CHAPTER III
RESEARCH METHODOLOGY

This chapter contains research methodology that are separated into several sections such as information of microorganisms, chemicals, instruments, raw material, the experimental framework and procedures of experiment respectively.

3.1 Microorganisms

A pure strain of *Alcaligenes latus* TISTR 1403, gram negative, aerobic bacteria, rods or cocobacilli and occurring singly in pairs or rarely in chains is used in this study. It was purchase from Thailand Institute of Scientific and Technological Research (TISTR). The lyophilized bacteria was re-activated in shake flask (250 mL) containing 100 mL of nutrient broth (NB) at 35°C. The bacteria was maintained on nutrient agar plate at 4°C until needed. In Figure 3.1 (A) the bacteria was streaked on the agar and (B) shows pairs on chains of *A. latus*.

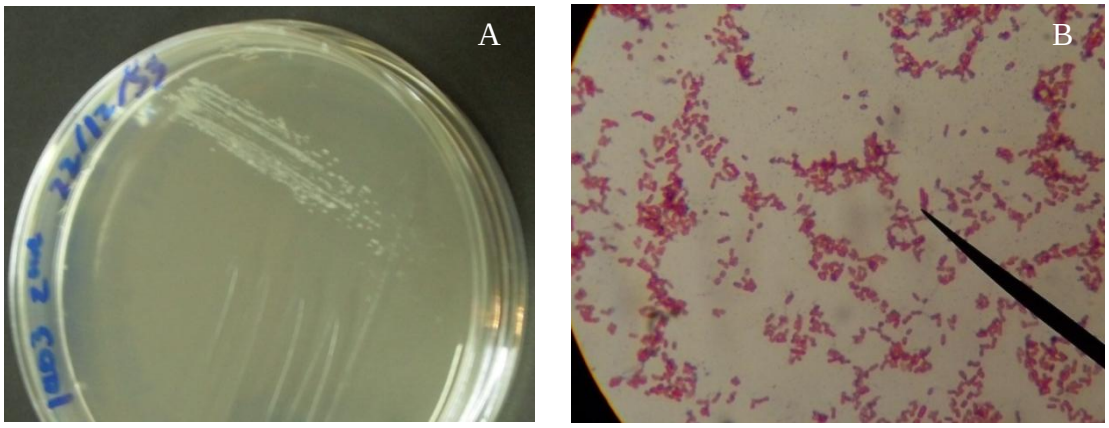

Figure 3.1 Morphology of *Alcaligenes latus* TISTR 1403 (A) on nutrient agar plate and (B) gram stain under light microscopy

3.2 Chemical and instruments

3.2.1 Chemicals

All chemical used in this study are analytical grade. The details of them and the suppliers are shown as follow:

Chemicals	Company
Chloroform ($CHCl_3$)	Lab scan, Ireland
Sulfuric acid (H_2SO_4)	Merck, Germany
Dipotassium phosphate (K_2HPO_4)	Ajax Finechem, New Zealand
Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$)	Ajax Finechem, New Zealand
Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$)	Ajax Finechem, New Zealand
Ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$)	Ajax Finechem, New Zealand
Cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$)	Sigma-Aldrich, Germany
Manganese sulfate tetrahydrate ($MnSO_4 \cdot 4H_2O$)	Ajax Finechem, New Zealand
Copper(II) sulfate pentahydrate (CuSO4·5H2O)	Ajax Finechem, New Zealand
Diamonium sulfate ($(NH_4)_2SO_4$)	BDH, England
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$)	Ajax Finechem, New Zealand
Potassium dihydrogen phosphate (KH_2PO_4)	Ajax Finechem, New Zealand
Boric acid (H_3BO_3)	BDH, England
Manganese(II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$)	Sigma-Aldrich, Germany
Hydrochloric acid (HCl)	Lab Scan, Ireland

