CHAPTER IV
RESULTS AND DISCUSSION

This chapter contains results and discussion obtained from this research. It is separated into 5 sections. The first section involved the characterization of sugarcane juice. Section 4.2 showed the isolation and screening of PHAs biopolymer producing microorganisms. Thirdly, growth monitoring and cultivation of the efficient isolates to accumulate and produce PHAs biopolymer were revealed. In the forth section concerned the production of PHAs by both pure strain of *Alcaligenes latus* TISTR 1403 and the isolated strain. In addition, the capability of their PHAs production also was compared. Section 4.5 statistical optimization of process parameters for the production of PHAs in maximum PHAs production strain was considered. Finally, the production of PHAs in a large scale was further investigated and discussed.

4.1 Characterization of sugarcane juice

Sugarcane juice was separated into two sets; first was untreated by heat and another was treated set by heat treatment are shown in Figure 4.1. Physical and chemical properties of the juice are shown in Table 4.1.

Table 4.1 Properties of sugarcane juice

Properties	Untreated by heat	Treated by heat
pH	4.9-5.2	5.2-5.4
°Brix	17.2-17.8	17.4-17.8
Total sugar[a]	150.50 gL^{-1}	170.83 gL^{-1}
Total sugar[b]	146.32 gL^{-1}	160.20 gL^{-1}
Glucose	9.25 gL^{-1}	14.01 gL^{-1}
Fructose	6.20 gL^{-1}	5.71 gL^{-1}
Sucrose	130.87 gL^{-1}	140.49 gL^{-1}

[a] was measured by Phenol-sulfuric acid

[b] was measured by High performance liquid chromatography (HPLC)

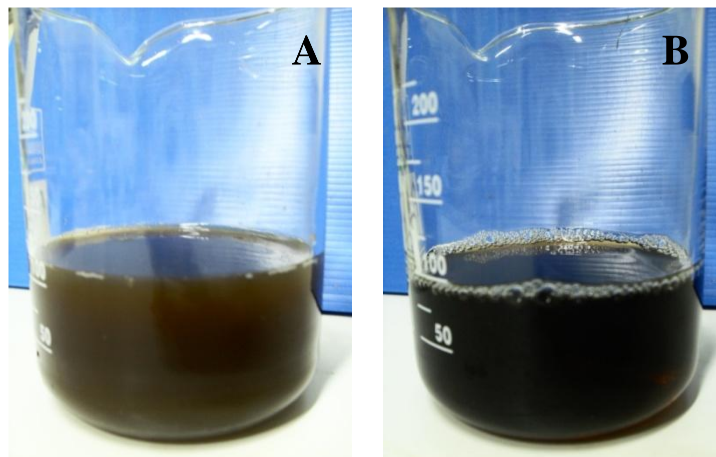


Figure 4.1 Sugarcane juice. (A) untreated by heat (B) treated by heat

Sugarcane juice after treating by heat treatment at temperature about 110°C, it was changed not much for physical properties. It appeared to clear and darkness in color. Considering to the chemical properties, during heat treatment, it was considerable loss of low molecular weight carbohydrates leaded to affect on fructose and glucose. For example, in the blanching of carrots and swedes (rutabagas), there was a loss of 25% and 30% of these carbohydrates. In peas, green beans and Brussels sprouts the loss was less pronounced - about 12% by blanching and 7-13% for boiling (Campbell, Junshi, 1994). Later, Beegom et al. (1995) also reported that the loss of glucose and fructose at boiling was higher than that of sucrose.

Use of heat treatment to treat sugarcane juice was clearly affected to its clarity and total sugar concentration. Thus, the temperature used to sterile raw material of sugarcane juice should be carefully considered. Sumaya-Martinez et al. (2005) reported that high temperature could affect to Maillard reaction on browning reaction. Previously, Baisier, Labuza (1992) also reported that temperature is one of the most important parameters that affects the reaction rates and aroma characteristics of foods. This reaction called as the Maillard reaction that is usually found in food processing or storage. It involves sugars, amino acids or proteins, that are condensed and progress to complex network of reaction products, influenced by many factors such as temperature, pH and sugar (Ajandouz et al., 2006; Jing, Kitts, 2002). Recently, Lan et al. (2010) concluded the Maillard reaction that produced volatile compounds of low molecular mass, non-volatile colored compounds of intermediate molecular mass and

brown substances of high molecular weight. The advanced Maillard reaction consists of dehydratation and fission of the Amadori product into furfurals (Hodge, 1953). It was condensed with themselves or with aldehydes formed by Strecker degradation of amino acids and form brown pigments (Morales,Van Boekel, 1997). It was implied the bacterial growth may be inhibited by compound from the reaction. In addition, sugar also can be formed with amino acid or protein to complex substance. Thus, it resulted in another way effected on substrate consume.

4.2 Isolation and screening of PHAs producing microorganisms

4.2.1 Sample collection and characteristics of isolates

Soil sample was collected from sugarcane plantation while sugarcane juice also was used to isolate the PHAs producing microorganisms. Sources of isolation, amount of isolates found and also the characteristics of the isolates when they were tested on sucrose yeast extract agar and stained with Sudan black B are shown in Table 4.2.

Table 4.2 Source of collection and characteristics of the isolates

Source of sample	Amount of isolates
Soil from sugarcane plantation (SV)	9
Sugarcane juice (SC)	27
Characteristics of the isolates	
Grow on sucrose yeast extract agar	36
Staining with Sudan black B	17
Stained at 24 hr	10
Stained at 48 hr	7

The results obtained that there were 36 isolated strains can be grown on sucrose yeast extract medium. However, 17 isolated strains could give a black color after staining by Sudan black B. Although, only 4 isolated strains were chosen to monitor growth condition after staining for 48 hr. Because these strains were stained by Sudan black B after cultivation time at 24 hr and gave more black color in their cells. It implied that they were reached to accumulate of PHAs inclusion in a short time less than other strains. The evidence of Sudan black B staining on PHAs inclusion was reported by Legat et al. (2010). They mentioned that the simple method was applied to screen for the presence of PHAs in *Halococcus* and other haloarchaeal species by Sudan black B dye and observed by phase contrast microscopy. Previously, Burdon (1946) also reported that PHAs inclusions can be stained with Sudan black B indicating that they are of a lipid nature. Therefore, this simple method was chosen to monitor of PHAs accumulation in the cell by phase contrast microscopy in the study.

4.2.2 Growth monitoring and cultivation condition

The 4 selected isolates coded as SV13, SV19, SC114 and SC126 were studied in gram stain, micro and macroscopic morphology under microscope techniques. The results obtained are shown in Table 4.3

Table 4.3 Characteristics of isolated strains

Characteristics	**SV13**	**SV19**	**SC114**	**SC126**
Gram stain	positive	positive	positive	negative
Microscopic morphology	rod shape, pair or short chain	rod shape, long chain	rod shape, single or short chain	coccus to rod shape, single or short chain
Macroscopic morphology	large raised opaque white colonies, irregular margins	large raised opaque white colonies, irregular margins	convex opaque white colonies, smooth margins	small convex opaque yellow colonies, smooth margins

All 4 isolated strains are bacteria which are grown under aerobic condition at 37ºC and can be utilized sucrose as a carbon source. It should be noted that sucrose yeast extract medium is used for cultivation of bacteria. They can utilize sucrose as a carbon source as a selective medium. Three gram-positive bacteria (SV13, SV19 and SC114) and one gram-negative bacteria (SC126) were cultivated in 250 mL flask containing of 100 mL nutrient broth and incubated at 37°C shaking at 200 rpm for 48 hr to monitor growth by measuring optical density and results shown in Figure 4.2.

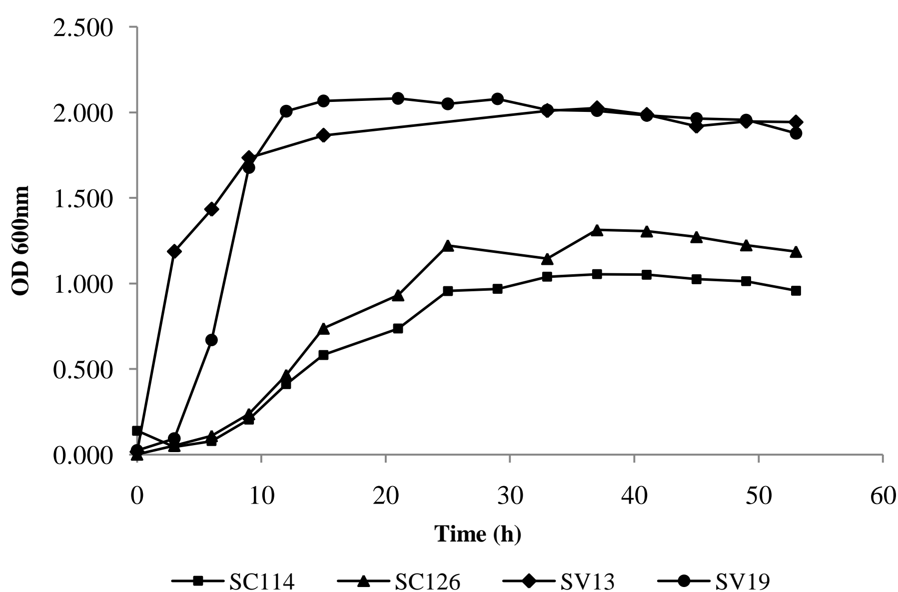


Figure 4.2 Growth of isolated strains. After cultivation in 250 mL flask containing of 100 mL nutrient broth at 37°C and 200 rpm of agitation rate

4.2.3 Growth monitoring and cultivation condition

Only three gram-positive bacteria (SV13, SV19 and SC114) and one gram-negative bacteria (SC126) were chosen and cultivated in 250 mL shake flask with 100 mL minimal medium containing approximately 30 gL^{-1} initial total sugar, pH 7, incubated at 37ºC on rotary shaker with 200 rpm of agitation rate for 48 hr. Total sugar consumed, maximum cell dry weight (DCW) and maximum PHAs content were compared among them and the results showed in Table 4.4.

