# CHAPTER III
# BIOPOLYMER GERNERATION FROM SWEET SORGHUM JUICE (SSJ): SCREENING, ISOLATION, IDENTIFICATION AND FERMENTATIVE POLYHYDROXYALKANOATES PRODUCTION BY *Bacillus aryabhattai*

## 3.1 Introduction

The synthetic plastics obtained from petroleum have long been used by humans in daily life. They are non-degradable and cause the accumulation problem in environment. However, after the biopolymer (polyhydroxyalkanoates; PHAs) discovery by Lemoigne (1926) led to a wide study of bioplastic production. PHAs are defined as a kind of biodegradable plastic and biocompatibility, which can be produced from a numbers of microorganisms mostly bacteria. Typically, they can accumulate PHAs as energy storage when the microbial cells encountered with nutrient imbalance condition such as limitation of nitrogen and phosphorus meanwhile also in the presence of excess carbon source (Anderson & Dawes, 1990). Previous studies reported that there were various bacterial strains such as *Alcaligenes* spp. (Grothe et al., 1999; Kaewkannatra et al., 2008) and *Bacillus* spp. (Kulpreecha et al., 2009) that can synthesize and accumulate PHAs in their cell. Moreover, various soil and marine environments are also found as promising sources of PHAs-producing bacteria. Many researchers attempted to isolate soil or marine bacteria to produce PHAs from inexpensive raw material such as jatropha biodiesel waste, molasses, starch and sugar cane juice, etc.

Sweet sorghum (*Sorghum bicolor* L. Moench) is a cheap agricultural feed crop. The juice obtained from sweet sorghum stem contains of high total sugar content in forms of sucrose and also other sugars in small amounts of glucose and fructose. The properties of sweet sorghum juice (SSJ) can be used as an ideal carbon source medium for microbial growth to produce added value products such as bioethanol (Loapaiboon et al., 2009), hydrogen (Antonopoulou et al., 2008), lactic acid (Hetényi et al., 2010) and recently unsaturated fatty acids likes docosahexaenic acid (DHA) (Liang et al.,

2010). In this research study, we attempted to isolate utilising SSJ bacteria from soil environments and to investigate a potential use of the SSJ as a sole carbon source and produce PHAs via batch fermentation by isolated bacteria and compared to PHAs producing referenced strains of *A. eutrophus*, *A. latus* and *Hydrogenophaga* sp., respectively. In addition, yields and their productivity were also evaluated.

## 3.2 Materials and Methods

### 3.2.1 Collection of soil samples

Soil samples were collected from sugarcane plantation in several places nearby sugar cane factory, Chaiyabhumi province, Thailand. The samples were preserved at 4°C prior to use.

### 3.2.2 Preparation of sweet sorghum juice (SSJ)

Sweet sorghum KKU 40 was kindly provided from Faculty of Agricultural Science, Khon Kaen University, Thailand. The stem was milled and the juice was obtained after filtering through cotton sheet. The juice then was kept at - 20 $^{o}$C for prevention of microbial contamination.

### 3.2.3 Screening of utilising sweet sorghum juice bacteria

One gram of soil was weighed and added into 250 mL flask containing 100 mL nutrient broth (NB). Flasks were incubated in orbital shaking incubator at 35 $^{o}$C and 200 rpm for 24 h. The medium was diluted by 10-folds in sterile normal saline prior to spreading on solid minimal salt media on Petri dish containing sweet sorghum juice as a sole carbon source supplemented with a solution of 0.5 mg Nile blue A in dimethyl sulfoxide (DMSO) to give a final concentration in media of 0.5 μg/mL. The plates were incubated at 35 $^{o}$C for 24 h. Several PHA-producing colonies of soil bacteria were observed under UV light (Spiekermann et al., 1999). Only positive single colonies were selected and re-streaked on solid minimal salt media containing sweet sorghum juice. After 48 h, PHAs-producing bacteria were confirmed by Sudan black B staining (Schlegel, Lafferty, & Krauss, 1970) and PHAs granules were observed by microscopic technique. The different strains were selected by their genera morphology and polymer accumulation behavior under light microscope.