Disodium molybdate tetrahydrate ($Na_2MoO_4.4H_2O$)	Ajax Finechem, New Zealand
Sodium hydroxide (NaOH)	BDH, England
Poly(3-)hydroxybutyrate	Fluka, USA
Sudan black B ($C_{29}H_{24}N_6$)	Fluka, UK
Glucose	Ajax Finechem, New Zealand
Peptone powder	Hiemedia, India
Beef extract powder	CRITERION^{TM}, USA
Yeast extract	Hiemedia, India
Ethanol (C_2H_5OH)	Commercial
Sodium hypochlorite (NaClO)	Commercial
Sucrose	Commercial

3.2.2 Instruments

Instruments	Model/Company
Incubator shaker	VS-8480SFN
Centrifuge	TGL-16C
Spectrophotometer	Shimadzu UV-1601
Spectrophotometer	T60 Visible
High Performance Liquid Chromatography	Shimadzu
pH meter	Senz pH (Digital pH Tester)
pH meter	pH/Ion 510
Laminar air flow	CTL 120 BT
Hot air oven	LDO-060E (Labtech)
Desiccator	Lio LAB
Vertex mixture	LMS Mixer VTX-3000L
Chemical fume hood	Greentech
Fermentor	Biostat B
Hand refractometer	N-1α/Atago

Microscopy	ZEIZZ Primo Star
Autopipette 100 µL	Biohit
Autopipette 1000 µL	Pipetman (Gilson)
Autopipette 5000 µL	Pipetman (Gilson)
Autoclave	LAC-5040S (Labtech)
Hotplate stirrer	LMS-1003 (Labtech)
Refrigerator	Sanyo
Analytical balance	Sartorius BP221S
Precision Scale	Sartorius BP3100S

3.3 Raw material

The sugarcane juice as shown in Figure 3.2 was kindly provided from sugar cane industry (Mitraphol Research and Development, Co., Ltd, Chaiyaphum province, Thailand). The juice then was kept at -20°C in freeze refrigerator to prevent others microbial contamination. In addition, the juice was thawed and then sterilized by autoclave at temperature of 110°C for 20 min prior to use.

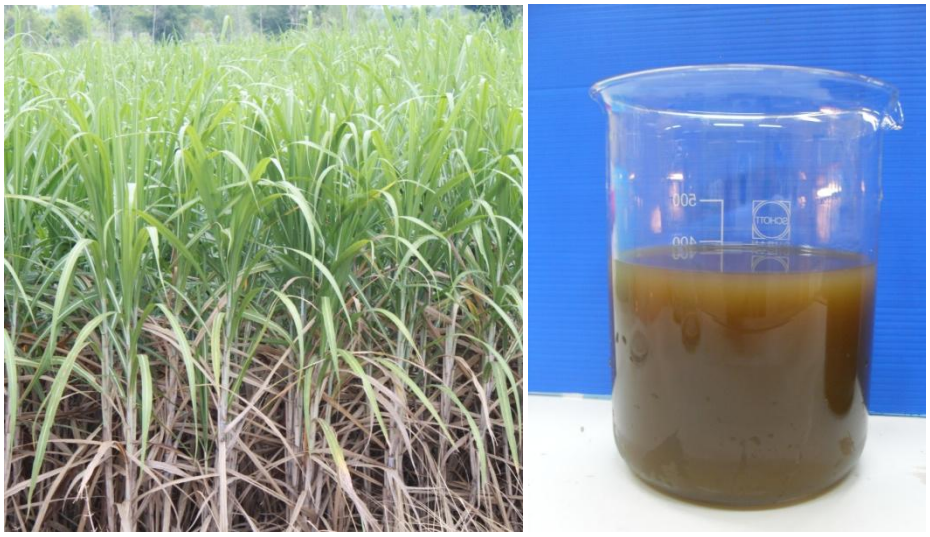

Figure 3.2 Sugarcane plant and its juice

3.4 Experimental framework

In this study the experimental frame work was scoped and showed in Figure 3.3. Firstly, the raw material of sugarcane juice was characterized in its physical and chemical properties. The pure bacterial strain was maintained in the nutrient broth coupling with the isolation and screening steps of the PHAs biopolymer producing bacterial form environments was investigated. Then, growth conditions and cultivations of both pure and isolated strains were carried out in flask scale. In addition, screening factors in fermentation process parameters was done by Plackett and Burman design while the process optimization of the PHAs production was investigated by response surface methodology (RSM) using central composite design (CCD). Then, the optimal condition obtained was further investigated in large scale of 5 L fermentor. Finally, the data obtained from sequent step of the experiment were analyzed using standard methods. The flow chart of experimental study and framework is shown in Figure 3.3.