Table 4.4 Parameters measured among the isolates

parameters	SV13	SV19	SC114	SC126
Total sugar consumed (gL^{-1})	11.346	12.521	5.769	14.402
Maximum DCW (gL^{-1})	4.0	2.2	2.7	1.9
Maximum PHAs ($\mu g\ mL^{-1}$)	19.128	22.081	3.221	18.523

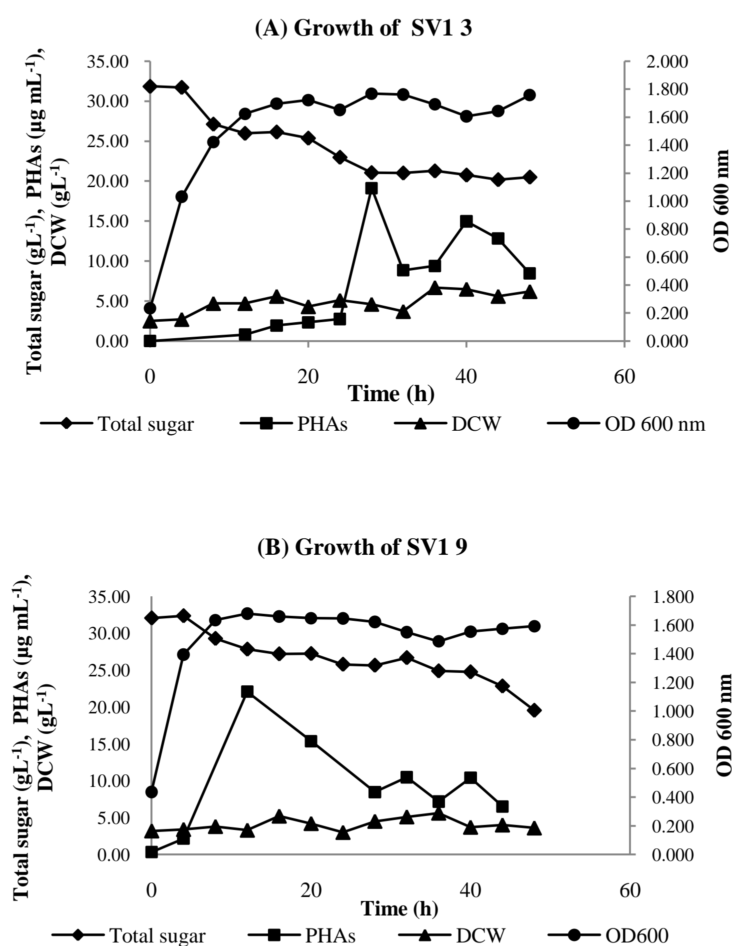


Figure 4.3 (A-D) Growth profile of isolated bacterial strain (SV13, SV19, SC114 and SC126) that presented PHAs granule by staining with Sudan black B. Growth profile was monitored total sugar consumption, PHAs in form of PHB by crotonic acid assay, DCW and optical density by measuring culture medium absorbance at 600 nm.

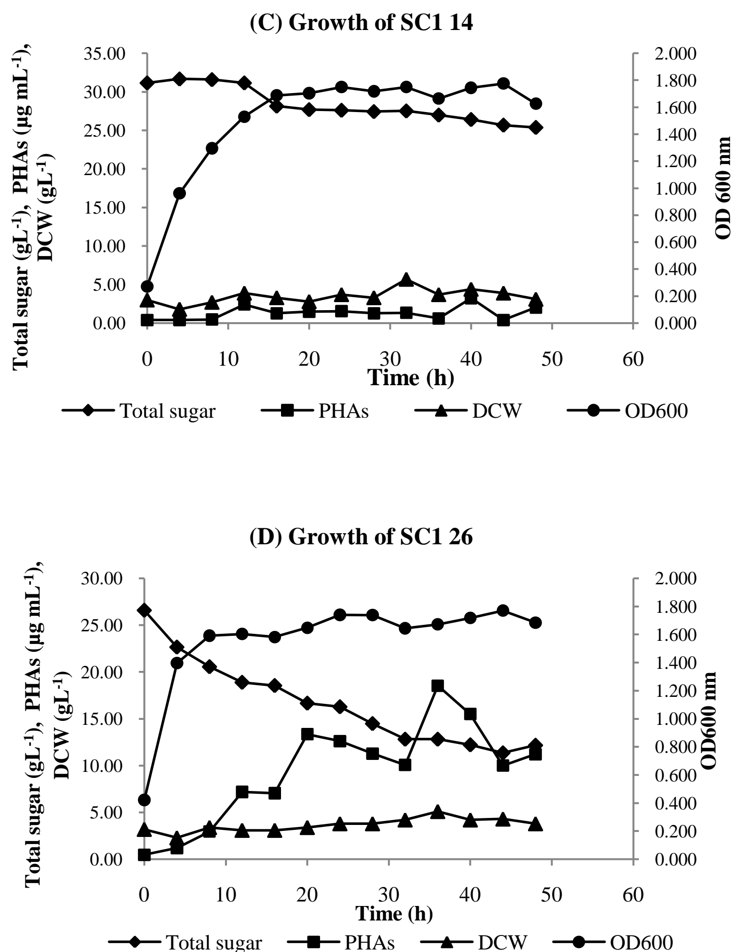


Figure 4.3 (A-D) Growth profile of isolated bacterial strain (SV13, SV19, SC114 and SC126) that presented PHAs granule by staining with Sudan black B. Growth profile was monitored total sugar consumption, PHAs in form of PHB by crotonic acid assay, DCW and optical density by measuring culture medium absorbance at 600 nm. (Cont.)

In Figure 4.3 (A-D), the results showed the efficient SV13, SV19 and SC126 isolated strains. They produced nearly maximum PHAs at around 18-22 µg mL^{-1}. However, only SV13 and SV19 were chosen to monitor growth condition and production of PHAs biopolymer. Since, the isolate SC126 showed in a poor growth rate when compared to SV13 and SV19. Moreover, it was weakly strain that leads to easier contaminate and inhibits of growth by other strains.

Growth profile was confirmed that the efficient isolated strain SV13 was better than SV19. Figure 4.4 (A) the production of PHAs in SV13 including replication1 (Rep1) and replication 2 (Rep2) was higher than SV19 while Figure 4.4 (B) the total sugar consumption of both strains were slight decreased in similar trends. However, only the isolated strain SV13 was chosen to identify and produce PHAs. In addition, the comparison between pure bacterial strains is also carried out in further study.

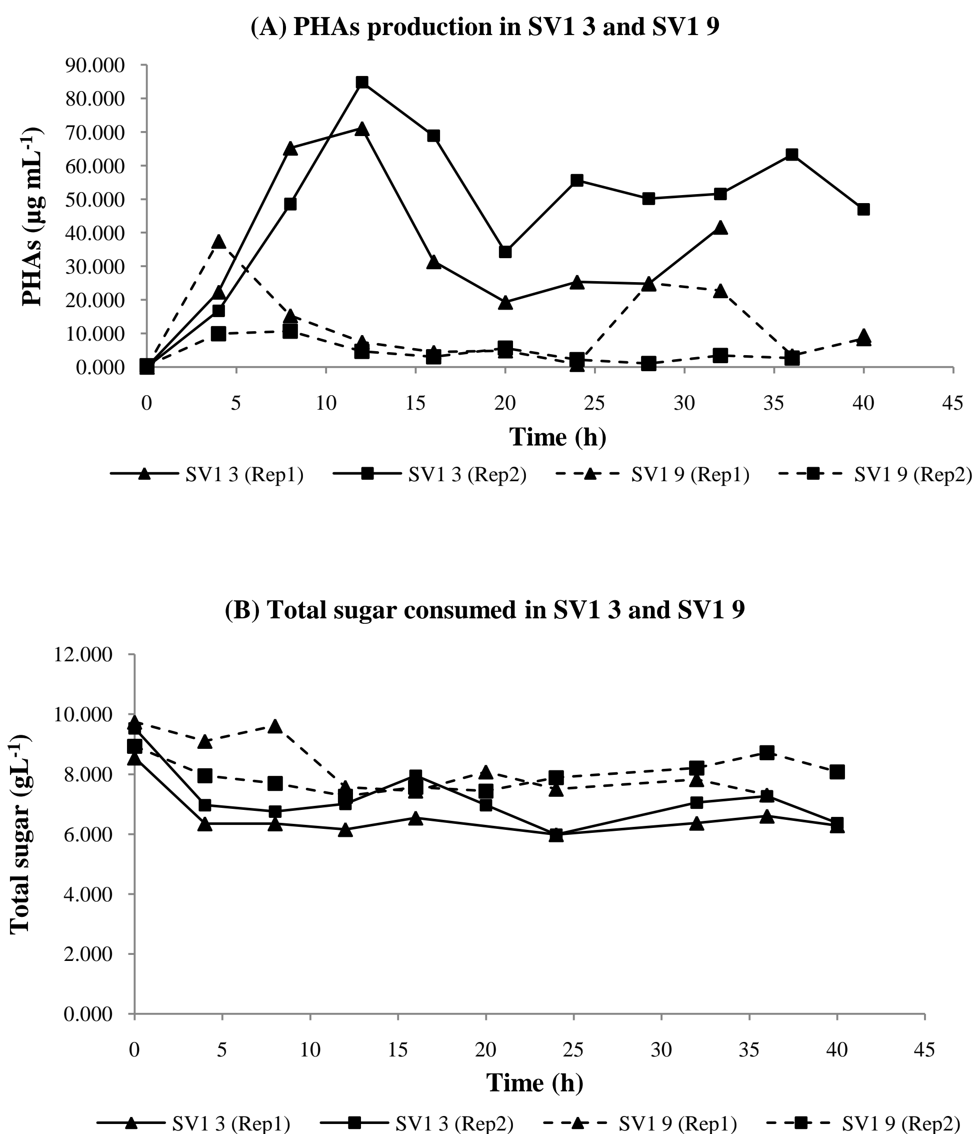


Figure 4.4 (A-B) Comparison of PHAs production and total sugar consumed by isolate SV13 and SV19. Minimal medium with initial total sugar 10 gL^{-1}, pH 7, 37°C and 200 rpm for 40 hr of cultivation.

