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Optimization of Polyhydroxyalkanoates (PHA) Production From Liquid Bean Curd Waste by Alcaligenes Latus Bacteria

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ABSTRACT

Study to obtain the optimum condition for the production of PHAs by *Alcaligenes* latus was carried using liquid bean curd waste as low cost carbon source. A Response surface method with two factors i.e. the initial sucrose concentration (15 g/L, 20 g/L, and 25 g/L) and time of incubation (48 hours, 60 hours, and 72 hours) was employed. The yield was taken after the inoculation A. *latus* on to the liquid bean curd waste. The results obtained from the study are computed with Design expert 7.1.3. The optimum condition found were the initial sucrose concentration of 25 gr/l and time of incubation of 60 hours 18 minute which producing 2.48 gr/l PHA and the dry cell concentration was 66.56%. The functional group of the PHA granule was identified as C=O by Fourier transforms infrared (FTIR).

Key words: Polyhydroxyalkanoates, liquid bean curd waste, *Alcaligenes latus*, carbon source, FTIR**.**

1. INTRODUCTION

Petroleum is considered as the principal source of most chemical industrial products such as polymers and plastics. Nearly $24 billion (US) worth of hydrocarbon feedstock are used annually in the chemical industry (1). Recently, due to the decreasing of fossil fuel resources, industrial sector and government agencies search for renewable feedstock's to replace petroleum as sources for production of Poly-ß-hydroxyalkanoates (PHA). Bean curd is one of the most promising feedstock's to replace petroleum to produce PHA. It is an important food which has gained consumer acceptance and used as vegetable protein source mainly for poor people.

However there is several problems face by most of SME's bean curd industry due to the liquid waste released during the course of processing. The high BOD 2,000 – 3,000 mg/L and COD 3,000 – 7,000 mg/L are blamed to cause environmental problem. The use of liquid bean curd waste as source of the structural polyester through microorganisms metabolisms is one of the solution overcome the extent of pollution problem. The conversion of liquid bean curd waste for the production of PHA is thought beneficial not only to reduce the extent of pollution problem but also to increase the economical value of the waste.

Poly-ß-hydroxyalkanoates (PHA) is linier polyester produced naturally by microorganism through sucrose and metabolism (2). Based on the unit length of the polymer component PHA could be classified into two groups i.e the short chain length PHA (poly HASCL) and PHA medium chain length (Poly HAMCL). The short chain length composed by three or five carbon atom namely 3HB (hydroxybutirat) unit and 3HV (hydroxyvalerat). The medium chain PHA consists of six or more carbon atom, consisting of 2 HV unit. The two PHA types is called Hybrid PHA short and medium chain PHA (3).

PHAs are produced and accumulated in most bacteria under unfavorable condition of limited essential nutrients in the presence of excess carbon sources. Several factors need to be considered in the selection of the bacteria for PHAs production at industrial scale. The ability of the bacteria to utilize cheap carbon sources is very important because the cost of substrates will significantly affect the overall production cost. The production of 3-hidroxybutirat/3 hidroxylpropionat is considered similar with the pure poly (3-hydroxybutirat/4-hydroxypropionat) in the medium containing disacharida as carbon source. Kim and Lenze (3) stated the limiting nutrient of *A. latus* is amonium salt.

The aim of this study is the find out the best combination treatment between sucrose concentration and time of incubation for the production of PHA by *Alcaligenes latus,* the yield of dry cell concentration.

2. MATERIALS AND METHODS

PHA production.

The experiment was carried out at Bioindustry and Waste Management Laboratory, Brawijaya University, Malang, Indonesia. Strain of *Alcaligenus latus* obtained from Biological Division, Airlangga University, Surabaya, Indonesia. This culture was maintained in slant Nutrient Agar and sub culturing every two weeks in Nutrient Broth and incubated at 35^0C prior to the experiment. The culture of *A. latus* was grown on sterile media Nutrient Broth, incubated at 35^0C for 24 hours. The media for propagation

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was the same with the fermentation media and volume of culture for propagation was 10 % of the volume for media fermentation.
The adapted culture was grown in the 100 ml medium for three days, and observed according to the experiment design. The initial sucrose concentration as well as the C/N ratio was 10:1, the pH is 6.0 and then agitated at 150 rpm.
Observations carried out are the dry cell concentration (Gravimetric method), the PHA concentration within the cell, the PHA yield which obtained after the division of PHA concentration and the dry cell concentration and spectral analysis by FTIR spectrophotometer.
As media for fermentation the sterilized liquid soybean curd waste was inoculated with cell of *A.latus*. The liquid bean curd waste properties are presented in Table 1.

