

Optimum Growth Conditions and pH Control Solution for PHB Biosynthesis in *A. eutrophus*

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Abstract: Optimum growth conditions and pH control solution were obtained in the biosynthesis of PHB (poly- β -hydroxybutyrate) with *Alcaligenes eutrophus*. Optimum carbon and nitrogen sources were fructose and $(\text{NH}_4)_2\text{SO}_4$, respectively, and optimum additive was 1:1 mixture of yeast extract and polypeptone. To select the optimum pH control solution, various pH control solutions (NaOH, KOH, Na_2CO_3 , and NaOH + KOH) were tested. Na_2CO_3 solution was found to be the best out of the four solutions tested, because CO_2 generated from Na_2CO_3 could be used as a carbon source. Mixture of NaOH and KOH showed better results than the solution of NaOH or KOH alone. This could be due to the balanced amount of cations (Na^+ and K^+), which might promote not only the permeation of substrates, but also pumping out Na^+ ions.

Keywords: PHB, *A. eutrophus*, pH control solution.

1. Introduction

Poly- β -hydroxybutyrate (PHB), a biodegradable plastic made by microorganisms, is being spotlighted in the various applications for its advantages and versatilities. Although the greater part of PHB's are made by *Alcaligenes eutrophus*, *Alcaligenes latus* or recombinant *Escherichia coli* is sometimes used.

The pH control is one of the important factor in the biosynthesis of PHB. The pH in the fermentation of *Alcaligenes* generally decreases due to the byproducts produced in TCA cycle in the metabolism. Methods for maintaining constant pH are (1) using buffer solution (phosphate buffer is preferred for *Alcaligenes*, because the optimum pH is about 7 for this case) or (2) using a pH controller with acid and base such as HCl and NaOH.

A high concentration of phosphate buffer impedes the cell growth, hence the concentration of potassium

phosphate must be lower than 5 g/L [1]. This low concentration reduces the capacity of buffer, and this is the reason that phosphate buffer is not acceptable for the pH control in the PHB synthesis. Generally, the concentration of byproducts produced by TCA cycle exceeds the buffering capacity of 5 g/L potassium phosphate solution.

The pH control by acid and base can fix up the above problems, but other problem arises that the activities of enzymes are reduced by adding acid or base, because HCl and NaOH are known as a denaturant of proteins. Therefore, many researchers have concentrated their work on the selection of pH control solution. In *Alcaligenes*, only alkali solution is required because byproducts decrease pH.

Following pH control solutions can be used for the synthesis of PHB with *A. eutrophus*:

(1) NaOH: A simple and cheap pH control medium, basis for the other control solutions.

(2) KOH: Addition of NaOH results in the increase of Na^+ ions, and these ions enter the cell together with substrates (glucose, fructose, and sucrose, etc.). These permeated Na^+ ions are pumped out again by

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ethylster was employed as an internal standard. Shimadzu GC-7A with FID detector was used for analysis, and the cells were pre-treated with 1,2-dichloroethane, HCl, and n-propanol prior to injection.

3. Results and Discussion

3.1. The Optimum Growth Conditions

The optimum conditions were evaluated from the one-stage batch fermentations, and the results are shown in the followings.

(1) Optimum carbon source: The known carbon sources for *Alcaligenes eutrophus* are fructose, glucose, and lactic acid. Each concentration of carbon sources was fixed at 20 g/L and 1 g/L of $(\text{NH}_4)_2\text{SO}_4$ was used as nitrogen source. No additive was added. The resulting dry cell weight and $Y_{P/X}$ were summarized in Table 3, and fructose was revealed as an optimum carbon source.