4.2.4 Batch fermentation of sugarcane juice by efficient isolates strain

The bacterial strain SV13 was chosen to produce PHAs in a 250 mL Erlenmeyer shake flask containing of 100 mL minimal medium via batch fermentation. The modified production medium composed of approximately 10 gL^{-1} sugarcane juice as a carbon source and initial pH 7. The culture in the log phase as the inoculums at 10% (v/v) was added into the flask. Then, it was incubated at 37ºC in rotary shaker with 200 rpm of agitation rate for 40 hr. The variations of growth, total sugar, DCW and PHAs are shown in Figure 4.5.

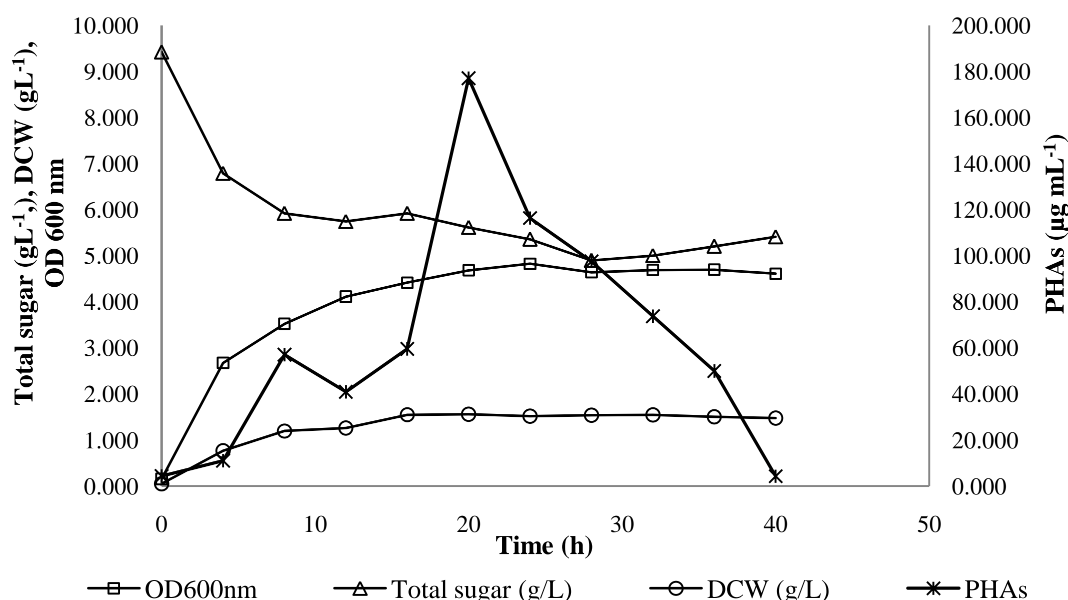


Figure 4.5 Growth profile and monitoring of total sugar consumed, dry cell weight (DCW) and PHAs as functions of time during batch fermentation by SV13

In Figure 4.5, it was indicated that the isolated SV13 is a growth associated with PHAs producing strain. Considering, the values of total sugar consumption and optical density (OD600 nm), it was found that the maximum PHAs was obtained after stationary phase. However, at the late stationary phase, PHAs were decreased due to PHAs granule degradation in bacterial cell to employ as a carbon source. This phenomena was confirmed by staining with Sudan black B as showed in Figure 4.6. The native strain of SV13 showed in different after staining by Sudan black B dye at 16, 20, 24 and 32 hr during cultivation. When considered in total sugar concentration,

it was remained until the end of cultivation although, in the early cultivation, it was rapidly decreased until 10 hr. Here after, it was slowly decreased and still remained at about 5 gL^{-1}. According to sugarcane juice composes of many kinds of sugar such as glucose, fructose, sucrose, maltose, lactose and xylose. Therefore, bacteria can not completely utilize the sugar and then leads to the remaining of concentration of total sugar contained in production medium.

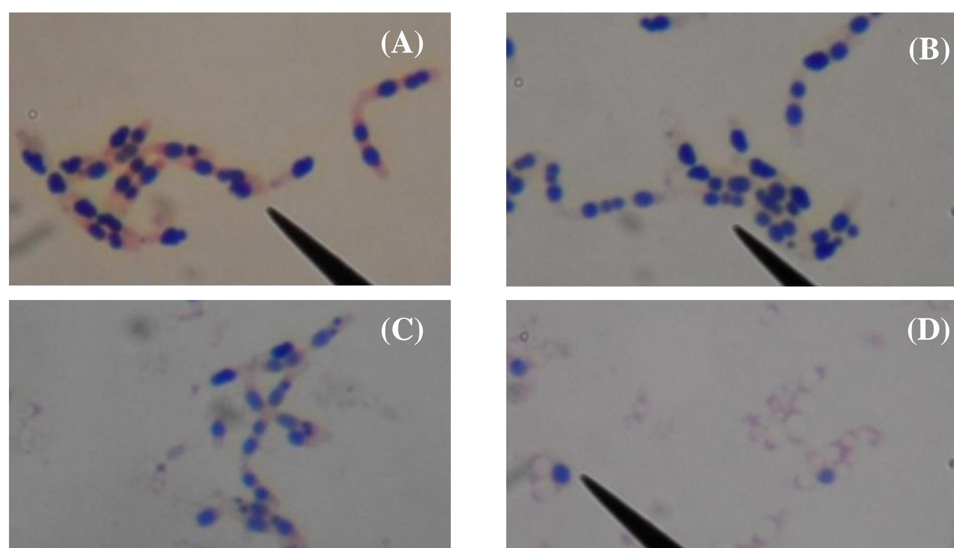


Figure 4.6 SV13 was stained by Sudan black B dye under light microscopy during cultivation times (A) at 16 hr (B) at 20 hr (C) at 24 hr (D) at 32 hr

The production of PHAs biopolymer of SV13 is summarized in Table 4.5. The maximum biomass yield, PHAs yield and productivity were obtained at 20 hr. The PHAs contents was decreased after 24 hr that was related to the results from Sudan staining and growth profile described as previous. However, at 32 hr, PHAs inclusions perhaps were utilized as a carbon source leaded to appear clear inclusions in bacterial cells. Since, PHAs are functioned as intracellular carbon-storage compound that can be used when carbon becomes a limiting resource. The pattern of PHAs synthesis was related to McCool et al. (1996) who reported PHAs accumulation reached a maximum rate at late log/early stationary phase which was observed in minimal and rich media. Actually, the intracellular accumulation of PHAs enhances the survival of several bacteria under environmental stress conditions imposed in

water and soil (Kadouri et al., 2005; Zhao et al., 2007). The accumulation of PHAs in *Azotobacter vinelandii* was utilized as a carbon and energy source during encystment (Lin, Sadoff, 1968; Segura et al., 2003). The evidence has been provided suggesting of spore formation and germination as well as cyst production may be related to PHAs accumulation and utilization. PHAs are maximally accumulated just prior to the formation of spores and are degraded during sporulation process in *Bacillus cereus* (Wu et al., 2001; Kominek, Halvorson, 1965) and *Clostridium botulinum* (Emeruwa, Hawirko, 1973). Thus, the appropriate period of 20 hr to harvest cell mass for recovery of PHAs biopolymer in next study, was chosen. In addition, Grothe et al. (1999) reported that the suggesting critical importance of timing to harvest to prevent the hydrolysis of PHB in the stationary phase that affect to lose of PHB.

Table 4.5 Summary of PHAs[a] production by isolated bacterial strain SV13

Time (hr)	**Total sugar consumed (S)** (gL^{-1})	**DCW (X)** (gL^{-1})	**PHAs (P)** (gL^{-1})	**Biomass yield ($Y_{X/S}$)** (g DCW/g total sugar consumed)	**PHAs yield ($Y_{P/S}$)** (g PHAs/g total sugar consumed)	**Productivity** ($g\ L^{-1}\ h^{-1}$)
0	0.0000	0.0483	0.0044	-	-	-
4	2.6361	0.7667	0.0110	0.2908	0.004178	0.00275
8	3.5034	1.1950	0.0570	0.3411	0.016273	0.00713
12	3.6820	1.2583	0.0409	0.3417	0.011107	0.00341
16	3.5034	1.5483	0.0595	0.4419	0.016982	0.00372
20	3.8095	1.5583	0.1770	0.4090	0.046465	0.00885
24	4.0646	1.5183	0.1163	0.3735	0.028614	0.00485
28	4.5238	1.5383	0.0975	0.3400	0.021553	0.00348
32	4.4218	1.5444	0.0737	0.3493	0.016675	0.00230
36	4.2177	1.5017	0.0500	0.3560	0.011845	0.00139
40	4.0136	1.4750	0.0043	0.3675	0.001071	0.00011

[a] PHAs content was analyzed in form of PHB by crotonic acid method

Considerately, total sugar in production medium was not run out by bacteria. This may be due to the fact that sugarcane juice composed of many kinds of sugar such as glucose, fructose, sucrose, maltose, lactose and xylose. Most bacteria preferably utilize monosaccharides of glucose, fructose and etc in a firstly priority. The concentration of total sugar in production medium was still remained and expected that were di, tri or multi-saccharides.

4.2.5 Identification and characterization of SV13

Preliminary characteristics of SV13 were considered following to the Bergey's manual of systematic bacteriology (Brenner et al., 2005). It was revealed that the cells form in rod-shaped, majority gram-positive, or positive only in early growth stages. They can motile by flagella typically lateral and can be formed as endospores, heat-resistant and can be strict aerobes or facultative anaerobes. The maximum temperature for vegetative growth ranges from 35-45°C and minimum range from 10-20°C. The minimum pH value for growth varies from about 7.5-8 to 2.0. During early growth stage, the *Bacillus cereus*'s cell on glucose agar contains of lipid globules with largely poly-β-hydroxybutyrate. Then, the isolated strain SV13 was also identified by using 16S rDNA gene sequence analysis and was certified by Thailand Institute of Scientific and Technological Research (TISTR, Thailand). It was confirmed that it was closely to *Bacillus cereus* 99% identity. In Figure 4.7 showed morphology of SV13 on nutrient agar plate that appeared a large white colony, irregular margins and morphology under light microscopy. It was a gram-positive and rod shape with a pair or short chain.