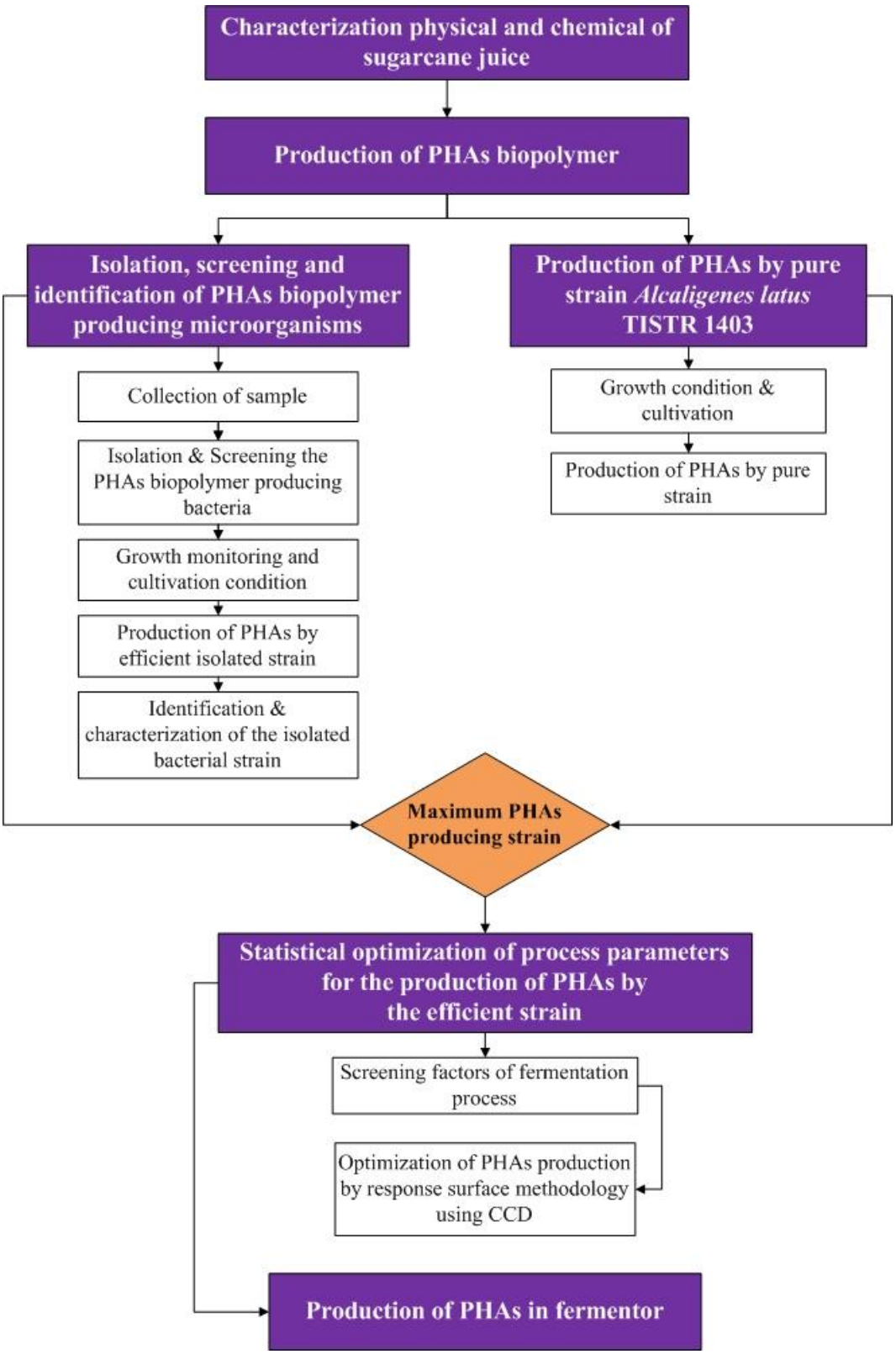

Figure 3.3 Flow chart of experimental design and framework

3.5 Procedures

3.5.1 Characterization of sugarcane juice

Some physical and chemical properties of sugarcane juice were characterized as followed;

3.5.1.1 Determination of Total sugar using the phenol-sulfuric acid assay (Dubois et al, 1956) (See Appendix B)

3.5.1.2 Determination of Total soluble solid measured as ºBrix using Hand refractometer which measured gram sucrose per 100 g solution (Zoecklein et al., 1995)

3.5.1.3 Determination of pH using pH meter (Zoecklein et al., 1995)

3.5.1.4 Determination of type and sugar content using High Performance Liquid chromatography (HPLC)

3.5.2 Isolation, screening and identification of PHAs producing microorganisms

3.5.2.1 Collection of soil samples

Soil samples were collected from an agricultural area in Khon Kaen province. The serial dilution technique was used to isolate microorganisms from the soil samples. In brief, soil sample of 1 g was suspended in 9 mL sterile distilled water. Then, mixed solution was poured onto agar plate and immediately spread over the surface using a sterile glass spreader. It should be noted that the suitable dilution should not contain of microorganisms less than 300 colonies. If it is more, the higher dilution is required whist slighted colonies should be kept maintenance on nutrient agar. Then, the utilization of sucrose as a carbon source was tested. Sucrose yeast extract agar was used for selecting capable utilized sucrose isolates. All isolates were streaked on plate and incubation at 37ºC for 24-48 hr (Finkler et al., 2005).

3.5.2.2 Screening of PHAs producing bacteria

The isolated bacteria that utilized sucrose as a carbon source were tested in their capability for PHAs accumulation by Sudan black B dye. Lipid granule of PHAs produced in bacterial cell was observed under light microscopy after staining with black color.

3.5.2.3 Growth monitoring and cultivation condition