Table 3. Effects of Carbon and Nitrogen Sources

		Dry cell weight (g/L)	$Y_{P/X}$ (%)
Carbon sources	Fructose	11.9	21.6
	Glucose	11.9	19.4
	Lactic acid	8.5	21.7
Nitrogen sources	$(\text{NH}_4)_2\text{SO}_4$	21.5	24.1
	$(\text{NH}_4)_2\text{HPO}_4$	20.3	19.5
	NH_4Cl	20.5	20.3

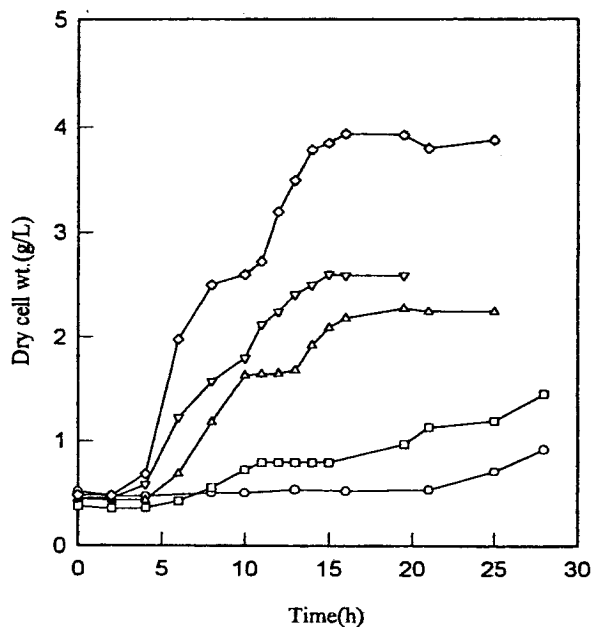


Figure 1. Time courses of dry cell weight on various additives. Fructose 20 g/L, $(\text{NH}_4)_2\text{SO}_4$ 1.0 g/L, additives 1.0 g/L each. ○ no additive, □ beef extract, △ polypeptone, ▽ yeast extract, ◇ yeast extract + polypeptone.

(2) Effect of additives (organic nitrogen sources): Yeast extract, polypeptone, and beef extract were tested as additives. Each concentration was 1 g/L, and the results were shown in Figure 1. Lag time was reduced from 21 h (no additive) to 4 h (beef extract, polypeptone) or 2 h (yeast extract). Secondary lag phase was observed for all additives, and this phase was the point where the additives were all consumed and started to adapt to nitrogen source. Same trends were observed with *A. latus* (in ref. [4]). The specific growth rates were in the following order; yeast extract (0.25 h^{-1}) > polypeptone (0.21 h^{-1}) > beef extract (0.07 h^{-1}), and the specific growth rate with beef extract was much lower than yeast extract or polypeptone. Therefore we used the 1:1 mixture of yeast extract and polypeptone and we could get the highest growth rate of 0.41 h^{-1} , so this mixture was selected as an optimum additive. To evaluate the optimum concentration, 2, 5, 8 and 10 g/L (each) of mixed additives were used, and 8 g/L was revealed as an optimum, as shown in Table 4. Secondary lag phase was not observed in this case, probably because sufficient amount of additives.

(3) Optimum inorganic nitrogen source: $(\text{NH}_4)_2\text{SO}_4$, $(\text{NH}_4)_2\text{HPO}_4$, and NH_4Cl were tested for the nitrogen source. Each concentration of nitrogen sources was fixed at 1 g/L and 20 g/L of fructose was used as carbon source. Mixed additives of yeast extract and polypeptone was added with the concentration of 8 g/L each. The results are also shown in Table 3, and $(\text{NH}_4)_2\text{SO}_4$ was revealed as an optimum nitrogen source. The effect of the variation of $(\text{NH}_4)_2\text{SO}_4$ concentration was also investigated, but the results were almost the same as shown in Table 5. This

Table 4. Effect of Concentration of Mixed Additives

Yeast extract + polypeptone	Dry (g/L) cell weight (g/L)	$Y_{P/X}$ (%)	Specific growth rate (h^{-1})
2+ 2 g/L	9.0	8.9	0.23
5+ 5 g/L	10.6	23.6	0.35
8+ 8 g/L	20.5	19.5	0.56
10+10 g/L	12.7	21.3	0.23

Note. Fructose 20 g/L, $(\text{NH}_4)_2\text{SO}_4$ 1.0 g/L.

Table 5. Effect of Nitrogen Concentration

$(\text{NH}_4)_2\text{SO}_4$ (g/L)	Dry cell weight (g/L)	$Y_{P/X}$ (%)
0	23.9	29.7
0.1	23.9	23.0
1	23.7	21.1
2	23.7	19.4
5	23.1	19.9

Note. Fructose 20 g/L, yeast extract 8 g/L, polypeptone 8 g/L.