### 3.2.4 PHAs production from sweet sorghum juice

The fermentative utilisation of sweet sorghum juice was studied by inoculating the selected strains in comparison with 3 referenced PHAs-producing strains as *A. latus*, *A. eutrophus* and *Hydrogenophaga* sp. in culture media containing sweet sorghum juice. Each strain was cultured in nutrient broth and incubated in orbital shaking at 200 rpm, 35$^{o}$C for 24 h prior use. All inoculums were adjusted to give the final optical density at 600 nm (OD600 nm) in culture media of 0.1 in 250 mL Erlenmeyer flask containing 100 mL sterilized mineral salt media composting of (unit per liter); 20 g total sugar of sweet sorghum juice; 1.5 g $KH_2PO_4$, 3.57 g $Na_2HPO_4$, 0.2 g $MgSO_47$ $H_2O$; 1 g $(NH_4)_2SO_4$ and 1 mL trace element solution prepared according to the method described by Grothe, et al (1999). In addition, dry cell weight (DCW) was measured. 1 mL the broth was withdrawn and centrifuged at 8,000 rpm for 10 min. After centrifugation, cell pellet was washed twice with distilled water, dried at 90 °C overnight and cooled down in desiccators till constant weight was obtained.

### 3.2.5 Recovery and quantification of PHAs

After fermentation, PHAs were then extracted from wet cell pellet. PHAs concentration was determined using method as described by Law and Slepecky (1961).

### 3.2.6 Identification of bacteria by 16S rDNA gene

The genomic DNA of the S4 strain was prepared following the standard protocol described by Katsura, Kawasaki, Potacharoen, Saono, Seki, Yamada, et al, (2001). The 16S rDNA gene amplification was carried out by Taq polymerase with the forward primer: 5'-GAG TTT GAT CCT GGC TCA G-3'and reverse primer: 5'-GTT ACC TTG TTA CGA CTT-3'. The amplification program employed by DNA Engine Dyad® Thermal Cycler (Bio-Rad Laboratories) comprised of 1 cycle at 94°C for 3 min, 25 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and elongation at 72°C for 2 min, followed by a final amplification step at 72°C for 3 min. The amplification products were purified by using Qiagen PCR purification kit and performed on an ABI Prism® 3730XL DNA Sequence (Applied Bio-systems, Foster City, California, United States of America).

The 16S rDNA gene sequence analysis was carried out using NCBI-BLAST (National centre for Biotechnology Information (NCBI) http://www.ncbi.nml.nih.gov) program.

### 3.2.7 Phylogenetic tree analysis

The DNA sequences determined and obtained from databases were aligned with a program CLUSTAL X (version 1.8) in BioEdit Program. Alignment gaps and unidentified bases were eliminated. Distance matrices for the aligned sequences were calculated using the Kimura's two-parameter method (Kimura, 1980). Phylogenetic trees of 16S rDNA genes were constructed by the neighbor-joining method of Saitou and Nei (1987). The robustness for individual branches was estimated by 1000 replications bootstrapping with the program MEGA Version 4.0.

## 3.3 Results and Discussion

### 3.3.1 Bacterial isolates

Various soil bacteria were able to grow on solid sweet sorghum juice medium with Nile blue A stain. These dyes could diffuse into the cytoplasm of the colonies during growth and subsequently into the PHAs inclusions. The positive PHAs-producing colonies were exposed UV illuminator at the wavelength 280 - 360 nm and shows brighten colonies (Spiekermann et al., 1999). In the present study, we have chosen only eighteen strong bright colonies and then were re-streaked on solid nitrogen-limited medium. After 48 h, each colony was observed under light microscope. The differences genera cell morphologies of bacteria were investigated and it could be categorized into three groups. The isolates named as S4, S6 and S11 were selected from 18 colonies showed as Figure 3.1.

In Figure 3.1, the isolates S4, S6 and S11 show strong bright colony on solid sweet sorghum juice medium with Nile blue A. They were re-streaked on solid sweet sorghum juice medium and compared with three PHAs-producing strains (*A. eutrophus*, *A. latus* and *Hydrogenophaga* sp.). After 48 h, the colonies on cultivating plate were observed and isolate S4, S6 and pure culture of *A. eutrophus* and *Hydrogenophaga* sp. able grew well on solid sweet sorghum juice medium (Figure 3.1b-c). Moreover, the isolates S4, S6, *A. eutrophus* and *Hydrogenophaga* sp. were exhibited very strong bright colonies (Figure 3.1b). In contrast, the isolate S11 grows slowly and *A. latus* gradually grows on sweet sorghum juice, its shows negative score

on solid medium with Nile blue A which could be due to some inhibitor from either dye or sweet sorghum juice.

