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Production of Pha (Poly Hydroxy Alkanoates) by *Ralstonia Eutropha* on Hydrolysed Sago Starch as Main Substrate using Fed-Batch Cultivation Method

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Abstracts

The objective of this research was to examine the aplication of a fed batch cultivation method to produce PHA by *Ralstonia eutropha* using hydrolysed sago starch as the main substrate. The results showed that a higher formation and accumulation of PHA could be achieved through fed batch cultivation at the dilution rate of 0.1./h. The fed batch cultivation with feeding of substrate containing only hydrolysed sago starch as carbon source resulted the highest specific product yield of 76.54 % and product (PHA) concentration of 3.72 g/L.

Keywords: Fed batch cultivation method - Ralstonia eutropha - hydrolysed sago starch - PHA (Poly Hydroxy Alkanoates) - bioplastic

1.0 Introduction

Discharging of plastics and other petrochemical based polymers at their end use has caused serious environmental problems since they cannot be easily degraded by natural microorganims existing in the environment. In addition, petrochemical as its raw material is unrenewable natural resources whose reserves are diminishing rapidly. The increasing concern on quality and sustainability of the environment has led to the investigation on alternative sources of plastic from renewable resources which is biologically degradable by existing natural microorganisms.

Poly-β-Hydroxyalkanoates (PHA) is one of the promising alternatives. Compared to other biopolymers such as starch and protein, PHA has many advantages. This polymer is hydrophobic, resistat to humidity and has low permeability against oxygen [1]. This polymer has obtained an increasing attention to be used as material for coating, carrier, diposable products, surgical strings, and packaging materials[2, 3].

Eventhough PHA has many advantages, however, its commercialisation is hindered by economic problems. Its production cost is relatively higher than that of petrochemical based plastics, leading to a higher market price. Reduction of production cost is possible through the use of cheaper cultivation substrates, improvement of the bacterial strain, development of a more productive and more effisien cultivation process, and development of a more economic down stream processing.

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One of cheaper candidates for cultivation substrate is hydrolysed sago starch. Sago starch is a cheap and abundantly available source of hydrolysed starch, especially in the tropical regions. The use of hydrolysed sago starch as cultivation substrate for PHA production hopefully can reduce the production cost while giving an added value to sago starch.

PHA is produced by various bacteria as carbon and energy stock in cytoplasm at unbalanced conditions, such as limitation of essensial nutritions such as nitrogen, phosphor, and oxygen, and excess of carbon [2, 4]. Application of fedbatch strategy in which excess carbon is fed while limiting other essential nutrients may, therefore, induce the formation and accumulation of PHA.

The purpose of this research has been to investigate the possibility of using hydrolysed sago starch as main substrate for *Ralstonia eutropha* to produce PHA. The specific objective is to investigate the application of fedbatch strategy to induce the formation and accumulation of PHA.

2.0 Materials and Methods

2.1. Batch Cultivation

The *Ralstonia eutropha* IAM 12368 was obtained from IAM Culture Collection, Institute of Molecular and Cellular Biosciences, the University of Tokyo, Japan. Batch cultivations were carried out in 2L bioreactor (working volume of 1 L) for 96 hours, at pH 6.9, temperature 34°C, aeration rate of 0.2 vvm. Hydrolysed sago starch with the initial total sugar of 25 g/L is used as carbon source. $(NH_4)_2HPO_4$ is used as nitrogen source at the C/N ratio of 10:1. Other media components in 1 L are 5.8 g K_2HPO_4, 3.7 g KH_2PO_4, 10 ml $MgSO_4$ solution 0.1 M and 1 ml microelement solution. Biomass, product (PHA) and residual sugar concentration were measured in every 12 hours. Growth kinetics parameters were calculated.