Previously, Wang, Bakken (1998) reported the supporting data for PHAs production in terrestrial environments. They screened 63 soil bacteria for PHAs production and concluded that the strains capable of producing PHAs were not necessarily superior to the lack of ability. Survival ability was strain-specific and depended on the growth conditions prior to starvation state. Most PHAs-producing bacteria were found to belong to *Pseudomonas* and *Bacillus.* In addition, the accumulation of PHAs in *Bacillus* strains isolated from soil were investigated and reported by previous studies (Wang, Bakken, 1998; Yilmaz et al., 2005; Arshad et al., 2007).

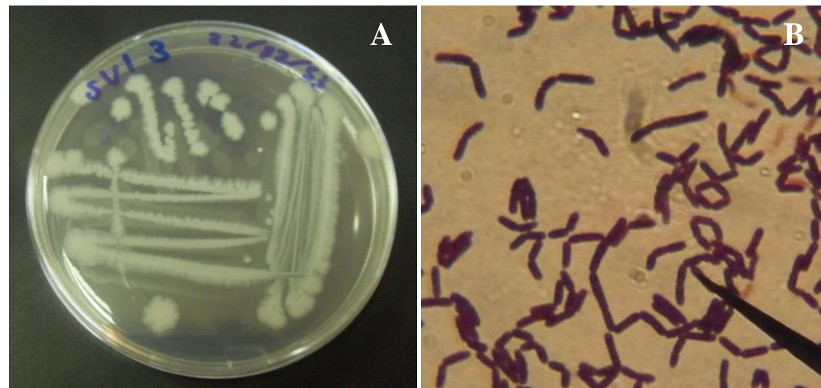


Figure 4.7 Morphology of SV13 (A) morphology of SV13 on nutrient agar plate (B) morphology under light microscopy

Steps of isolation and screening of PHAs biopolymer producing microorganisms as described in the section 4.2 are summarized by flow chart and shown in Figure 4.8

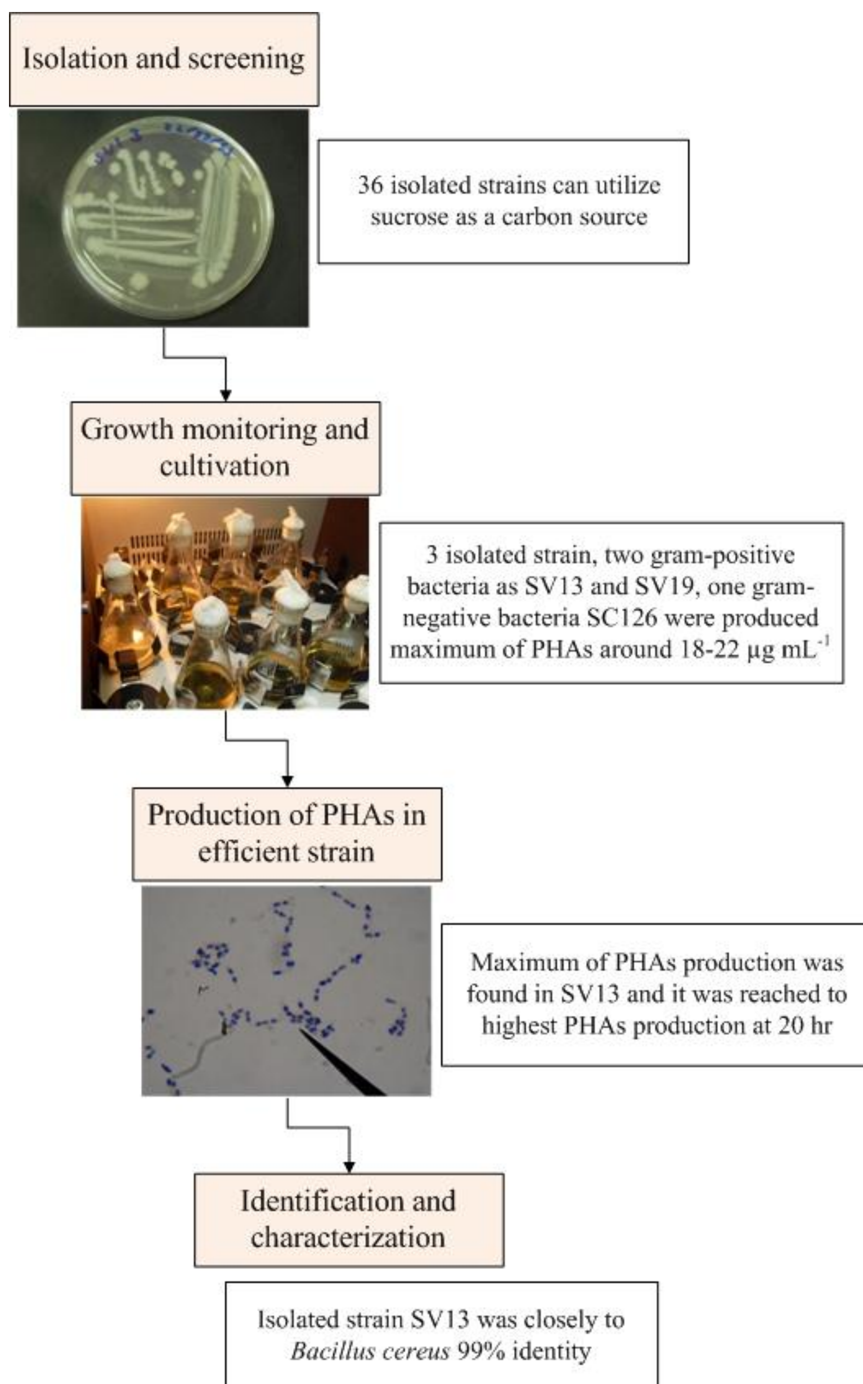


Figure 4.8 Flow chart of isolation and screening steps for PHAs biopolymer producing microorganisms

4.3 Production of PHAs by pure strain of *Alcaligenes latus* TISTR 1403

4.3.1 Cultivation and growth monitoring

A pure bacterial strain of *Alcaligenes latus* TISTR 1403 was purchased from Thailand Institute of Scientific and Technological Research (TISTR). The bacterial strain was cultivated in nutrient broth to re-activate. Then, it was investigated a growth profile in nutrient broth as shown in Figure 4.9 (A). The preliminary production of PHAs biopolymer was studied in a 250 mL shake flask containing 100 mL minimal medium, 10 gL^{-1} initial total sugars, pH 7. The inoculums 10% (v/v) from nutrient broth in log phase was inoculated. Then it was incubated at 35ºC in rotary shaker with 200 rpm agitation rate for 40 hr. The growth profiles results are shown in Figure 4.9 (B).

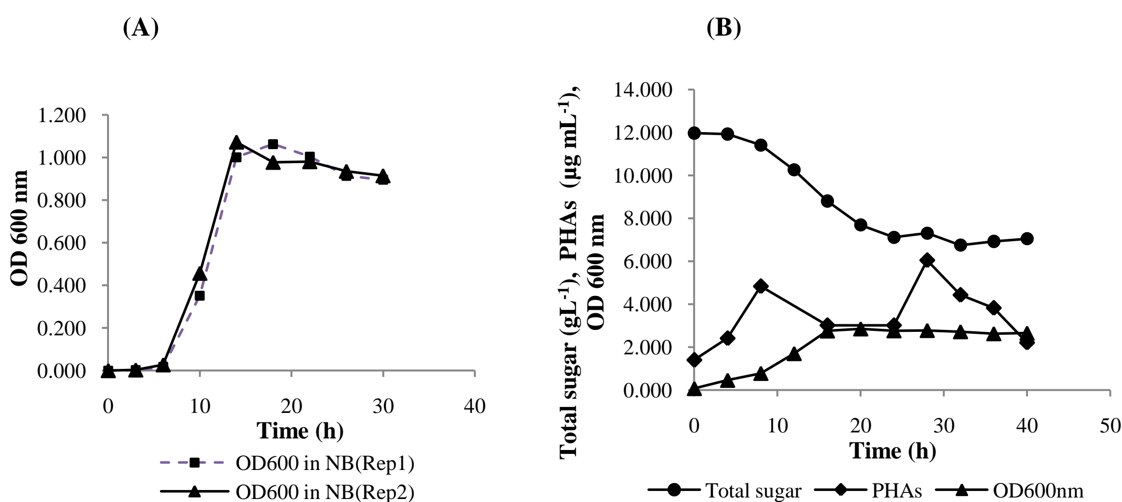


Figure 4.9 Growth profiles of *Alcaligenes latus* TISTR 1403, (A) in nutrient broth (B) in minimal medium

The growth profiles indicated that *A. latus* TISTR 1403 reached to stationary phase at 14 hr while total sugar consumed was decreased until 14 hr then it become steady. The production of PHAs was accumulated when the cell reached to stationary phase. In early cultivation time, total sugar consumed was rapidly decreased while PHAs content was increased. It was implied that this bacteria strain is growth-associated production of PHA. The results obtained in this study are in agreement with previous studied by Nath et al. (2008). They reported some microbial strains of

A. latus and *Methylobacterium* sp. ZP24 that showed the ability to accumulate PHAs during their growth. . In addition, it should be noted that growth profiles of the strains used should be confirmed during PHAs production.

4.3.2 Batch fermentation of sugarcane juice *Alcaligenes latus* TISTR 1403

The inoculums 10% (v/v) of *A. latus* TISTR 1403 was inoculated into 250 mL Erlenmeyer flask containing 100 mL minimal medium. It was composed of approximately 10 gL^{-1} sugarcane juice as a carbon source and the initial pH was controlled at 7. Then the flasks were incubated at 35ºC on rotary shaker with 200 rpm of agitation rate for 40 hr. The sample were withdrawn every 4 hr. The variations of growth, total sugar, DCW and PHAs contents were monitored and showed in Figure 4.10.