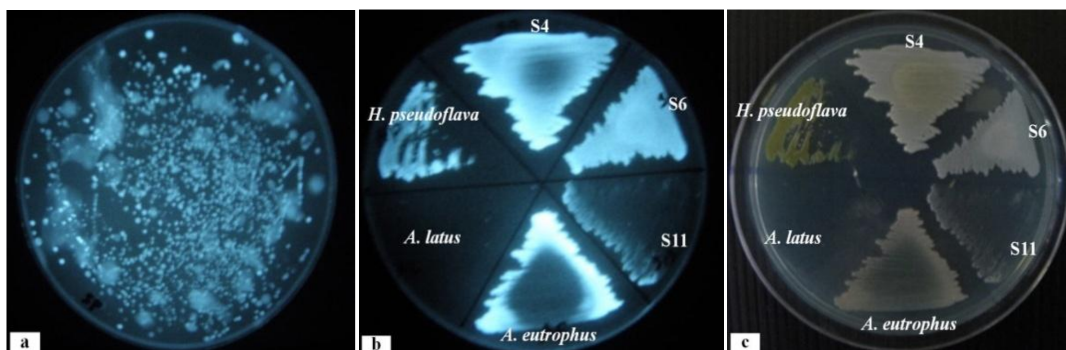


**Figure 3.1** Variable colonial bacteria on solid sweet sorghum juice medium with Nile blue A (a) Isolation of PHAs-producing bacteria from soil, (b) the isolates S4, S6, S11 and 3 referenced bacterial strains (*A. eutrophus*, *A. latus* and *Hydrogenophaga* sp.) under UV light and (c) the isolates S4, S6, S11 and 3 referenced bacterial strains under visible light

### 3.3.2 PHAs production from sweet sorghum juice

In this study, the three isolates and three pure referenced bacteria were used in the fermentation of sweet sorghum juice medium containing approximately 20 g/L of total sugar to confirm PHAs production. All strains could grow in sweet sorghum juice due to sweet sorghum juice usually contain high composition of sucrose and a trace of glucose and fructose levels. The reason as above it is generally accepted as easier to ferment sweet sorghum juice by various bacteria. To confirm the PHAs production, the isolates S4, S6 and S11 and three pure referenced bacterial strains were stained with Sudan black B when they were cultured in sweet sorghum juice medium at suitable phase accumulation of PHAs. In Figure 3.2, all 6 bacterial strains showed black granules when Sudan black B was used to stain .The color stain can be attached to lipophilic-material as PHAs which are accumulated in their cell.

As a results shown in Table 3.1, the isolates S4 and S6 were promising PHAs-producing bacteria. The PHAs productions from those isolates were 1.74 and 1.48 g/L with PHAs contents of 57.62 % and 51.56 % PHAs/CDW, respectively.

Moreover, the isolates S4 and S6 showed in higher productivity of 0.126 and 0.082 g/L.h as compared to the isolate S11 and the pure referenced strains of *A. eutrophus*, *A. latus* and *Hydrogenophaga* sp. PHAs concentration obtained from these strains were 0.29, 0.91, 0.21 and 0.35 g/L, respectively. In addition, the PHAs content were also lower (21.17, 38.72, 20.33 and 31.53 % PHAs/CDW, respectively) than those of the isolates S4 and S6.