2.2 Fed batch cultivation

Fed batch cultivations were carried out in a 2 L bioreactor with the initial volume of 1 L. Feeding of substrates at certain dilution rate were conducted at the beginning of stationary phase [5]. The treatments carried out were as follows:

(Ao) Limitation of oxygen supply at stationary phase, without substrate feeding;
(F1) Feeding of complete media with the same composition as batch media,with aeration;
(F2) Feeding of hydrolsed sago starch, with aeration;
(F3) Feeding of hydrolysed sago starch without aeration;
(F4) Feeding of hydrolysed sago starch +$MgSO_4.7H_2O$, with aeration;
(F5) Feeding of hydrolysed sago starch+$MgSO_4.7H_2O$ without aeration;
(F6) Feeding of hydrolysed sago starch+ $MgSO_4.7H_2O$+$(NH_4)_2HPO_4$ with aeration;
(F7)Feeding of hydrolysed sago starch+ $MgSO_4.7H_2O$+$(NH_4)_2HPO_4$ without aeration.

The effects of different substrate feedings and/or oxygen limitation were investigated by measuring biomass concentration (g dry cell/L) and product concentration (g dry PHA/L). Specific yields of PHA (percent of g dry PHA/g dry cell) were then calculated. PHA was obtained by the NaOH digestion method. (Lee *et al.*, 1999)[4]. Experiments were repeated at least two times and duplicated samples were taken in every sampling.

3.0 Results and Discussion

3.1. Batch Cultivation

The growth and substrate consumption in batch cultivation are plotted in Fig 1. The results show that the the growth of *R. eutropha* on hydrolysed sago starch at the initial total sugar of 25 g/L and C/N ration of 10:1, enter a stationary phase after 48 hours of cultivation (Fig 1) with the μ of 0.05/h. This information was used to determine the time to start feeding and the dilution rate of the feeding at the fed batch cultivation. At the end of batch cultivation, it yielded 4.41 g dry cell/L and 1.44 g dry PHA/L (32.65% of biomass). Other parameters obtained were yield biomass per substrate (Y x/s) = 0.15 g dry cell/g sugar, and substrate efficiency ($\Delta S/So$) reached 99%.

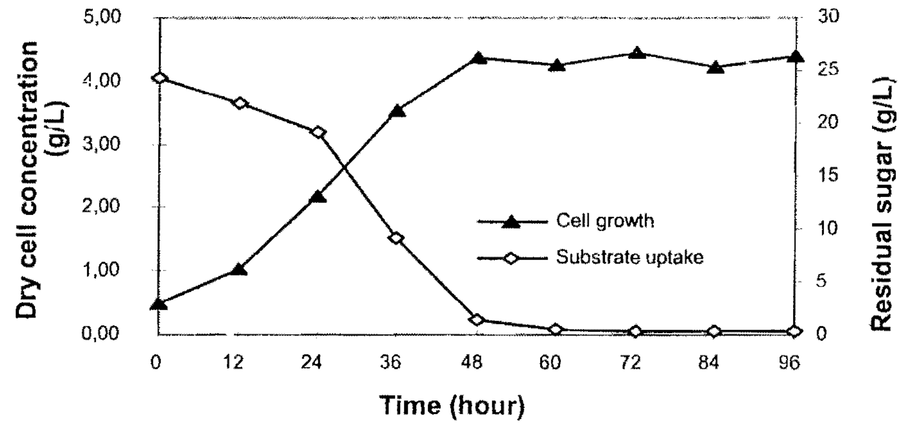

Fig 1. Growth and substrate consumption of *R. eutropha* in batch cultivation with the initial hydrolysed sago starch concentration of 25 g/L, and C/N ratio of 10:1.

3.2. Fed-Batch Cultivation

Based on the previous research results in batch cultivation, feedings of substrates and/or limiting the aeration were started from 48th hour. The hypotheses to be tested whether feeding of carbon source and/or limiting other essential nutrients (nitrogen, oxygen, phosphate) at stationary phase can induce formation and accumulation of PHA.

The results (Table 1.) show that, in all treatments, feeding of substrates and/or limiting the aeration can induce the formation and accumulation of PHA as indicated by the increasing specific yield of PHA and PHA concentration. These treatments may have caused unbalanced growth in which the carbon source was in excess while other essential nutrients (nitrogen, phosphat, oxygen) were limited. Compared to control (batch cultivation), feeding of hydrolysed sago starch only (F2) gave the best results, namely increasing the PHA formation as indicated by the increasing of specific product yield from 32.65% to 76.54% (increasing of more than two folds), and increasing the product accumulation as indicated by the increasing of product concentration at the end of fed batch from 1.44 g/L to 3.72 g/L (increasing of more than two folds). In addition, biomass was only slightly increased from 4.41 g/L to 4.86 g/L (increasing about 10%). Comparison between all treatments for biomass, product and specific product yield is summarized in Table 1.