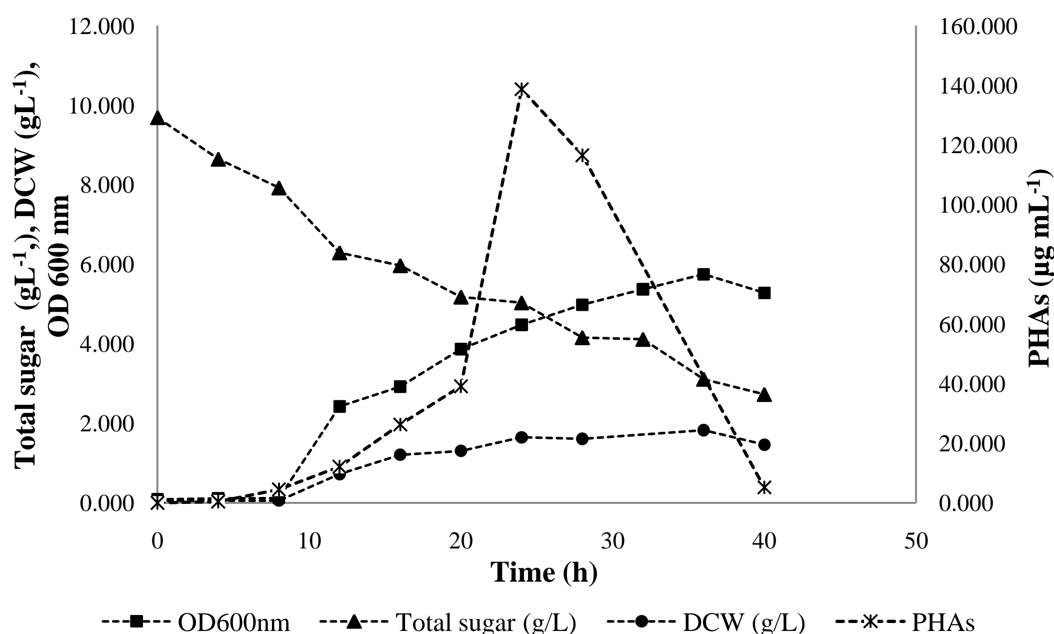


Figure 4.10 Growth profile and monitoring of total sugar consumed, dry cell weight (DCW) and PHAs as functions of time during batch fermentation by *Alcaligenes latus* TISTR 1403

The production of PHAs was slightly increased from 10 to 20 hr and reached the maximum at 138.73 μgL^{-1} within 24 hr during stationary phase. On the other hand, total sugar consumed was decreased until end of fermentation. However, in the

late phase of cultivation, PHAs content was sharply decreased. Since, the bacterial cell has to degrade PHAs to utilize as an energy source necessarily to survive during it came up to the last of growth cycle. Generally, PHAs were observed when they were associated with sporulation of bacteria. In some case, PHAs can be formed in other lipids especially for non-spore forming bacteria (Emeruwa, Hawirko, 1973). For many bacteria are accumulated PHAs to serve in both carbon and energy source during starvation such as *Alcaligenes latus* (Hrabak, 1992). Therefore, in this case the depletion of total sugar concentration as a carbon source leaded to decrease PHAs content. The DCW and optical density were slightly increased along the growth cycle although they become slightly decreased at the end of cultivation time. This resulted from the degradation of PHAs. In addition, the production of PHAs of *A. latus* TISTR 1403 is summarized in Table 4.6. Maximum PHAs yield and productivity were obtained at 24 hr. It could conclude that it is a suitable time to harvest cell mass for extraction of PHAs biopolymer.

Table 4.6 Summary of PHAs[a] production by *Alcaligenes latus* TISTR 1403

Time (hr)	**Total sugar consumed (S)** (gL^{-1})	**DCW (X)** (gL^{-1})	**PHAs (P)** (gL^{-1})	**Biomass yield ($Y_{X/S}$)** (g DCW/g total sugar consumed)	**PHAs yield ($Y_{P/S}$)** (g PHAs/g total sugar consumed)	**Productivity** ($g\ L^{-1}\ h^{-1}$)
0	0.00000	0.05556	0.00000	-	-	-
4	1.04592	0.05778	0.00040	0.05524	0.000385	0.000101
8	1.76871	0.05778	0.00450	0.03267	0.002544	0.000562
12	3.40136	0.72222	0.01209	0.21233	0.003554	0.001007
16	3.72449	1.20889	0.02626	0.32458	0.007049	0.001641
20	4.51531	1.30667	0.03908	0.28041	0.008655	0.001954
24	4.65986	1.64667	0.13873	0.35337	0.029772	0.005781
28	5.53571	1.60889	0.11651	0.29063	0.021046	0.004161
36	6.58163	1.82667	-	0.27754	0.011845	-
40	6.96429	1.46222	0.00517	0.20996	0.001071	0.000129

[a] PHAs content was analyzed in form of PHB by crotonic acid method

The results obtained were coincided with the previous preliminary studies. Extensively study of *A. latus* strain can be used to produce PHAs by using molasses or sugar syrup as carbon source. Under batch fermentation process, *A. latus* ATCC 29713 was reported to produce PHAs up to 63% of dry cell mass after 93 hr fermentation (Grothe et al., 1999) while Yu et al. (1999) studied the production of PHAs from *A.latus* DSM 1124 by using low cost of substrate as waste material such as soya waste. The PHAs content was obtained at 33% and 71% (weight) in case of soya waste and malt waste. These were implied that PHAs content produced by bacteria were related to raw material used. Yezza et al. (2007) were successfully to produce PHB by *A. latus* ATCC 29714 from maple sap. They used initial concentration of maple sap 20 gL^{-1} for 27 hr cultivation. The productivity obtained at 0.34 $g\ L^{-1}\ h^{-1}$ that was greater than this study. According to properties of raw materials between sugarcane juice and maple sap are different especially after heat treatment by sterilization. Maple sap showed a light yellow color while sugarcane juice was dark brown and the inhibitors were occurred from sterilization process that are interfered and caused to lower production of PHAs in case of sugarcane juice was used as carbon source.

4.4 Statistical optimization of process parameters for the production of PHAs in the efficient strain

The process to optimization, Plackett and Burman design was used to screen factors for fermentation process. After screening factors, response surface methodology (RSM) by central composite design (CCD) was used to optimize the process. The response value of PHAs production (µg mL^{-1}) and dry cell weight, DCW (gL^{-1}) were measured.

4.4.1 Screening factors of fermentation process parameters

Five variable factors such as initial total sugar, pH, agitation rate, inoculums size and nitrogen were chosen to screen the effective factors by using responses of Plackett and Burman design. The data obtained are shown in Table 4.7-4.8.

Table 4.7 Experimental design and responses of Plackett and Burman design on five variables

Replication 1

	Factors										Responses	
	A		B		C		D		E			
	Initial Total sugar (gL^{-1})		Inoculums (% (v/v))		Agitation rate (rpm)		pH		Nitrogen (gL^{-1})		PHAs (μg mL^{-1})	DCW (gL^{-1})
	Code	value	Code	value	Code	value	Code	value	Code	value		
1	+	50	+	20	+	300	-	6	+	2.0	444.668	3.9300
2	+	50	+	20	-	100	+	9	-	0.0	14.370	1.7300
3	+	50	-	5	+	300	-	6	-	0.0	17.325	2.3500
4	-	10	+	20	-	100	-	6	+	2.0	106.702	1.1900
5	+	50	-	5	-	100	+	9	+	2.0	23.100	2.3100
6	-	10	-	5	+	300	+	9	+	2.0	5.305	0.3800
7	-	10	+	20	+	300	+	9	-	0.0	9.334	1.0800
8	-	10	-	5	-	100	-	6	-	0.0	62.114	1.0500

Replication 2

	Factors										Responses	
	A		B		C		D		E			
	Initial Total sugar (gL^{-1})		Inoculums (% (v/v))		Agitation rate (rpm)		pH		Nitrogen (gL^{-1})		PHAs (μg mL^{-1})	DCW (gL^{-1})
	Code	value	Code	value	Code	value	Code	value	Code	value		
1	+	50	+	20	+	300	-	6	+	2.0	461.456	3.7300
2	+	50	+	20	-	100	+	9	-	0.0	7.991	1.4900
3	+	50	-	5	+	300	-	6	-	0.0	30.553	2.1000
4	-	10	+	20	-	100	-	6	+	2.0	58.018	0.9600
5	+	50	-	5	-	100	+	9	+	2.0	27.733	1.9700
6	-	10	-	5	+	300	+	9	+	2.0	9.804	1.6200
7	-	10	+	20	+	300	+	9	-	0.0	29.412	1.0500
8	-	10	-	5	-	100	-	6	-	0.0	54.727	0.4600

Table 4.8 The results of data analysis (*t*-value) on the effect of factors in fermentation process on PHAs production and biomass (DCW) in the isolated strain SV13

Replication 1

Factors	PHAs		Confidence level	DCW		Confidence level
	Effect	*t*-value		Effect	*t*-value	
Initial total sugar	79.002	0.825		1.655	3.436*	95 %
Inoculums	116.805	1.220		0.460	0.955	
Agitation rate	67.587	0.706		0.365	0.758	
pH	-144.66	-1.511*	70 %	-0.755	-1.567*	70 %
Nitrogen	119.199	1.245*	65 %	0.400	0.830	

Replication 2

Factors	PHAs		Confidence level	DCW		Confidence level
	Effect	*t*-value		Effect	*t*-value	
Initial total sugar	93.943	0.875		1.300	4.587*	95 %
Inoculums	108.515	1.011		0.270	0.953	
Agitation rate	95.689	0.891		0.905	3.193*	90 %
pH	-132.475	-1.234*	65 %	-0.280	-0.988	
Nitrogen	108.582	1.011		0.795	2.805*	85 %

In Table 4.8, the data of replication 1 was shown that, pH and nitrogen are factors that directly affect for PHAs production at 70 and 65% confidence levels while initial total sugar and pH were also indirect affected to DCW at 95 and 70% confidence levels. The results were indicated that, increasing of nitrogen, PHAs production was increased meanwhile decreasing of pH, the production of PHAs was increased. However, increasing of initial total sugar, the DCW was increased although it was not directly affected to PHAs production. While, in case of the replication 2

showed in the same effect of pH on PHAs as in replication 1. Moreover, agitation rate and nitrogen were affected to DCW at 90 and 85% confidence levels. These implied that as agitation rate and nitrogen increased resulted in an increasing of the DCW.