The isolated bacteria stained by Sudan black B was cultivated on sucrose yeast extract agar plate. After incubation at 37ºC for 24 hr and 48 hr, they again were stained by Sudan black B dye to detect lipid granule. The distinct stains with dye in the short time isolation were chosen to monitor growth phase in nutrient broth. The selected isolates were inoculated into 250 mL Erlenmeyer flask containing 100 mL of minimal medium (modified production medium) and incubated at 37ºC in rotary shaker with 200 rpm of agitation rate and for 48 hour. Samples were collected very 4 hr to estimate the production of PHAs biopolymer, total sugar consumption and biomass using several standard technique as growth profile isolates were monitored the optical density by measuring culture's absorbance at 600 nm, total sugar concentration and PHAs content were measured using phenol sulfuric, crotonic acid assay method (See Appendix C). Dry cell weight was determined by centrifugation of cell mass at 10000 rpm for 5 min, washing the cell with distilled water for twice and drying at 70°C until the cell reached constant weight. The isolates that gave the maximum of PHAs content was chosen to further study.

3.5.2.4 Production of PHAs by the isolated strain

Inoculums of 10% (v/v) were inoculated into 250 mL Erlenmeyer flask containing 100 mL of minimal medium (modified production medium) which was added by sterilized sugarcane juice with the initial total sugar approximately 20 gL^{-1}. Then, it was incubated at 37ºC in rotary shaker with 200 rpm of agitation rate for 48 hr. Samples were collected to estimate the production of PHAs biopolymer. Growth profile of the isolates was monitored in optical density by measuring culture medium absorbance at 600 nm, total sugar concentration was measured using phenol sulfuric method while PHAs content was considered by crotonic acid assay. Dry cell weight was determined by centrifugation of cell mass at 10000 rpm for 5 min. Then, it was washed twice with distilled water and dried at 70°C until constant weight.