Table 1. Composition of liquid soybean curd waste

SME's	BOD (mg/L)	COD (mg/L)	pH	SS (mg/L)	DO (mg/L)
A	4,266	5,9336	3.47	629.29	1.57
B	4,100	5,2680	3.56	642.68	1.93
C	2,833	3,0676	3.84	615.59	2.20
D	3,000	3,1573	3.77	619.94	2.10
E	5,800	7,6333	3.66	801.88	1.20
F	3,500	4,5663	3.93	716.22	1.30
G	4,566	6,6793	3.80	755.93	1.50
H	4.433	6,4266	3.83	752.29	1.50

The formulation of media for fermentation was carried out by mixing of sucrose, liquid bean curd waste and urea in such away to get the initial C/N ratio of 10:1. The assumption used in this formulation was that Carbon concentration (C) at sucrose is 42.05% from the sucrose total and the Nitrogen concentration (N) in the liquid bean curd waste is 0.229 g/L and the amount N in of urea is 46.67%. Prior to be used, the media was sterilized at 121^{0}C for 15 minutes. Observations carried out were BOD, COD, SS, pH and DD.

Laboratory analysis

Over the course of the culture period, samples with a range of PHB contents were collected aseptically and analyzed by IR spectroscopy and GC. For the IR spectroscopy analysis, 1 cm^3 of a culture was collected and centrifuged at 13,000 x g for 5 min. The growth medium was removed by washing, re-suspended in isotonic saline, and then was centrifuged. The final pellet was re-suspended in 80 ml of isotonic saline, and 20 ml of this solution was deposited onto a type KRS-5 (thallium bromide-iodide crystal) IR-transparent substrate. Additional serial dilutions of the remaining solution were prepared, and 20-ml portions were deposited. The deposits were dried in vacuum desiccators for 10 to 15 min before the spectra analyzing. For the GC analysis, 10-20-cm^3 portions of cultures were collected at the same time that samples were collected for the IR spectroscopy analysis. These samples were centrifuged at 13,000 X g for 5 min, the supernatants were removed, and the remaining pellets were freeze-dried and stored at -20°C until analysis.
Spectra were recorded with a Bruker model IFS-55 FTIR spectrometer coupled to a Bruker IR microscope fitted with a liquid N2-cooled mercury-cadmium-tellurium detector. The Bruker system was controlled with an IBM-compatible PC running OPUS, version 2.2, software. Absorbance spectra was collected at wave number values between 3,650 and 700 cm^{-1} with spectral resolution of 8 cm^{-1}, and 10 scans were co added and averaged. A Blackman-Harris four-term apodization function was used along with a zero-filling factor of 2.
To minimize differences between spectra due to baseline shifts, the spectra were corrected by using the Rubber Band algorithm of the OPUS, version 2.2, software and 200 baseline points and excluding the CO_2 bands. Spectra were normalized to the amide I band at 1,654 cm^{-1} to account for any differences in deposit thickness. Six to 12 spectra were recorded for each sample deposit to assess precision and to ensure that representative spectra of each sample deposit were collected.

Statistical analysis

This study was conducted using response Surface Method with two factors namely Sucrose Concentration (S) and Time of Incubation (T). The sucrose concentration (S) was used as the first factor with consentration of: (1) 15 g/l (X1 = -1); (2) 20 g/l (x2 = -1) and (3) 205 g/l (X1 = 0) and Incubation Time (T) was used as the second factor with level of: (1) 48 h (X2 = -1); (2) 70 h (X2 = -1); and (3) 72 h (X2 = 0). The statistical analysis was conducted with Design Expert 7.1.3.