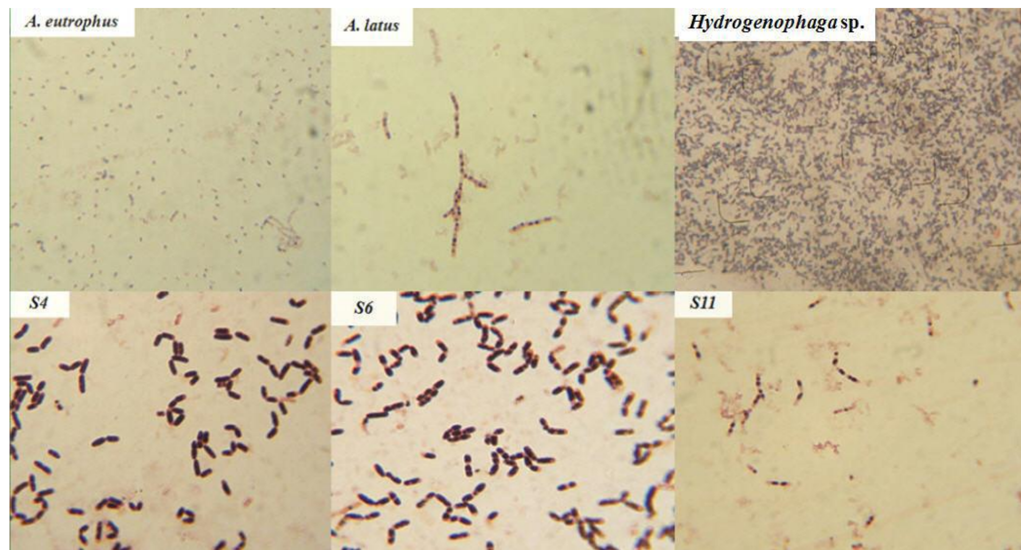


**Figure 3.2** Micrographs of bacteria containing PHAs inclusions, rod shape in black color represents PHAs granules

**Table 3.1** PHAs production by various isolates and 3 pure referenced strains of bacteria

| Isolates | Nile blue A | Sudan black B | CDW (g/L) | PHAs (g/L) | PHAs Yield (%) | PHAs Productivity (g/L.h) |
|---|---|---|---|---|---|---|
| S4 | +++ | +++ | 3.02 | 1.74 | 57.62 | 0.097 |
| S6 | +++ | +++ | 2.07 | 1.48 | 51.56 | 0.082 |
| S11 | ++ | ++ | 1.30 | 0.29 | 21.17 | 0.0088 |
| *The referenced strains* | | | | | | |
| *A. eutrophus* | +++ | ++ | 1.6 | 0.91 | 38.72 | 0.038 |
| *A. latus* | - | + | 1.3 | 0.21 | 20.33 | 0.0029 |
| *Hydrogenophaga* sp. | ++ | ++ | 1.11 | 0.35 | 31.53 | 0.0049 |

Growth curve of the isolate S4 grown on mineral salt medium containing 20 g/L total sugar in the SSJ as a carbon source is shown in Figure 3.3 The results illustrated that the production of PHAs by isolated S4 were rapidly increased during the exponential phase (3-15 h) with increasing of DCW. PHAs accumulation reached a maximum of 57.62 % by DCW at 18 h of fermentation.

### 3.3.3 Identification of the isolated strain S4

The isolated S4 was identified using the full length sequences 16S rDNA technique and compared to those available in the public databases. It was found that, the S4 was closely related to that bacterial strain *Bacillus aryabhattai* (99.70 % similarly). The phylogeny based on these full length 16S rDNA sequences and related *B. aryabhattai* is shown in Figure 3.4. Previous studies have reported that *Bacillus aryabhattai* was isolated from cryotubes used to collect air samples from upper atmosphere at altitudes of between 40-41 km (Shivaji, Chaturvedi, Begum, Pindi, Manorama, Padmanaban, et al., 2009). The strain was gram-negative and rod-shaped cells. Colonies on nutrient agar are peach-colored, entire, round, flat and 5–8 mm in diameter. Cells produce ellipsoidal and central endospores and are motile. Growth occurs at 10–37 $^{o}$C and pH 6–10 (Shivaji et al., 2009). They can use various carbon sources for their growth. Recently, it could also be isolated from soil (Lee, Ka, & Song, 2012). It could produce other phytohormones such as indole butyric acid,

gibberellins, and abscisic acid (Lee et al., 2012). Guan, Cho, and Lee (2011) reported that this strain exhibited the best growth and protease activity under cultivation with nutrient agar containing 2 % (w/v) skim milk. Interestingly, the results obtained from this work indicated that *B. aryabhattai* was also discovered in soil. It was firstly found to be a PHAs-producer. Moreover, it can also accumulate PHAs in their cells and grow well on the SSJ more than other referenced PHAs-producing strains. Another important observation found in this study is that the PHAs production by *B. aryabhattai* was found to be growth associated product. Generally, the strain was Gram-positive, lack of endotoxin as lipo-polysacharide which is pryogenic in human beings (Singh, Patel, & Kalia, 2009). This feature is undesirable for biomedical applications of the PHAs. It should be stated that *B. aryabhattai* can be used as novel strain for PHAs production instead of others Gram-negative bacteria.

