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FERMENTATION OPTIMIZATION FOR PRODUCTION OF POLYHYDROXYBUTYRATE (PHB) BY NEWLY ISOLATED *AZOTOBACTER VINELANDII* KDP

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ABSTRACT

Producing the biodegradable plastic is the most important processes nowadays because to overcome the recent issues concerning the global environment and solid waste management. The optimum conditions for PHB production are more important to enhance the production by microorganism. In the present investigation newly isolated *Azotobacter vinelandii* KDP culture conditions were optimized to produce PHB. The optimum pH and temperature of media for biomass and PHB production by *A. vinelandii* KDP was 7.0 and 35^0C respectively. Maximum biomass (2.67g/l) and PBH (2.18g/l) production obtained at 5g/l sucrose contained media. 2.0 mM/l NH_4Cl was the best concentration for maximum yield of PHB (1.28g/l). Optimum concentration of trace metal selenium for maximum yield of biomass and PHB production was 80µg/l. Under this optimum condition *A. vinelandii* produced two fold PHB.

KEYWORDS: ***Azotobacter vinelandii,*** **polyhydroxybutrate, pH, selenium, sucrose, optimization.**

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INTRODUCTION

The rapid increase in human population has raised the global consumption of goods, thus increasing the volume of non-biodegradable residues, especially plastics. Plastic products are not biodegradable; they are extremely persistent and accumulate in the ecosystem, resulting in a significant burden on solid waste management. These growing piles of resistant waste constitute a severe environmental problem of soaring impact. It is very difficult to reduce the consumption of plastic products due their versatile properties, but it is possible to replace petroleum based plastics with alternative materials that have polymer like properties and that degrade after being discarded. Therefore, there is a need to study and develop biodegradable polymers with plastic like properties (Braunegg et al., 2004a; Chanprateep, S. 2010). Some of the biodegradable plastics are under development include poly- β- hydroxybutyrate (PHB), polylactides, aliphatic polyesters, polysaccharides, and copolymers. Among these biopolymers, poly- β -hydroxybutyrate (PHB) can help to overcome the problem of pollution caused by petrochemical based plastics (Lee, 1996). Poly-β-hydroxyl butyrate (PHB) is readily biodegradable plastic in both aerobic and anaerobic conditions. PHB has many applications in medicine, veterinary practice, tissue engineering materials, pharmaceutical, food packaging and agriculture (van der Walle et al., 2001; Zinn et al., 2001; Luengo et al., 2003; Chen and Wu, 2005; Bucci et al., 2005).

PHB has recently attracted much commercial interest as a plastic material because its physical properties are remarkably similar to polypropylene and polyethylene. PHB is produced by many bacteria as granular inclusions in the cytoplasm (Poirier *et al.,* 1995; Lee and Chang, 1995b). These are produced either as monomers or copolymers, depending upon the bacterial strain, genotype of the organism and cultivation condition including nutritional supplements in the fermentation medium like mixture of carbon source or limiting specific nutrient source (Doi, 1990; Glazer and Nikaido, 1995; Lee, 1996). The importance of investigating novel strains lies in the possibility of replacing well-known industrial production strains with new ones, aspiring to a more productive and efficient polymer production process. Current studies report the isolation of new PHA producing species from extreme environments, and some of them might replace well-established, industrially implemented microorganisms in the near future (Quillaguaman, et al., 2010).

So far, PHAs are not competitive with petroleum-based polymers in economic terms due to their high production costs (Choi and Lee, 1999). The development of economically viable biopolymers can replace conventional plastics that are currently in use. Therefore, efforts are focused on improving the production steps that generate the major part of costs. Recent studies attempt to solve the most costly factors (feedstock, polymer extraction and microorganism efficiency) by investigating the use of cheaper carbon sources (Koller, et al., 2007), novel polymer isolation methods, different fermentation strategies (Oliveira et al., 2007), and discovering new microorganisms (Tian et al., 2009). There are a number of literature reviews on the selection of suitable carbon sources for efficient PHA production, for which the total cost of bioprocessing must meet economic requirements. The most frequently reported factor that influences the price of PHA is the cost of the carbon source. Fortunately, most microorganisms are saprophytes that can metabolize a wide range of carbon sources. However, the selection of carbon sources should not focus only on market prices but also on availability and global price consistency. In addition, inexpensive carbon sources such as agricultural wastes and industrial by-products may incur additional costs due to pre-treatment steps, extended cultivation times, and purification. Simple carbon sources such as sugar and starch from crops seem to be superior to complex carbon sources, but they are also a primary source of human food and

animal feed (Chanprateep, S. 2010; Langevelda et al., 2010; Lenz, and Merchessault, 2010)

