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Effects of nitrogen and phosphorus on PHA production by *Azotobacter vinelandii* UWD

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INTRODUCTION

Azotobacter vinelandii UWD has been reported to accumulate PHA during exponential growth phase without the nutrient limitation unlike other PHA-producing microorganisms such as *Alcaligenes eutrophus* and *Pseudomonas* spp. [1]. *A. vinelandii* UWD successfully produced PHA from unrefined sugar sources including cane and beet molasses, malt extract, fish peptone, and corn syrup [1, 2]. This shows that the cost of the fermentation feedstock for *A. vinelandii* UWD can be considerably lowered by using these inexpensive substrates. In this study we determined the optimum phosphorus and nitrogen content for PHA production by *A. vinelandii* UWD.

MATERIALS AND METHODS

A mutant strain UWD of *Azotobacter vinelandii* (ATCC 53799) was cultured and maintained in a glucose medium containing 30 $g \cdot L^{-1}$ of glucose and mineral salt solution of the following composition ($g \cdot L^{-1}$) : KH_2PO_4 0.3, $MgSO_4 7H_2O$ 0.3, $CaSO_4 \cdot 2H_2O$ 0.015, CH_3COONH_4 1.1562, ferric citrate 0.01029, $Fe_2SO_4 \cdot 7H_2O$ 0.0075, $Na_2MoO_4 \cdot 2H_2O$ 0.00036, and yeast extract 1. The pH of the glucose medium was adjusted to 7.3 by 0.5 N NaOH solution. Fermentation was performed in duplicate at 30°C in 250ml flasks using 100 ml of working volume. The shake flask culture was continued for 18-52 hours at 200 rpm.

The effect of C:P and C:N ratios on PHA production was studied using mineral salt solution containing 30 $g \cdot L^{-1}$ of glucose. The C:P and C:N ratios in glucose medium varied from 41:1 to 330:1 and from 17:1 to 137:1, respectively by adding phosphate buffer or NH_4Cl. The C:N and C:P ratios were based on mole quantities.

Minerals contained in swine wastewater were analyzed by an inductively coupled plasma atomic emission spectroscopy (HP 4500, Hewlett Packard, U.S.A.). The amount of alkanonates in the swine wastewater was determined by gas chromatography (HP 5890, Hewlett Packard, U.S.A.). PHA was extracted from the dry cells by acid-propanolysis [3]. As a standard the pure PHA copolymer powder (MW, 643,000; 19.1 mol% of HV; Marborough Biopolymers) was used. Residual cell mass (RM) was calculated by substracting the PHA dry mass from the total dry cell mass. The concentration of glucose was measured using a glucose analyzer (YSI model 27, U.S.A.).

RESULTS AND DISCUSSION

Swine wastewater contains phosphorus and nitrogen at 89:1 of C:P and 11:1 of C:N. The effects of phosphorus and nitrogen on the cell growth and PHA production were investigated by adding or reducing phosphorous and nitrogen (Tables 1 and 2). As compared to phosphorous experiments (incubation time 48 h) shorter incubation time (24 h) was used for nitrogen experiments to avoid the effect of rapid pH drop due to faster cell growth.

For phosphorous experiments, maximum PHA production was obtained at 165:1 of C:P, and dry cell mass and PHA content were 8.0 $g \cdot L^{-1}$ and 72%, respectively. The rate of PHA production was 0.12 $g \cdot L^{-1} \cdot h^{-1}$. At phosphorus concentrations over the optimum value the cell mass and PHA production decreased. Especially, large amount of phosphorous depressed PHA formation. The rate of PHA production at 165:1 of C:P ratio was 0.12 g-PHA$\cdot L^{-1} \cdot h^{-1}$, but at 41:1 of C:P ratio decreased to 0.03 g-PHA$\cdot L^{-1} \cdot h^{-1}$.

In swine wastewater the amount of carbon and phosphrous were 490 mM and 5.53 mM, respectively, resulting in C:P ratio of 89:1. As compared to the optimum C:P ratio (165:1) (Table 1) the swine waste contained 2.56 mM of phosphorous in excess. The amount of carbon requirement based on the optimal C:P ratio was 911 mM, which means that 421 mM of carbon needs to be supplemented. This can be possibly accomplished by using carbon rich food processing wastes.

For nitrogen experiments, higher nitrogen ratio increased cell growth, PHA production, and yields. These results indicate that a high nitrogen ratio could stimulate PHA production as well as cell growth. The optimum value of C:N was found to be 22:1 producing 5.8 g$\cdot L^{-1}$ of cell mass and 64% of PHA content. In swine wastewater the amount of carbon and nitrogen were 490 mM and 44.9 mM, respectively, resulting in C:N ratio of 11:1. As compared to the optimum C:N ratio (22:1) the swine wastewater contained 22.3 mM of nitrogen in excess. The amount of carbon making swine wastewater optimal with respect to C:P ratio was 988mM. This means that carbon content needs to be doubled by using waste materials rich in carbon. Similar results have been reported by Page (1). When complex nitrogen sources such as peptones, yeast extract, casitone were added to the glucose medium, PHA formation was stimulated, and also higher nitrogen ratio promoted PHA formation.

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Table 1. Effect of phosphorus on PHA production in glucose medium (30 $g \cdot L^{-1}$)

C:P [a]	DCW ($g \cdot L^{-1}$)	PHA (%)	PHA ($g \cdot L^{-1}$)	PHA ($g \cdot L^{-1} \cdot h^{-1}$)
330:1	6.5	54	3.5	0.07
165:1 [a]	8.0	72	5.7	0.12
110:1	6.5	41	2.7	0.06
82:1	5.0	36	1.8	0.04
55:1	4.3	30	1.3	0.03
41:1	4.3	29	1.3	0.03

[a]This C:P ratio corresponds to the value of the mineral salt solution supplemented with 30 $g \cdot L^{-1}$ of glucose.

All cultures were incubated for 48 h.

Table 2. Effect of nitrogen on PHA production in glucose medium (30 $g \cdot L^{-1}$).

C:N	DCW ($g \cdot L^{-1}$)	% PHA (dry wt)	PHA ($g \cdot L^{-1}$)	PHA ($g \cdot L^{-1} \cdot h^{-1}$)
137:1	0.7	16	0.11	0.01
66:1 [a]	2.0	37	0.74	0.02
45:1	2.5	41	1.03	0.02
34:1	3.3	56	1.87	0.07
22:1	5.8	64	3.73	0.08
17:1	5.5	65	3.54	0.07

[a]This C:N ratio corresponds to the value of the mineral salt solution supplemented with 30 $g \cdot L^{-1}$ of glucose.

All cultures were incubated for 24 h.