Identification

Figure 2 shows that the stained culture is a gram positive bacterium as it appears to be in dark purple colour as it retained the crystal violet dye. The presence of the thick cell wall in the gram positive bacterium is the reason for retaining the purple coloured dye. Besides that, the culture appeared to be in a rod shaped and scattered individually. 16S rDNA sequencing analysis data from Macrogen Inc. shows that the strain is 99% similar to those *B. subtilis*. Thus, this strain was designated as *Bacillus subtilis* UniMAP-KB01. According to the literature, *B. subtilis* is a gram positive, aerobic bacterium and capable to produce spores in extreme conditions for survival. There are a few studies reported on

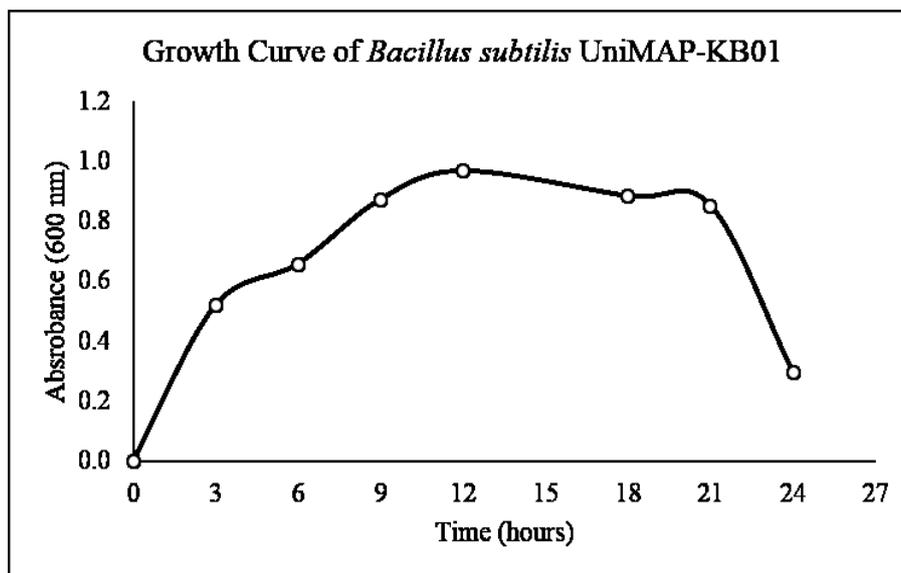
the isolation of *B. subtilis* from gastrointestinal tract of pig [14], cow dung [15] and fish [16]. This cellulase producing microbe enables animals and fish to digest the cellulose present in the plant materials consumed. This supported the potential of *B. subtilis* to be considered as cellulase producer as it excrete mostly extracellular cellulase [17].



**Figure 2.** Gram staining of *Bacillus subtilis* UniMAP-KB01 viewed under light microscope.

### 3.2. Growth Profiling and Kinetic Studies

Figure 3 shows the growth profile of *B. subtilis* UniMAP-KB01. The lag phase was not observed in this growth curve as the optical density readings increased intensely from 0 until 12 hours of incubation time. This indicates the cells were in exponential phase. Then, the stationary phase was observed lasted for 9 hours before the optical density reading started to drop at 21 hours of incubation time. This showed the culture entered the death phase as the nutrients depleted and toxic accumulated. It was deduced that the starter culture for the rest of the experiment prepared at exponential phase.



**Figure 3.** Optical density versus incubation time for 24 hours.

Figure 4 shows the non-linear growth kinetic graph of *B. subtilis* UniMAP-KB01 generated using POLYMATH software.  $X_i$  exp denotes the experimental values of microbial biomass concentration.

The  $X_t$  calc represent concentration of biomass at specific time calculated by the model equation as in Eq. 1, using  $\mu = 0.0577$  and  $X_0 = 0.5191$ .