After using the Plackett and Burman design to screen all 5 factors and found that 4 factors as pH, nitrogen, initial total sugar and agitation rate that were affected on both PHAs production and DCW. Although, the initial total sugar showed in the maximum confidence level in both replications (up to 95%) however, in practical, it could not control or set up to desired value following the experimental design because of the limitation of the equipments. It also significantly affected on the DCW but was not direct to the PHAs. In addition, both pH and nitrogen showed in the confidence levels that were lower than other factors in both replications. However, they showed an effect directly on PHAs in replication 1 and indirectly on PHAs but directly on the DCW. It also should be noted that since, the design used for screening in this study could not explain the interaction between factors (Wiriyacharee, 2004). Most studies that use this design to screen the factors should not only consider just high confidence level but also lower confidence level but directly affected on the product requirement. Therefore, in this case only 3 factors as pH, nitrogen and agitation rate were chosen to continue the process optimization by using the Central Composite Design or CCD.

Previously study, Grothe et al. (1999) reported that the initial pH of culture medium was significantly affected to PHB and biomass productivities and optimal initial pH is 6.5 that is neutral to weakly acid that related to this study.

4.4.2 Optimization of PHAs production using central composite design (CCD)

After screening, response surface methodology (RSM) by using central composite design (CCD) was desired to investigate the optimization of PHAs production. The factors as agitation rate, pH and nitrogen were used on this step. The Design-Expert version 6.0.10® software was used for Design of Experiments (DOE). Design of experiments and response values are shown in Table 4.9 and analysis of variance is shown in Table 4.10.

Table 4.9 Central composite design of variables for process optimization of strain SV13 measured response of PHAs

Run	Code Factors			Actual Factors			Response (Y)
	A	B	C	A	B	C	
	Agitation rate (rpm)	pH	Nitrogen (gL^{-1})	Agitation rate (rpm)	pH	Nitrogen (gL^{-1})	PHAs ($\mu g\ mL^{-1}$)
1	-1.000	-1.000	-1.000	140.55	6.60	1.00	231.440
2	1.000	-1.000	-1.000	259.45	6.60	1.00	431.938
3	-1.000	1.000	-1.000	140.55	8.40	1.00	66.613
4	1.000	1.000	-1.000	259.45	8.40	1.00	357.370
5	-1.000	-1.000	1.000	140.55	6.60	3.00	161.698
6	1.000	-1.000	1.000	259.45	6.60	3.00	560.032
7	-1.000	1.000	1.000	140.55	8.40	3.00	89.847
8	1.000	1.000	1.000	259.45	8.40	3.00	436.980
9	-1.682	0.000	0.000	100.02	7.50	2.00	22.020
10	1.682	0.000	0.000	299.98	7.50	2.00	551.303
11	0.000	-1.682	0.000	200.00	5.99	2.00	491.940
12	0.000	1.682	0.000	200.00	9.01	2.00	134.430
13	0.000	0.000	-1.682	200.00	7.50	0.32	291.160
14	0.000	0.000	1.682	200.00	7.50	3.68	164.518
15	0.000	0.000	0.000	200.00	7.50	2.00	415.250
16	0.000	0.000	0.000	200.00	7.50	2.00	416.060
17	0.000	0.000	0.000	200.00	7.50	2.00	443.997

Table 4.10 ANOVA results for response surface quadratic model of PHAs production

Source	Sum of squares	df	Mean square	*F*-value	Prob>*F*[a]	
Model	473400.43	9	52600.05	14.66386	0.0009	significant
A	331230.06	1	331230.1	92.34046	< 0.0001	significant
B	78522.86	1	78522.86	21.89064	0.0023	significant
C	196.40	1	196.4003	0.054753	0.8217	
A^2	21198.77	1	21198.77	5.909803	0.0454	significant
B^2	13021.57	1	13021.57	3.630159	0.0984	
C^2	46408.04	1	46408.04	12.93765	0.0088	significant
AB	190.69	1	190.6885	0.05316	0.8242	
AC	8078.00	1	8077.996	2.251987	0.1771	
BC	247.45	1	247.4462	0.068983	0.8004	
Residual	25109.37	7	3587.052			
Lack of fit	24573.53	5	4914.707	18.34417	0.0525	not significant
Pure error	535.83	2	267.9166			
Total	498509.80	16				

[a] Prob > *F* less than 0.0500 indicate model terms are significant.

Final equation in terms of coded factors:

$$Y\ (PHAs) = +\ 423.48 + 155.74*A - 75.83*B - 3.79*C - 43.36*A2 - 33.99*B2 - 64.16*C2 + 4.88*A*B + 31.78*A*C + 5.56*B*C$$

Where Y is response, i.e. PHAs production and A, B, C are coded in terms of variables, i.e. agitation rate, pH and nitrogen, respectively. Analysis of variance (ANOVA) in Table 4.10 are presented "Prob>*F*" of model less than 0.0500. It is indicated that the model were significant and A, B, A^2, C^2 were also significant for

model terms. The Model *F*-value of 14.66 interpreted that the model was significant and contained only 0.09% chance for a "Model *F*-Value". This large could occur due to noise effect. The *F*-value of “Lack of fit” showed at 18.34. It was implied that the lack of fit was not significant relative to the pure error. There was 5.25% chance that was high enough to occur noise effect. The "Pred R-Squared" of 0.6152 was not close to the "Adj R-Squared" (0.8849) as one might normally expected. These may indicated that a large block effect or a possible problem with model or data. However, the coefficient determination of R-Squared (R^2) was 0.9496 that eqivalent to 94.96% variability in the response that could be explained by this model. It was implied that this model can be used to navigate the design space.

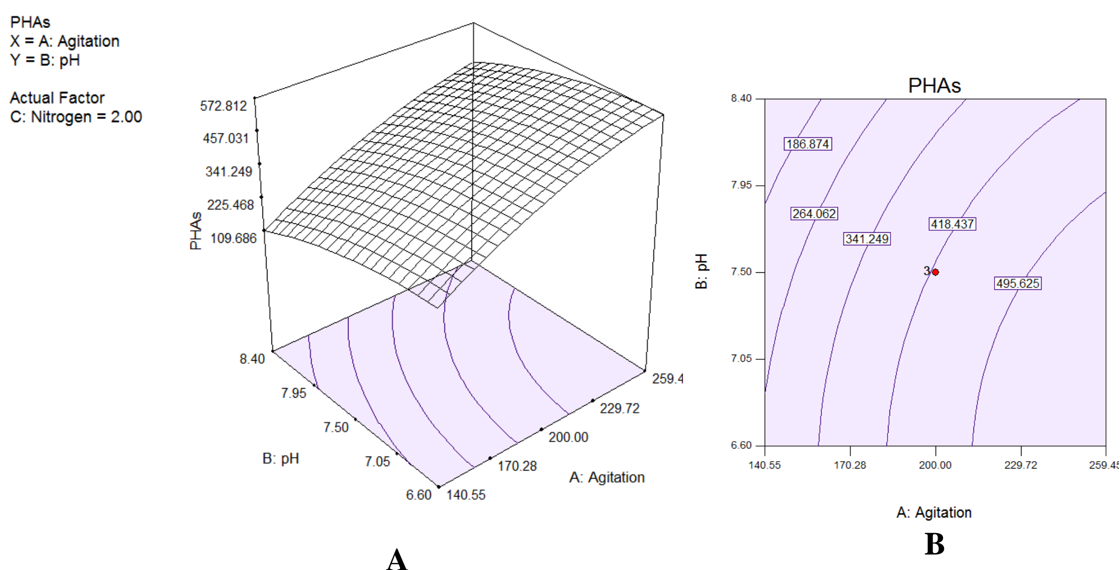
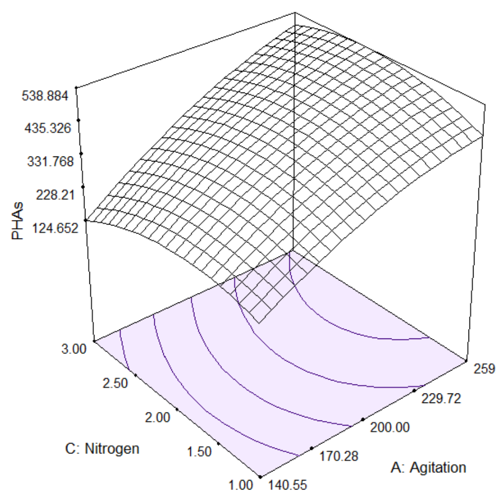


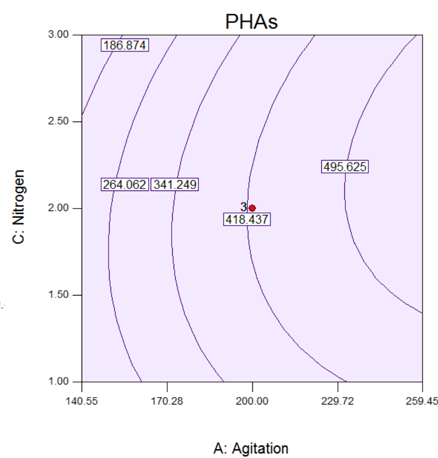
Figure 4.11 The 3D response surface and contour plots: (A) and (B) are interactive effects of agitation rate and pH with 2.0 gL^{-1} of nitrogen; (C) and (D) are interactive of agitation rate and nitrogen with pH at 7.0; (E) and (F) are interactive of pH and nitrogen with 200 rpm of agitation rate.

PHAs
X = A: Agitation
Y = C: Nitrogen

Actual Factor
B: pH = 7.50



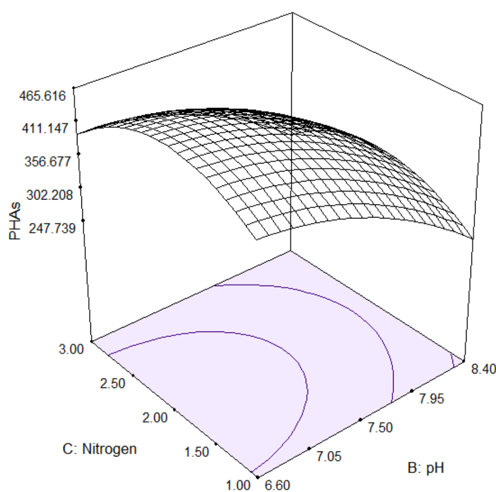
C



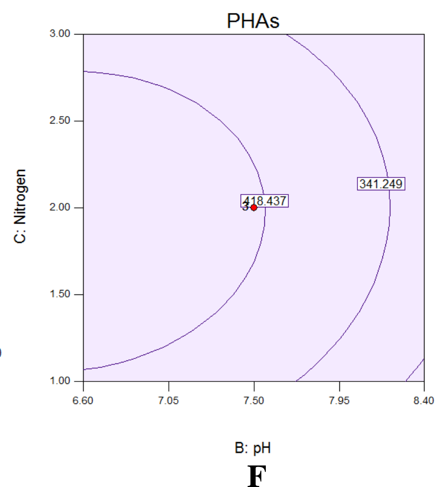
D

PHAs
X = B: pH
Y = C: Nitrogen

Actual Factor
A: Agitation = 200.00



E



F

Figure 4.11 The 3D response surface and contour plots: (A) and (B) are interactive effects of agitation rate and pH with 2.0 gL^{-1} of nitrogen; (C) and (D) are interactive of agitation rate and nitrogen with pH at 7.0; (E) and (F) are interactive of pH and nitrogen with 200 rpm of agitation rate. (Cont.)