Table 1 Biomass, Product (PHA) and Specific Product Yield of PHA produced by R. *eutropha* in batch and fed batch cultivation with and without limiting essenstial nutrients.

Treatments	**Biomass (g dry cell/L)**	**Product (g dry PHA/L)**	**Specific Product Yield (% w/w)**
***Batch* (control)**	4,41 ± 0,83	1,44 ± 0,27	32.65 ± 6.56
Batch *Ao*	3,40 ± 0,39	1,81 ± 0,57	51,15 ± 12.32
Fed batch			
F1	3,34 ± 0,11	2,15 ± 0,03	64.37 ± 2.30
F2	**4.86** ± 0,14	**3,72** ± 0,24	**76,54** ± 5.41
F3	4,26 ± 1,45	2,68 ± 0,67	63.36 ± 2.29
F4	3,67 ± 0,28	2,12 ± 0,05	57.77 ± 4.61
F5	3,65 ± 0,10	2,18 ± 0,01	59,73 ± 1.66
F6	4,58 ± 0,24	1.85 ± 0.06	40.39 ± 2.49
F7	3,40 ± 0,01	1,62 ± 0,09	47,65 ± 2.65

Notes:
(Ao) Limitation of oxygen supply at stationary phase, without substrate feeding;
(F1) Feeding of complete media with the same composition as batch media, with aeration;
(F2) Feeding of hydrolsed sago starch , with aeration;
(F3) Feeding of hydrolysed sago starch without aeration;
(F4) Feeding of hydrolysed sago starch +$MgSO_4.7H_2O$, with aeration;
(F5) Feeding of hydrolysed sago starch+$MgSO_4.7H_2O$ without aeration;
(F6) Feeding of hydrolysed sago starch+ $MgSO_4.7H_2O$+$(NH_4)_2HPO_4$ with aeration;
(F7)Feeding of hydrolysed sago starch+ $MgSO_4.7H_2O$+$(NH_4)_2HPO_4$ without aeration.

The results show that the more complete the media is fed (F6 and F7), the lower the PHA is produced. On the contrary, the more limited the media is fed (F2 and F3) which was basically only hydrolysed sago starch as carbon source, the more the PHA is produced. Different from the limitation of nitrogen, phosphate, and sulphur, however, limitation of oxygen (Ao, F3, F5, F7) may have a negative impact to the production of cell and hence the PHA as a product .

Tanaka *et al.* (1993) who used the two stage cultivation method involving *Lactococcus lactis* IO-1 and *Alcaligenes eutrophus* to produce PHB using xylose as main substrate produced biomass of 8.5 g/L and PHB at the specific yield of 55% [6]. The specific product yield in this current research (76.54%) is higher than that of Tanaka et al (1993). However, it may be due to the biomass produced in this current research (4.86 g/L) is lower than that of Tanaka et al (1993).

Kim *et al.* (1994) produced PHB by *R. eutropha* using automatic fed batch technique with amonium limitation [7]. Glucose concentration was controlled at the range of 10-20 g/L. The maximum specific product yield obtained was also 76% with the productivity of 2.42 g/L.h. Compared to the results of Kim et al. (1994), specific product yield in this current research (76.54%) was nearly the same. Further, Ryu et al (1997) was able to obtain the specific product yield of up to 80% by using high density fedbatch of *Alcaligenes eutrophus* in a 60 L bioreactor [8].

4.0 Conclusions

These results have proven that hydrolysed sago starch can also be used as carbon source to produce PHA by *Ralstonia eutropha.* A higher formation and accumulation of PHA can be achieved by feeding of an excess carbon source while limiting other essential nutritions through fed batch cultivation method. Fedbatch cultivation strategy can increase both product concentration and specific product yield.

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