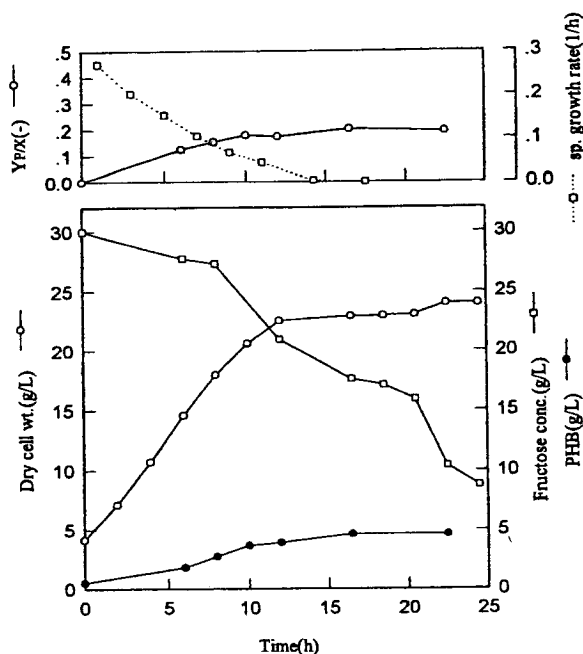


Figure 2. Time courses of dry cell weight, PHB, $Y_{P/X}$ and substrates of batch fermentation of *A. eutrophus* with 1 N NaOH control solution with additives of yeast extract and polypeptone, 8 g/L each.

could be mainly due to the fact that high concentration of additives (8 g/L each) took a role of nitrogen sources, and secondary lag phase was not observed.

(4) Comparison with the other results: Our $Y_{P/X}$ values did not exceed 0.3, but it is known that the maximum $Y_{P/X}$ can reach 0.8 (in ICI process) [15,16]. For the values of dry cell weight, our data were low as 30 g/L, comparing with the results of Kim and coworkers [17,18], 164 g/L. These low values were due to the facts that fructose was used instead of glucose, and that one-stage batch fermentation method was used instead of two-stage fed-batch fermentation.

3.2. The Effect of Various pH Control Solutions

In *A. eutrophus*, PHB synthesis is maximum in restricted nitrogen condition, so NH_4OH , which can be utilized as nitrogen source for *A. latus*, cannot be used as pH control solution. Thus NaOH, KOH, and Na_2CO_3 should be used in *A. eutrophus*. The pH control experiments in *A. eutrophus* were performed only in one-stage batch way. The results are as follows:

(1) NaOH: To maintain the constant pH of 7.0, a large amount of NaOH was required (55 mL of 1 N NaOH solution was consumed for 23 h). Figure 2 shows the time courses of dry cell weight, PHB, $Y_{P/X}$ and substrate concentrations with NaOH control. The final dry cell weight was 24 g/L after 23 h, and PHB concentration was 4.64 g/L ($Y_{P/X} = 0.193$). Initial

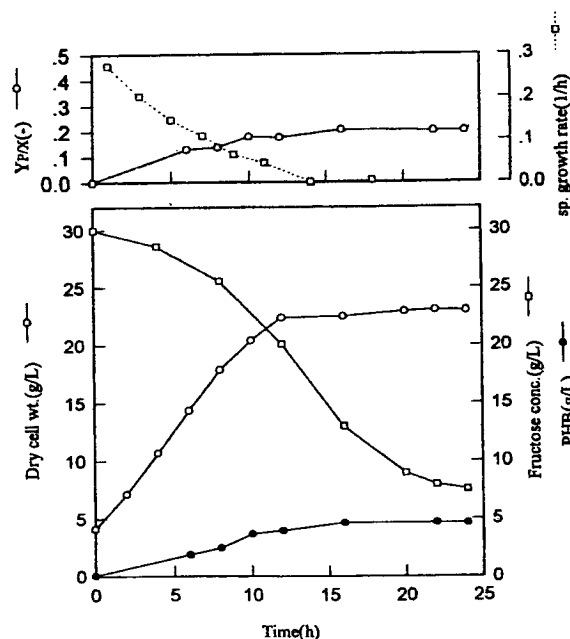


Figure 3. Time courses of dry cell weight, PHB, $Y_{P/X}$ and substrates of batch fermentation of *A. eutrophus* with 1 N KOH control solution with additives of yeast extract and polypeptone, 8 g/L each.