Considering, the response surface of PHAs production in Figure 4.11 revealed that the range of pH and agitation rate may not include the maximum area of PHAs production. It was suspected that trial new range of pH and agitation rate could be obtained and including all maximum surface of PHAs production. Therefore, the

investigation of agitation rate, pH and nitrogen were carried on trial a range of values that affected on the PHAs production. Then, they were optimized by using the CCD.

Table 4.11 Study on range of variable factors that effect to high PHAs production

Factors			**PHAs (µg mL^{-1})**		**High value of PHAs**
Agitation rate	pH	Nitrogen	Rep1	Rep2	
350	6.0	2.0	1045.024	964.948	Effect of agitation rate at 350 rpm
200	6.0	2.0	437.469	669.890	
300	6.0	0.5	979.223	983.076	Effect of nitrogen at 0.5 gL^{-1}
300	6.0	4.0	930.127	820.040	
300	5.0	2.0	1008.713	988.941	Effect of pH at 5.0
300	7.5	2.0	489.824	465.955	

Table 4.11 showed increasing and decreasing productivity trends when the factors values were varied. As agitaion rate increased, the PHAs production was increased. Meanwhile, pH and nitrogen decreased, the maximum PHAs was reached. However, due to the limitations of the equipment in the study,the agitation rate can not set the value over 350 rpm. Therefore, only pH and nitrogen were studied while agitation rate was set up consistantly at 300 rpm. The design experiment of CCD was shown in Table 4.12.

Table 4.12 Central composite design of variables for process optimization of strain SV13 measured response of PHAs

Run	Code Factors A	Code Factors B	Actual Factors A	Actual Factors B	Response Replication 1	Response Replication 2
	pH	Nitrogen (gL^{-1})	pH	Nitrogen (gL^{-1})	PHAs ($\mu g\ mL^{-1}$)	PHAs ($\mu g\ mL^{-1}$)
1	-1.00	-1.000	5.00	0.50	348.619	378.453
2	1.000	-1.000	6.50	0.50	538.122	482.044
3	-1.000	1.000	5.00	2.75	325.691	323.632
4	1.000	1.000	6.50	2.75	570.994	474.586
5	-1.414	0.000	4.69	1.63	236.188	248.895
6	1.414	0.000	6.81	1.63	440.608	416.851
7	0.000	-1.414	5.75	0.03	585.635	595.027
8	0.000	1.414	5.75	3.22	565.193	559.669
9	0.000	0.000	5.75	1.63	687.84	626.51
10	0.000	0.000	5.75	1.63	736.46	626.52
11	0.000	0.000	5.75	1.63	686.74	652.49
12	0.000	0.000	5.75	1.63	676.519	654.69

Table 4.13 ANOVA results for response surface quadratic model of PHB production in replication 1

Source	Sum of squares	df	Mean square	*F*-value	Prob>*F*[a]	
Model	283235.6	5	56647.11	64.70772	< 0.0001	significant
A	65503.82	1	65503.82	74.82468	0.0001	significant
B	44.96058	1	44.96058	0.051358	0.8282	
A^2	212012.8	1	212012.8	242.1812	< 0.0001	significant
B^2	25806.6	1	25806.6	29.47875	0.0016	significant
AB	778.41	1	778.41	0.889174	0.3821	
Residual	5252.584	6	875.4306			
Lack of Fit	3086.896	3	1028.965	1.425365	0.3889	not significant
Pure Error	2165.688	3	721.8958			
Cor Total	288488.2	11				

[a] Prob > *F* less than 0.0500 indicate model terms are significant.

Final equation in terms of coded factors:

$$Y \text{ (PHAs in Replication1)} = +696.80 +90.49*A -2.37*B -182.01*A^2 -63.50*B^2 +13.95*A*B$$

Where Y is response, i.e. PHAs production, A and B are code term variables, i.e. pH and nitrogen. Analysis of variance (ANOVA) in Table 4.13 are shown the “Prob>*F*” of model were less than 0.0500. It indicated that the model was significant and A, A^2, B^2 were significant for the model terms. The Model *F*-value of 64.71 implied that the model was significant. There was only a 0.01% chance that a "Model *F*-Value". This large could occur due to noise effect. The “Lack of fit” of 1.43 implied that the lack of fit was not significant relative to pure error. There was 38.89% chance that large could occur due to noise. The "Pred R-Squared" of 0.9106 showed reasonable and corresponded to the "Adj R-Squared" of 0.9666. The coefficient determination of R-Squared (R^2) was 0.9818 that was indicated that 98.18% variability in the response could be explained by this model. It was implied

that this model can be used to navigate the design space. The 3D response surface and contour plots of PHAs production in replication 1 between the interactive effect of pH and nitrogen are shown in Figure 4.12.

The results obtained clearly showed that pH was significantly affected on the PHAs production. This is in agreement with previous studies by Grothe et al. (1999) who reported that nitrogen was slightly affected for PHAs production because bacteria in genus of *Bacillus* can accumulate PHAs as a carbon source under non limiting nutrient (Chen, 2010).

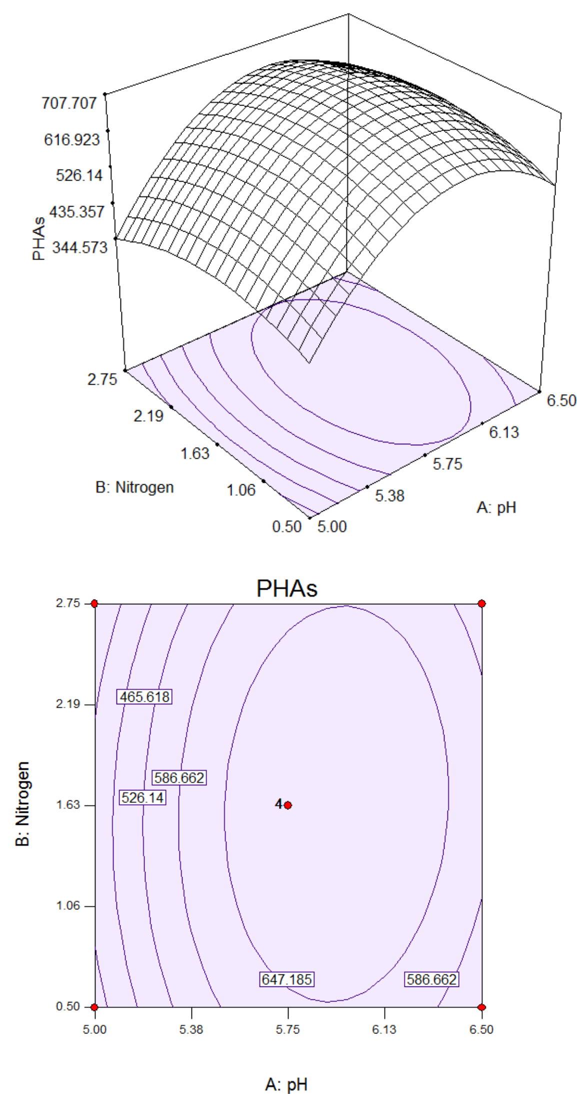


Figure 4.12 The 3D response surface and contour plots of PHAs production in replication 1: interactive effect of pH and nitrogen at 300 rpm of agitation rate.

Table 4.14 ANOVA results for response surface quadratic model of PHAs production in replication 2

Source	Sum of squares	df	Mean square	*F*-value	Prob>*F*[a]	
Model	204410.8	5	40882.16	60.41742	< 0.0001	significant
A	30266.69	1	30266.69	44.72942	0.0005	significant
B	1575.927	1	1575.927	2.328973	0.1778	
A2	171500.4	1	171500.4	253.4506	< 0.0001	significant
B2	11001.26	1	11001.26	16.25814	0.0069	significant
AB	560.8134	1	560.8134	0.828794	0.3977	
Residual	4059.971	6	676.6618			
Lack of Fit	3324.495	3	1108.165	4.520197	0.1235	not significant
Pure Error	735.4757	3	245.1586			
Cor Total	208470.8	11				

[a] Prob > *F* less than 0.0500 indicate model terms are significant.

Final equation in terms of coded factors:

$$Y \text{ (PHAs in Replication 2)} = +640.05 +61.51*A -14.04*B -163.70*A^2 -41.46*B^2 +11.84*A*B$$

Where Y is response, i.e. PHAs production, A and B are coded as term of variables, i.e. pH and nitrogen. Analysis of variance (ANOVA) in Table 4.14 are presented “Prob>*F*” of model that were less than 0.0500 indicated model were significant and A, A^2, B^2 were also significant for model terms. The Model *F*-value of 60.42 implied that the model was significant. There was only a 0.01% chance that a "Model *F*-Value". This large could occur due to noise. A value of “Lack of fit” at 4.52 implied thatthe lack of fit was not significant relative to pure error. There was 12.35% chance that large could occur due to noise. The "Pred R-Squared" of 0.8803 was reasonable and agreed with the "Adj R-Squared"of 0.9643. The coefficient determination of R-Squared (R^2) was 0.9805, indicated that 98.05% variability in the response could be explained by this model. It was implied that this model can be used

to navigate the design space. The optimial response surface area was shown in Figure 4.13.