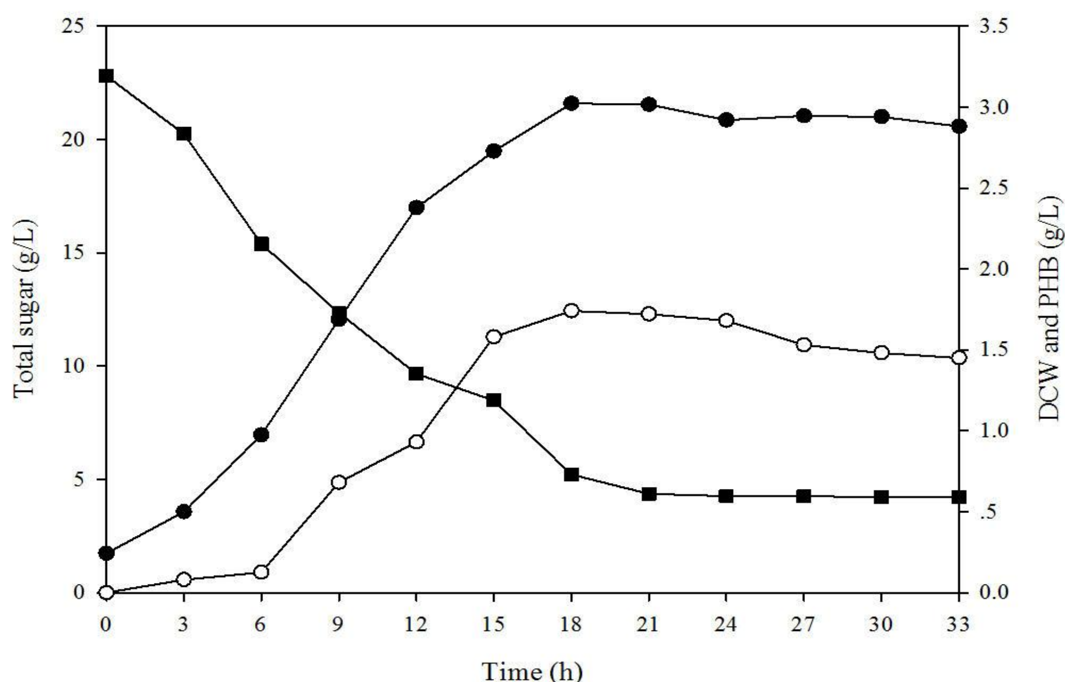


**Figure 3.3** Growth curve of isolate S4 grown on mineral salt medium containing the SSJ with 20 g/L total sugar as a carbon source. Close circle, DCW; open circle, PHAs; close square, total sugar concentration

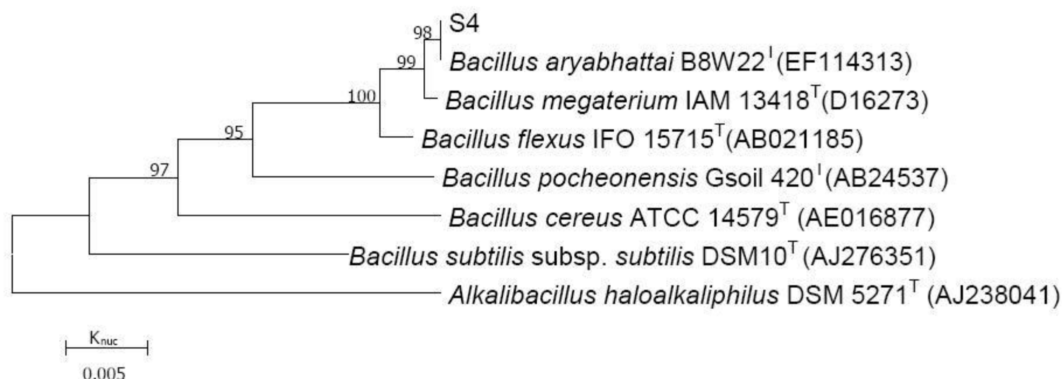


**Figure 3.4** Neighbour-joining phylogenetic tree reconstructed on the basis of 16S rDNA gene sequences showing the phylogenetic relationships between strain S4 and closely related to *Bacillus aryabhattai* B8W22$^T$ (GenBank accession no. EF114313)

## 3.4 Conclusion

Successfully, the isolate S4 identified as *Bacillus aryabhattai* showed the highest efficiency than other strains. It was found to be a growth-associated product synthesis strain. The results obtained from this study have clearly demonstrated that *B. aryabhattai* could directly use the SSJ as a carbon source and the potential use of sweet sorghum juice as a cheap raw material for producing higher value product of PHAs via bio-fermentation. Furthurmore, the optimal condition by using response surface methods (RSM) for *B. aryabhattai* is still further considered including the improvement of the processes such as batch and fed batch in larger scale of fermenter.

## 3.5 Acknowledgments

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