specific growth rate was lower than the other results. Linko and co-workers reported similar results for the same case; dry cell weight of 10 g/L, PHB of 6.9 g/L, and $Y_{P/X}$ of 0.69 after 48 h [6]. Lower $Y_{P/X}$ value is due to the disadvantages of batch fermentation, and lower initial specific growth rate could be explained by the inhibition of alkali solution.

(2) KOH: The amount of supplied 1 N KOH solution was 55 mL for 24 h, and this was the same as NaOH. The time courses were plotted in Figure 3, and the final dry cell weight was 24 g/L after 22 h, and PHB concentration was 4.66 g/L ($Y_{P/X} = 0.194$). No significant differences were found between NaOH and KOH, so it is concluded that osmosis by Na^+ ions does not affect the cell growth and the synthesis of PHB.

(3) Na_2CO_3 : The concentration of supplied Na_2CO_3 was 1 M, and the final dry cell weight was 30 g/L after 23 h, and PHB concentration was 8 g/L ($Y_{P/X} = 0.267$), as shown in Figure 4. These high values might result from the fact that CO_2 from Na_2CO_3 can be used as a carbon source. Increase of dry cell weight and PHB concentration implies that the effect of substrate inhibition was not significant.

(4) NaOH + KOH: To improve the accumulation of PHB, 1:1 mixed pH control solution (0.5 N NaOH + 0.5 N KOH) was used. The results are shown in Fig. 5, and the final dry cell weight was 26.6 g/L after 24 h, and PHB concentration was 6.64 g/L ($Y_{P/X} =$

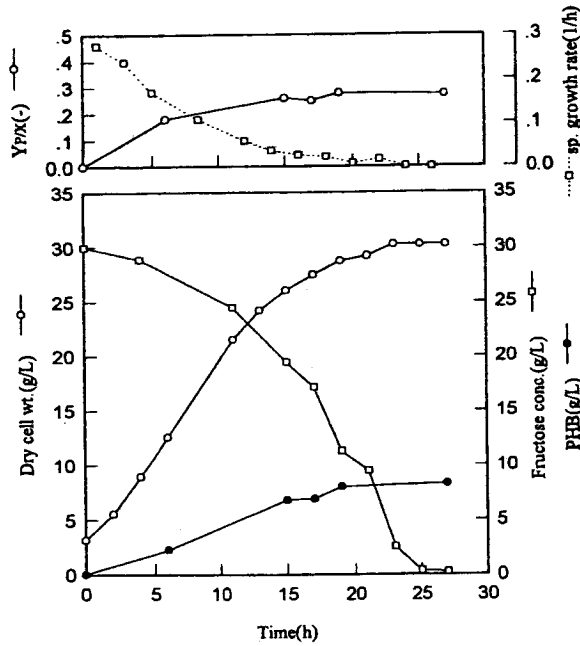


Figure 4. Time courses of dry cell weight, PHB, $Y_{P/X}$ and substrates of batch fermentation of *A. eutrophus* with 1 M Na_2CO_3 control solution. with additives of yeast extract and polypeptone, 8 g/L each.