3.5.2.5 Identification and characterization of the isolated strain

The basic morphological and physiological characterization of the efficient isolated strain was investigated follow the Bergey's manual of determinative bacteriology (Brenner et al., 2005) meanwhile for identification, molecular technique of 16S rDNA gene sequences was applied and certified by TISTR, Thailand.

3.5.3 Production of PHAs by *Alcaligenes latus* TISTR 1403

3.5.3.1 Growth condition and cultivation

A pure bacterial strain of *Alcaligenes latus* TISTR 1403 was inoculated into 250 mL Erlenmeyer flask containing 100 mL of nutrient broth and incubated at 35ºC in rotary shaker with 200 rpm of agitation rate for 40-48 hr to monitor the growth profile. Sample were collected every 4 hr to estimate the production of PHAs biopolymer. Growth profiles of *A. latus* TISTR 1403 was monitored as described in 3.5.2.3

3.5.3.2 Batch fermentation of sugarcane juice by *Alcaligenes latus* TISTR 1403

The inoculums 10% (v/v) was inoculated into 250 mL Erlenmeyer flask containing 100 mL of minimal medium (modified production medium) which added sterilized sugarcane juice with the initial total sugar approximately 10 gL^{-1}. The flasks were incubated at 35°C in rotary shaker with 200 rpm of agitation rate for 48 hr. Sample were collected to estimate the production of PHAs biopolymer. Growth profile of the isolates were monitored optical density by measuring culture medium absorbance at 600 nm, total sugar concentration by phenol sulfuric method, PHAs content by crotonic acid assay and dry cell weight was determined by centrifugation of cell mass at 10000 rpm for 5 min. Then, washed twice with distilled water and dried at 70°C until constant weight.

3.5.4 Statistical optimization of process parameters for the production of PHAs by the efficient strain

The statistical methodology was applied to screen factors affecting on the fermentation process by using a Plackett and Burman screening design and response surface methodology (RSM) with central composite design (CCD) for process optimization. In addition, only the most efficient strain between the pure strain and the isolated strain was chosen to carry out the production of PHAs under optimized condition by RSM.

3.5.4.1 Screening factors of fermentation process

Plackett and Burman design was used for screening main factors that effect for the production of PHAs. Five variable factors as initial total sugar, pH, agitation rate, inoculums size and nitrogen source were chosen to screen the effective factors. The range of variable parameters in term of coded and actual terms is shown in Table 3.1

Table 3.1 Plackett and Burman design of 5 variable factors

N=8	Factors							Response
	A	B	C	D	E	F	G	Y
Run	Initial Total sugar (gL^{-1})	Inoculums (% (v/v))	Agitation rate (rpm)	pH	Nitrogen (added) (gL^{-1})	Dummy	Dummy	PHAs ($\mu g\ mL^{-1}$)
1	+	+	+	-	+	-	-	
2	+	+	-	+	-	-	+	
3	+	-	+	-	-	+	+	
4	-	+	-	-	+	+	+	
5	+	-	-	+	+	+	-	
6	-	-	+	+	+	-	+	
7	-	+	+	+	-	+	-	
8	-	-	-	-	-	-	-	

Variable factors	Unit	Range of variables	
		Low level (-)	High level (+)
Initial total sugar	gL^{-1}	10.0	50.0
Inoculums	% (v/v)	5.0	20.0
Agitation rate	rpm	100	300
pH		6	9
Nitrogen	gL^{-1}	0.0	2.0

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

Response surface methodology (RSM) by using central composite design (CCD) was desired to optimization of PHAs production. The screened factors affecting on fermentation process were chosen to optimize. Design-Expert version 6.0.10® software for Design of Experiments (DOE) was used to design the experiment.

3.5.4.3 Validation of RSM optimization model

The multi linear regression model obtained from RSM should be tested validity of model. To validate the model, experiments were tested the production of PHAs using the optimized conditions. The adequate model could give the actual values were nearly predicted values which was indicated that the variability in the response could be explained by the model.