3. RESULTS AND DISCUSSION

Growth and PHA Production

A. latus began to grow after an adaptation phase of about 24 hours. At this time the cell had started to multiply and after 60 hours of incubation the multiplication of the cell was stop and decreased (Figure 1).

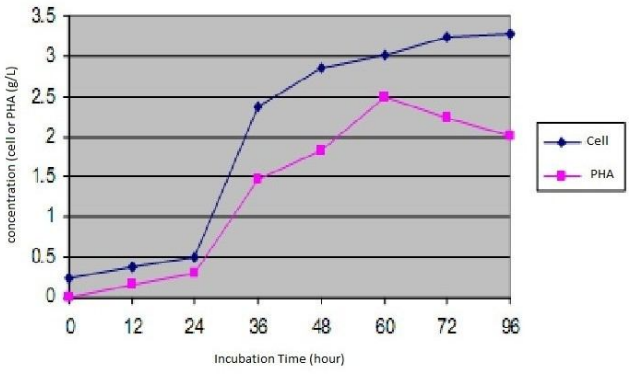

Figure 1 The growth and PHA production of *A. latus* on soybean curd waste.

The formation of PHA was presumably due to existence of carbon from sucrose as aan energy sources. The decreased of

PHA concentration after 60 hours of incubation may be due to the stationery phase of *A. latus*. In this condition PHA production was dissimilated by the cell as carbon source (3). The results of the functional circle analysis of the samples tested by FTIR spectrophotometer showed that 21 spectrum which was identified and 12 spectrum which could be detected its functional circle of PHA i.e. C-H, CH_2, C=O and C-O (Figure 2). The specific character of PHA molecule is the presence of C=O, -C-O-, and polymeric of –C-O-C- as well as C-H and C-C was presented in Table 2.

Table 2. The identified molecule based on wavelength by FTIR spectrophotometer.

No	wavelength (□□□cm^{-1})	Identified
1	3497.67	-OH
2	2925.81	-CH
3	1735.81	C=O ester
4	1642.24	C=O amida protein
5	1560.30	N-H amida protein
6	1419.51	-CH_3
7	1375.15	
8	1238.21	-C-O-

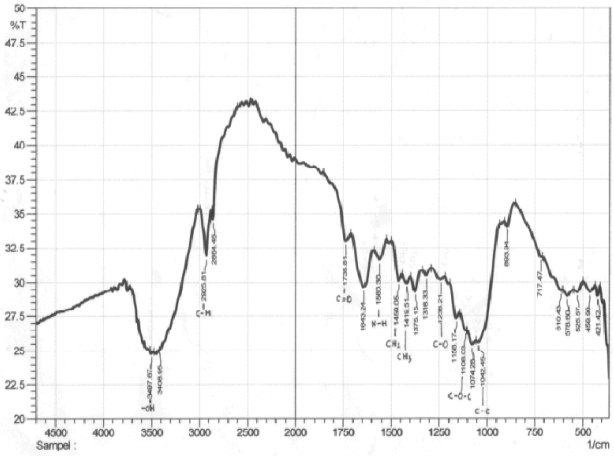

Figure 2. PHA sample analysis by FTIR spectrophotometer

Apparently the types of PHA obtained were different according to the carbon source utilized. In this study the carbon source was sucrose so the PHA produced was PHB. It is due to the result of spectra identified having functional circle of CH_3 at 1419 cm^{-1} wavelength. The prediction that the PHA obtained was PHB type consistent with results reported by Kanzis et al. (4) and Santhaman and Sasidharan(5)

Dry Cell Concentration

Figure 3 shows the dry cell production based on the change in sucrose concentration and time of incubation. The highest dry cell concentration (4.2 g/L) was obtained at an initial sucrose concentration of 27.07 g/L whereas the smallest was 1.60 g/L at an initial sucrose concentration of 12.93 and time of incubation of 60 hours. The increase in dry cell concentration is more affected by the ammount of sucrose added, whereas time of incubation has no significant effect (Figure 3). The addition of sucrose up to 25 g/l wss considered could be used to promote the microbial growth and had not yet become an inhibition factor. The incubation time of 48 hours was considered as the optimum time. The result was shorter than that obtained by Grothe and Christi (6) but consistent with that of obtained El-Sayed et al. (7)