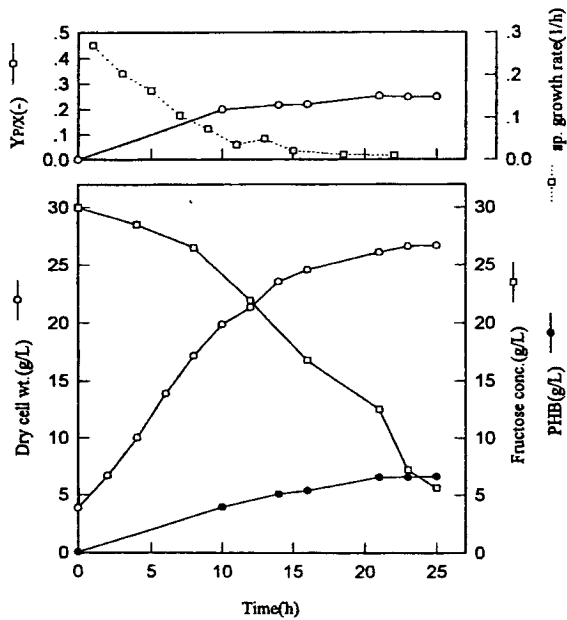


Figure 5. Time courses of dry cell weight, PHB, $Y_{P/X}$ and substrates of batch fermentation of *A. eutrophus* with 0.5 N NaOH + 0.5 N KOH control solution. with additives of yeast extract and polypeptone, 8 g/L each.

0.250). Although these values were smaller than Na_2CO_3 , they were improved over NaOH or KOH alone. These improvements could be explained by the scheme illustrated in Figure 6. In NaOH control,

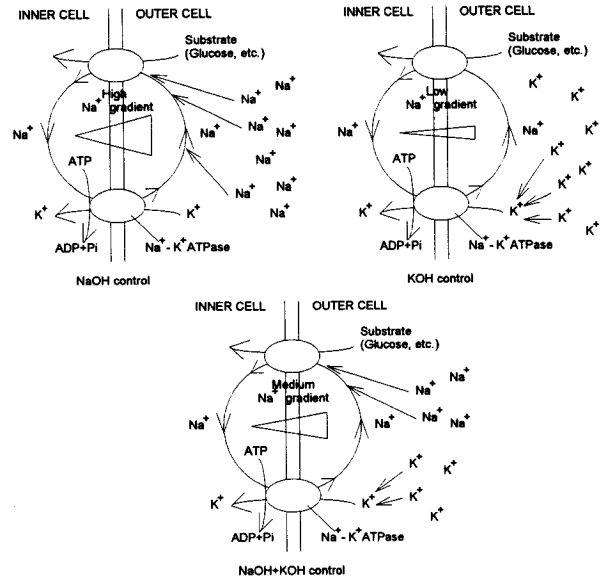


Figure 6. The effects of cations in *A. eutrophus* by various pH control solutions.

Table 6. Summary of Experimental Results on pH Control Solutions

	Dry cell weight	PHB conc.	$Y_{P/X}$ (%)
NaOH	24.0	4.64	19.3
KOH	24.0	4.66	19.4
Na_2CO_3	30.0	8.00	26.7
NaOH+KOH	26.6	6.64	25.0

excessive Na^+ ions result in high Na^+ gradient, so permeation of substrates by Na^+ ions is promoted. However, high energy is required in pumping out these excessive Na^+ ions, and the amount of K^+ ions is too low to exchange with Na^+ ions [1,19]. In KOH control, the condition is exactly reversal to NaOH control. Excessive K^+ ions promote the pumping of Na^+ ions, but the permeation of substrates is poor due to the low Na^+ gradient. 1:1 mixed solution of NaOH and KOH can fix up the above problems. Balanced amounts of Na^+ and K^+ ions promote not only the permeation of substrates, but also pumping out Na^+ ions. Mixed pH control solution may be effective not only in one stage batch, but also two stage fed-batch fermentation. The overall results are summarized in Table 6.

4. Conclusions

From the experimental results with varying substrates and pH control solutions, following results were obtained.

- (1) Optimum carbon and nitrogen sources were

fructose and $(\text{NH}_4)_2\text{SO}_4$, respectively. But the variation over nitrogen sources was insignificant, because high concentration of additives (8 g/L each) took a role of nitrogen sources. Optimum additive was 1:1 mixture of yeast extract and polypeptone.

(2) Optimum pH control solution was Na_2CO_3 , because CO_2 generated from Na_2CO_3 could be used also as a carbon source. This implies that the effect of substrate inhibition was insignificant.

(3) Mixture of NaOH and KOH showed higher dry cell weight and PHB concentration than sole NaOH or KOH. This could be mainly due to the balanced amount of cations (Na^+ and K^+), which promote not only the permeation of substrates, but also pumping out Na^+ ions.

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