3.5.5 Production of PHAs in a fermentor

The optimal condition obtained in flask scale was extended in a fermentor. Large scale of 5 L fermentor for PHAs production was designed by the condition obtained from RSM with 3 L working volume. The cell was harvested by centrifugation and cell pallets were obtained. Then, the cell pallets were dried and broken with 6% sodium hypochlorite (NaClO). Finally, PHAs were extracted from their cells. Some parameters were calculated as following biomass yield ($Y_{X/S}$), PHAs yield ($Y_{P/S}$), Productivity and Specific productivity.

3.5.5.1 Recovery of PHAs biopolymer

The gravimetric method was chosen to extract PHAs biopolymer. The description of flow chart are described in Figure 3.4. First step, the biomass was dried and broken by 6% sodium hypochlorite (NaClO). Then, it was extracted with hot chloroform and precipitated with chilled ethanol. The powder of PHAs was obtained.

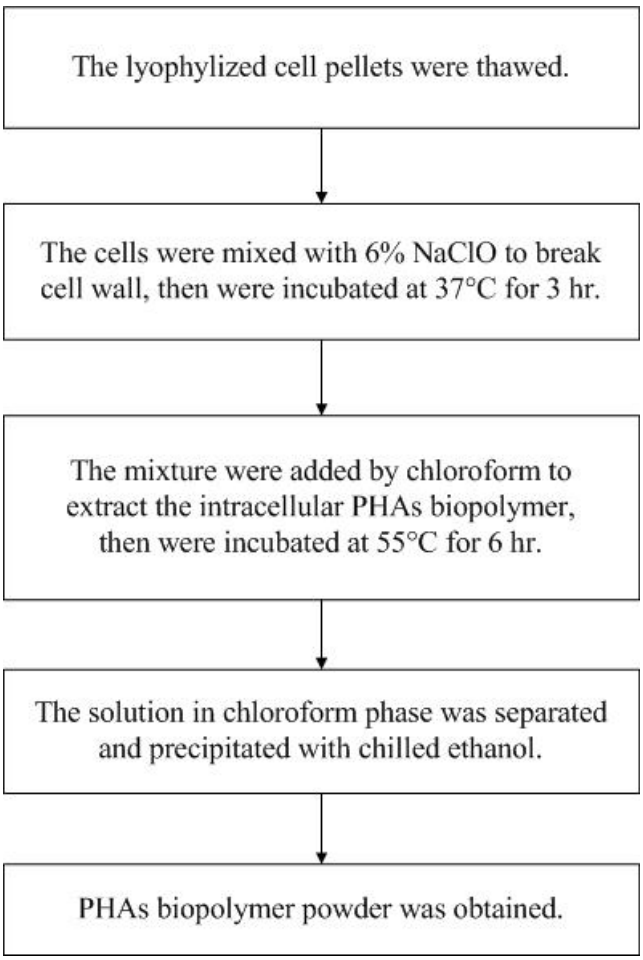

Figure 3.4 The extraction of PHAs biopolymer

3.5.5.2 Data analysis

1) Biomass yield ($Y_{X/S}$)

Amount of biomass yield was measured in term of dry biomass. Biomass yield was calculated by the ratio of dry biomass to the substrate consumed. (gram DCW produced per gram substrate consumed, g g^{-1})

2) PHAs yield ($Y_{P/S}$)

Amount of product classified in the term of product yield was calculated by the ratio of PHAs content to the substrate consumed. (gram PHAs produced per gram substrate consumed, g g^{-1})

3) Productivity

Productivity is defined as the ratio of output or product to the input or substrate required to produce the output or product during a given period of time. (gram PHAs produced per hour, g L^{-1} h^{-1})

4) Specific productivity

Specific productivity means the ratio of product and biomass yield per hour. It is not necessarily constant during batch culture. It depends on whether the product in linked to energy metabolism or not. (gram PHAs produced per gram DCW per hour, g g^{-1} h^{-1})