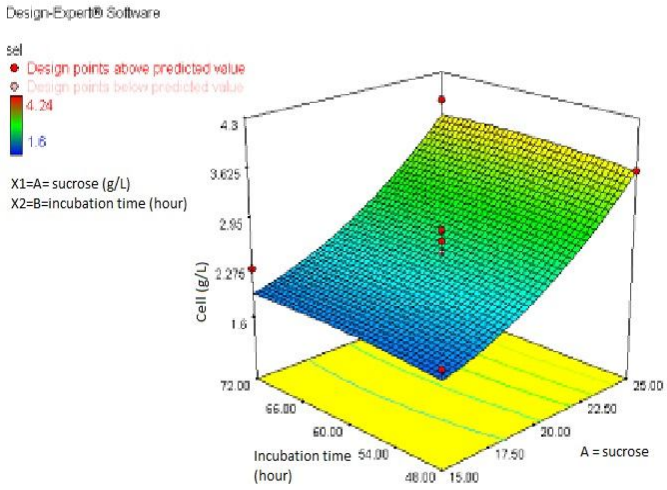

Figure 3. Dry cell production based on sucrose concentration and incubation time.

The yield of dry cell obtained could be detected through the following relationship:

$Y1 = 1{,}8247 – 0{,}2608X1 + 0{,}0500X2 + 0{,}0108X1^2$

Where Y1 is the dry cell concentration (g/L). X1 represent the initial sucrose concentration (g/L) abd X2 is the incubation time (hours). Based on the above equation it it found that the maximum dry cell concentration is depend to some extend on the initial sucrose concentration (X1) and the time of incubation (X2) is not significantly affected the yield.

Polyhydroxyalkanoates (PHA) Production

PHA production was influenced by sucrose addition and incubation time (Figure 4). The increase of PHA yield was liniery with the increase of sucrose addition. The time of incubation indicated a significant quadratic model. This is consistent with the finding proposed by Grothe and Chiste (6). *Alcaligenes* sp was capable to grow up to optical densities ranging from 8 to 20 and simultaneously accumulated the polyhydroxyalkanoate-(PHA-).). Generally, it could be noticed that the PHB content (%) in *A. latus* ATCC 29712 cells increased by about 48.13 % as compared with that accumulated in *R. eutropha* ATCC 17697 cells after 80 h of incubation time.

The highest yield of PHA was obtained at an initial sucrose concentration of 20 g/L and time of incubation of 60 hours and thelowest PHA yield (54.38%) was obtained at an

initial sucrose concentration of 20 g/L but the incubation time was 76 hours and 59 minutes (Table 3)

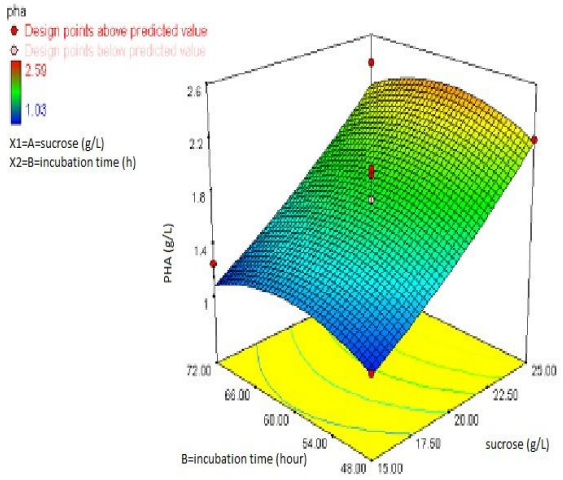

Figure 4. PHA production based on sucrose addition and incubation time.

Table 3 Yield of PHA based on the response of dry cells and PHA concentration.