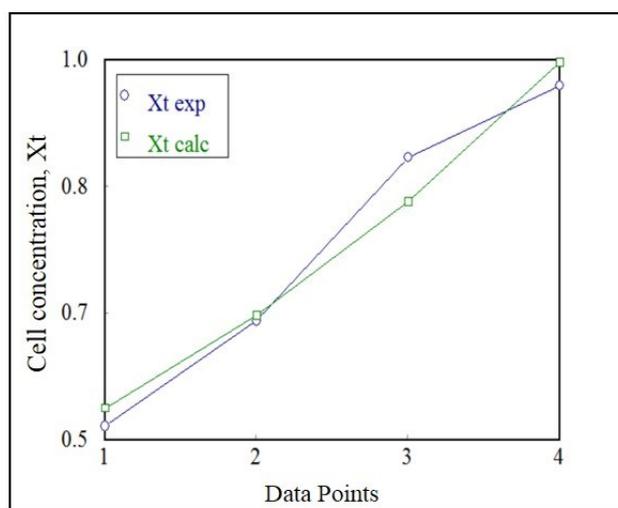
$$X_t = X_0 * e^{\mu * t} \quad (1)$$

$X_t$  = microbial biomass concentration at specific time

$X_0$  = initial microbial biomass concentration

t = time

$\mu$  = specific growth rate



**Figure 4.** Non-linear growth kinetic graph of *B. subtilis* UniMAP-KB01 generated using POLYMATH software.

From the same plot, the linear regression,  $R^2$  value was computed to be 0.9602, meanwhile, the adjusted  $R^2$  was 0.9404 which were highly acceptable. Besides that, the Root Mean Square Deviation, RMSD was 0.0176 and variance was 0.0025 which verifies the data obtained is significant and the errors are insignificant. Table 1 shows the data precision of *B. subtilis* UniMAP-KB01 generated using POLYMATH software.

**Table 1.** The data precision of *B. subtilis* UniMAP-KB01 generated using POLYMATH software.

Data	Value/Precision
Linear Regression, $R^2$	0.9602
Adjusted Linear Regression, Adj $R^2$	0.9404
Root Mean Square Deviation, RMSD	0.0176
Variance	0.0025

### 3.3. Hydrolysis Capacity Testing

Hydrolysis capacity testing showed the capability of the microorganism to degrade the cellulose. Thus, indirectly the ratio of cellulase activity can be estimated as cellulolytic index. Figure 5 shows the clear zone formed when stained with Gram's Iodine. The measurements of the clear zone diameter (1.70 cm) and colony diameter (0.60 cm) was used to calculate the cellulolytic index of *B. subtilis* UniMAP-KB01 which was  $2.83 \pm 0.46$ . The purpose of this testing is to screen the ability of a bacterium to produce the cellulase enzyme thus serves as a preliminary data before performing enzyme activity assay.



**Figure 5.** Halozone formation by Gram's Iodine assay of *B. subtilis* UniMAP-KB01.

### 3.4. Cellulase Activity

The FP Assay was carried out with crude enzyme of *B. subtilis* UniMAP-KB01. The Absorbance obtained at 540 nm was 0.015 as  $y$  which was substituted into the straight line equation  $y = 0.1163x$  obtained from the glucose standard curve. The corresponding concentration of glucose produced was 0.129 mg/0.5mL. The concentration was less than 2.0 mg, thus using the Eq. 2, Filter Paper Unit (FPU) was calculated which was 0.05 U/mL.

$$\text{FPU} = \text{mg glucose released} \times 0.185 \quad (2)$$

Research by Andriani and Park in 2006, stated that after the optimization of pH, temperature, carbon source, CMC concentration, nitrogen source and tryptone concentrations, the enzyme activity of *B. subtilis* TD6 was calculated to be 4.1 U/mL. The difference of the current result is huge when compared to previous mentioned study. However, the prolonged incubation time (144 hours) and optimized condition that was used by Andriani and Park explains the differences [16]. Thus, it was taken into consideration to optimize the fermentation condition before using it for industrial applications.

## 4. Conclusion

*Bacillus subtilis* UniMAP-KB01 was screened for cellulase production and concluded as it has potential for scale up cellulase production. Supplementary studies on the optimization of media condition, fermentation parameters, enzyme kinetics and enzyme immobilization studies using MWCNTs will be carried out for bioconversion of lignocellulosic waste into glucose. The produce glucose which will then be used as a precursor for biofuel production such as bioethanol.

## Acknowledgement

This study was financially supported by Universiti Malaysia Perlis Short Term Grant (STG) (A/C:9001-00519).

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