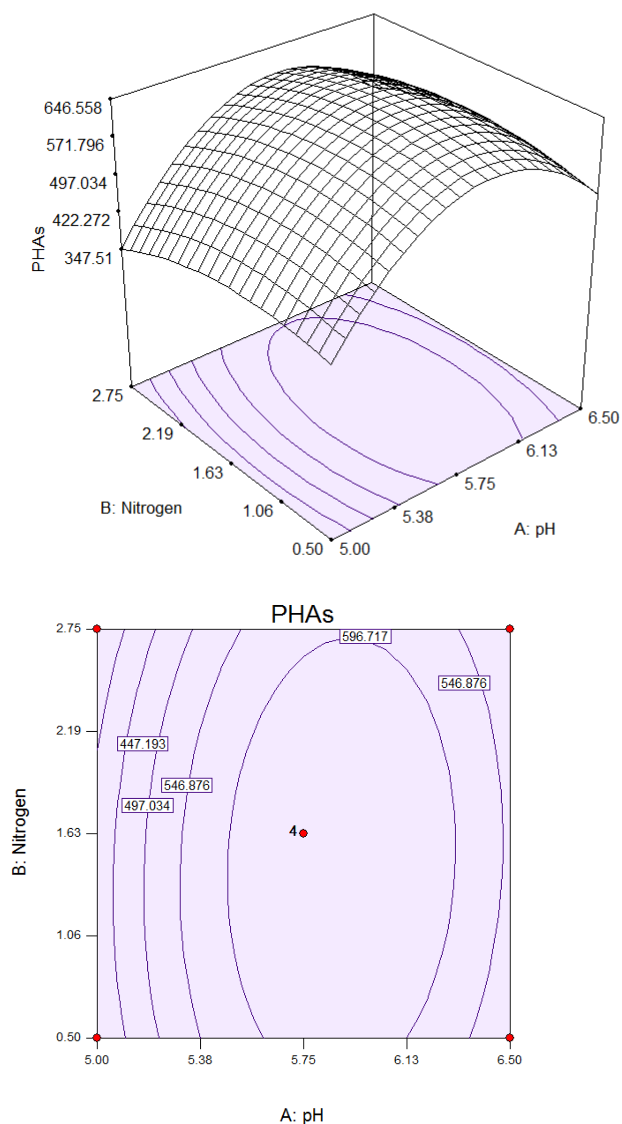


Figure 4.13 The 3D response surface and contour plots of PHAs production in replication 2: interactive effect of pH and nitrogen at 300 rpm of agitation rate.

4.4.3 Validation of response surface methodology (RSM) model

The validation of response surface model is shown in Table 4.15, pH and nitrogen values were chosen in the optimal response surface area from model and carried out in flask scale. The optimal condition that gave maximum PHAs production was designed by the RSM was further carried out in a flask and 5 L fermentor with 3 L working volume are shown in Table 4.16.

Table 4.15 Validation of RSM optimization model

pH	**Nitrogen**	**Condition**[a]	**Total sugar consumed** (gL^{-1})	**DCW** (gL^{-1})	**PHAs**[b] (gL^{-1})		
					Actual	Prediction (Equation in Rep1)	Prediction (Equation in Rep2)
5.9	1.46	Flask1	17.078	3.1907	0.6174	0.706	0.646
		Flask2	17.004	3.150	0.6178		
5.8	1.63	Flask1	15.423	2.9200	0.662	0.702	0.643
		Flask2	12.497	3.232	0.626		
5.8	0.03	Flask1	10.346	3.156	0.557	0.576	0.578
		Flask2	12.261	3.216	0.595		

[a] The initial total sugar was 50 gL^{-1}. The culture conditions were incubated at 37°C, 300 rpm agitation rate and 20% (v/v) inoculums.

[b] PHAs was analyzed in form of PHB.

Table 4.16 Verification of PHAs[a] production by SV13[b] using the optimize conditions

Scale	Total sugar consumed (gL^{-1})	DCW (gL^{-1})	PHAs (gL^{-1})	$Y_{P/X}$	$Y_{P/S}$	Productivity ($g L^{-1} h^{-1}$)	Specific productivity ($g g h^{-1}$)
Flask	17.35	3.1710	0.6174	0.1947	0.0356	0.0309	0.009736
	27.50	4.035	0.7913	0.196	0.0288	0.0396	0.009802
	31.41	4.211	0.7907	0.188	0.0252	0.0395	0.00938
Fermentor	15.72	1.0558	0.0174	0.0165	0.00111	0.0008	0.000824
	27.10	1.106	0.0219	0.0198	0.00081	0.0011	0.000990

[a] PHAs content was analyzed in form of PHB.

[b] The initial total sugar was 50 gL^{-1}. The culture conditions were incubated at 37°C, 300 rpm agitation rate and 20% (v/v) inoculums, pH 5.9 and 1.63 gL^{-1} nitrogen.

The result in Table 4.15 showed the optimum response surface model by using CCD design of 2 factors of pH and nitrogen. They gave the actual value nearly the prediction value observed by regression model. This is implied that the model can be applied for the production of PHAs. In Table 4.16, it was confirmed that the efficiency of this model for PHAs production under batch fermentation in flask scale. In contrast, the PHAs production in 5 L fermentor showed in lower value of PHAs yield. However, the results obtained in case of fermentation should be confirmed in further experiment.

4.5 Production of PHAs in fermentor

The production of PHAs using the optimize condition was designed by the RSM and recovery are shown in Table 4.17. The cells were harvested by centrifugation to obtain, cell pallets. After that, the cells were dried and disrupted with 6% NaClO and finally chloroform was added, the extracted PHAs were obtained that shown in Figure 4.14.

Table 4.17 Production of PHAs[a] using the optimize condition by SV13[b]

Scale	Time (hr)	Total sugar consumed (gL^{-1})	DCW (gL^{-1})	PHAs (gL^{-1})	$Y_{X/S}$	$Y_{P/S}$	PHAs (%w/w)	Productivity ($g\ L^{-1}\ h^{-1}$)	Specific productivity ($g\ g\ h^{-1}$)
Flask	20	27.5	4.035	1.546	0.1467	0.05621	38.31	0.0773	0.01916
Fermentor	20	27.101	1.106	0.021	0.0408	0.00077	1.89	0.0015	0.00095
	24	31.329	1.140	0.034	0.0364	0.00108	2.98	0.0014	0.00124

[a] PHAs was analyzed by recovery and extraction with chloroform.

[b] The initial total sugar was 80 gL^{-1}. The culture conditions were incubated at 37°C, 300 rpm agitation rate and 20% (v/v) inoculums, pH 5.9 and 1.63 gL^{-1} nitrogen.

The results showed the PHAs production obtained in a fermentor was lower than that found in the shake flask. It could conclude that regression model from batch fermentation in flask scale can not apply in a fermentor. This might be extended the working volume for PHAs production from 100 mL to 3,000 mL that caused the change of suitable time for cell harvesting. Furthermore, the mass transfer was also affected on PHAs production. Moreover, the Plackett and Burman design used for screening factors step showed the agitation rate that was significantly to DCW caused low density of cell than shake flask. It is recommended that agitation rate should be investigated. Although, the mathematic model for process optimization via response surface methodology can not apply in a fermentor scale. However, the best production of PHAs by using optimized area obtained in flask scale in this study that was similar to some previous studies as the production of PHAs by *Bacillus cereus* SPV under batch fermentation in shake flask occur in late stationary phase i.e. after 74 hr of growth. The products of 2.143 gL^{-1} DCW, 0.814 gL^{-1} PHAs and 38% (w/w) PHAs

were obtained when glucose was used as a carbon source while the DCW and PHAs were at 1.666 gL^{-1} and 0.640 gL^{-1} and 38.40% (w/w) PHAs when sucrose was employed as a carbon source (Valappil et al., 2007). Later, agro-industrial residues such as corn flour, wheat bran and cassava bagasse were hydrolyzed as substrate for *B. sphaericus* NCIM 5149. The production of 1.5 gL^{-1} DCW, 0.049 gL^{-1} PHAs and 3.3 % (w/w) was obtained when corn flour was used as carbon source while in case of cassava bagasse, 2.5 gL^{-1} DCW, 0.161 gL^{-1} PHAs and 6.4 % (w/w) PHAs were obtained (Ramadas et al., 2009).

Recently, the greater PHAs production by *B. cereus* YB-4 (6.1 gL^{-1} DCW, 23% (w/w) PHAs) was obtained after 24 hr batch fermentation in shake flask when glucose was used as a carbon source (Mizuno et al., 2010). In Figure 4.14 showed a biopolymer of PHAs obtained after extraction by using chloroform under optimized area from RSM via batch fermentation by SV13.

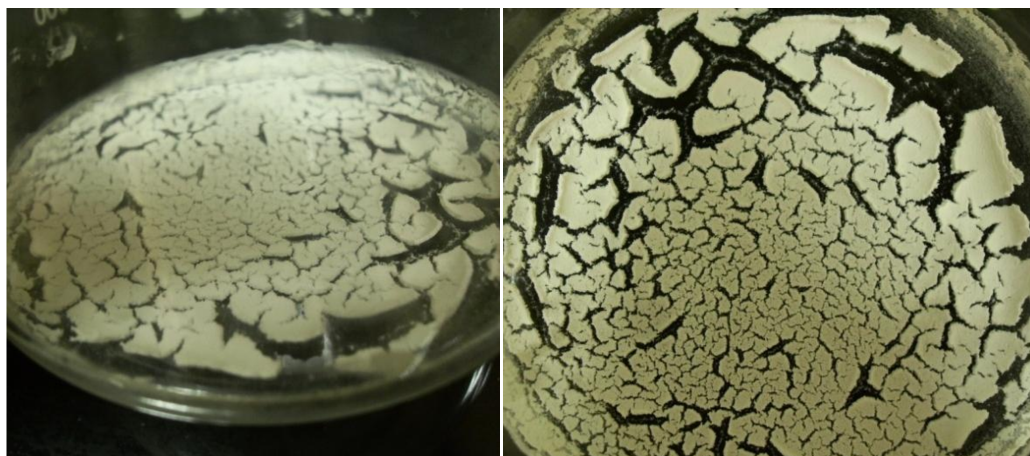


Figure 4.14 PHAs biopolymer in white solid obtained under optimized area from RSM via batch fermentation by SV13