Dry cell (g/L)	PHA (g/L)	Yield (%)
1.6	1.03	64.37
1.99	1.09	54.77
2.17	1.18	54.37
2.27	1.25	55.06
2.36	1.38	58.47
2.39	1.53	64.01
2.49	1.67	67.06
2.43	1.74	71.60
2.8	1.93	68.92
2.65	1.97	74.33
3.6	2.19	60.83
3.89	2.37	60.92
4.24	2.59	61.08

The increase in dry cell also increased the PHA yield (Table 3). However the yield decreased when the dry cells increased and followed with the increase of yield if the dry cell reached 2.65 g/L and then decreased again. This is a clear indication that the increase of dry cell was not always followed by the increase of PHA yield. Apparently the PHA yield is not only affected by the dry cell concentration but also the alvailable nutrient and the envirmental factor. Yamane *et al* (8) obtained tha the presentage of PHA (w/w) produced by *A. latus* was 50 % and Wang and Lee (9) reported that the production of PHB by *A. latus* at sucrose concentration of 30 g/L could reach by 80%. The PHB content also increased more rapidly under nitrogen-limited conditions. These results suggest that in A. latus the PHB synthesis rate and PHB content can be increased by applying nitrogen.

The result of optimization of dry cell and PHA concentration using Design Expert 4.1.3 presented in Table 4 shows that there are six optimally solution having similarly desirability score. Myer and Montgomery (10) stated that desirability function is used to determine the function of the optimum solution. The more close to one the more high the optimum precision is which has the similarly desirability score of 0.831 so that it could be stated that the degree of the precision as 83 %.

Table 4 Optimally solution results obtained by Design Expert 7.1.3

Sucrose concentration (g/l)	Incubation time (h)	Dry cell (g/)	PHA (g/l)	desirability	States
25	60 h, 59 m	3.69	2.38	0.83	Selected
25	61 h, 09 m	3.69	2.38	0.83	
25	60 h, 18 m	3.69	2.38	0.83	
25	60 h, 02 m	3.69	2.38	0.83	
25	58 h, 17 m	3.68	2.38	0.82	
25	69 h, 29 m	3.68	2.27	0.79	

The optimum node chosen for verification of the result as solution1, 2 and 3. Verification was done to prove whether verification result was the same with the optimum solution obtained from the computation. Based on the verification the optimum is obtained that the highest dry cell is the incubation time of 60 hours, 9 minutes. However, the largest PHA concentration was obtained at incubation time of 60 hours and 18 minutes. The results of optimum solution verification are given in Table 5.

Table 5. The verification results of optimum solution.

Sucrose concentration (g/L)	Incubation Time (h)	Dry cell (g/L)	PHA (g/L)	Yield (%)
25	60 h, 59 m	3.79	2.37	62.64
25	61 h, 09m	3.68	2.37	64.57
25	60 h, 18 m	3.73	2.48	66.56

The result presented in Table 3 show that solution 3 (initial sucrose concentration of 25 g/L and time of incubation of 60 hours 8 minutes) having the biggest PHA concentration (2.48 g/L) and the yield as was 66.56 %. The verification of the result of the yield is smaller in compared with the result of studied (74.34 %). This is due to *A. latus* culture used had already weak because being sub culturing for several times. This condition reduced the ability of *A. latus* to produce of PHA. Results obtained from this study or from verification were lower than that found by other worker (6), so that the yield of PHA obtained from this study has not yet being optimum. This might be due to the media for making PHA was whey or liquid bean curd waste which until now has never being used as media fermentation for PHA production. The cultivating conditions were agitated at a speed of 200 rpm, incubation temperatures of 30^{o}C for 48 h and 33^{o}C for 54 h when using *A. eutrophus and A. latus*, respectively. In the case of *A. eutrophus* the results revealed that the maximum concentrations of dry cell mass and PHB obtained were 0.920 g/L and 0.034 g/L, respectively. The yield and the productivity obtained were 0.037 g PHB/g dry cell and 0.0019/L/h, respectively. In comparison, *A. latus* showed the

maximum biomass concentration of 1.73 g/L and the PHB content of 0.68 g/L, which corresponded to the yield of 0.393 g PHB / g dry cell and the productivity of 0.0125 g/L/h (10)

4. CONCLUSION

The initial sucrose concentration affected not only the dry cell concentration but also the PHA yield produced by *A. latus*. The PHA (yield) obtained from the computation of optimization of two response is 74.34% and yield found based on the verification of optimally solution is 66.56%.
The highest PHA production was 2.48 g/L after 60 hours of incubation. The time of 60 hours was then considered as the central node for further study.

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