The production cost of any biotechnological process can be considerably reduced by process optimization (Sangkharak and Prasertsan, 2007). Reducing the costs of PHB production by optimizing fermentation process is the basic research objective for industrial application. Medium optimization refers to determining the appropriate nutrients and its concentrations that support the cell growth and or maximum production of a particular microbial product. Traditionally, medium optimization has been conducted in a sequence of shake experiments. A change in the concentration or nature of medium components is made and the resulting change in growth rate, cell density and product yield is compared to previous experiment. The fermentation medium needs to be formulated with inexpensive carbon and nitrogen sources, especially industrial byproducts, which can reduce the production cost and commercialization of the process (Zhang et al. 2009). The present investigation demonstrated abilities of newly isolated *Azotobacter vinelandii* KDP to produce maximum PHB in various culture conditions.

MATERIALS AND METHODS

Bacterial strain

Newly isolated gram-negative bacteria *Azotobacter vinelandii* KDP was used in this study and Culture was maintained on nutrient agar media incorporated with yeast extract (1.5 g/l), Beef Extract (1.5g/l), NaCl (5.0g/l), Agar (15g/l), tryptone (5 g/l), KH_2PO_4 (1 g/l), $MgSO_4$ $7H_2O$ (1 g/l), thiamine (1 g/l), glucose (5 g/l) and stored at 4ºC .

Batch fermentation

Batch fermentation was carried out in shake flasks using Burk's mineral salts medium, medium composition as follows: K_2HPO_4 0.8g/l, KH_2PO_4 0.2g/l, $MgSO_4.7H_2O$ 0.2g/l, $FeCl_3$ 0.1g/l, $Na_2MoO_4.2H_2O$ 0.1g/l, $CaSO_4.7H_2O$ 0.1g/l. The optimum conditions of fermentation media for PHB biosynthesis at different initial pH (6–8), temperature (25–50°C), and different concentration of sucrose (1 to 10 g/l), ammonium chloride (0.1 to 5.0mM) and selenium (10 - 100µg/l) were determined. Fresh inoculum was prepared for each experiment and 1% (v/v) of inoculum was used to inoculate the fermentation medium. Media was incubated in incubatory shaker (120 rpm) for 48hrs.

Biomass Estimation

Culture growth was measured by cell dry weight (CDW) determination. 10 ml culture was centrifuged at 10,000rpm for 10minutes and the cell pellet was resuspended in sterile distilled water and centrifuged again for washing. The twice washed cells were dried in a preweighed aluminum foil at 100^0C for 24hrs in hot air oven. The difference between the empty weight of foil and weight of the foil after drying gives the cell dry weight.

Isolation and Estimation of PHB

PHB was extracted from the cell masses by using modified Hypochlorite method (Rawte and Mavinkurve, 2002). At the end of incubation period of each experiment, 1 ml of cell suspension was centrifuged at 6,000 rpm for 15 min. The cell pellet was washed twice with 1 ml saline and again centrifuged. The cell pellet was then suspended in equal volume of sodium hypochlorite (5.5% active chlorine) and incubated at 45°C for 60 min.The extract was centrifuged for 20 minutes at 8,000 rpm and washed with water then with ethanol : acetone mixture (2:1). To get the purified PHB once again pellet was centrifuged at 8,000 rpm . Finally the polymer was converted into crotonic acid by treatmented with concentrated H_2SO_4, and estimated by UV-Visible spectrophotometer against a sulfuric acid blank. The absorbance was read at 235nm. By referring to the standard curve, the quantity of poly-ß-hydroxybutyrate produced was determined.

Statistical Analysis

All the experiments were repeated three times and Correlations analysis (Karl Pearson) was performed to find the degree of relationship

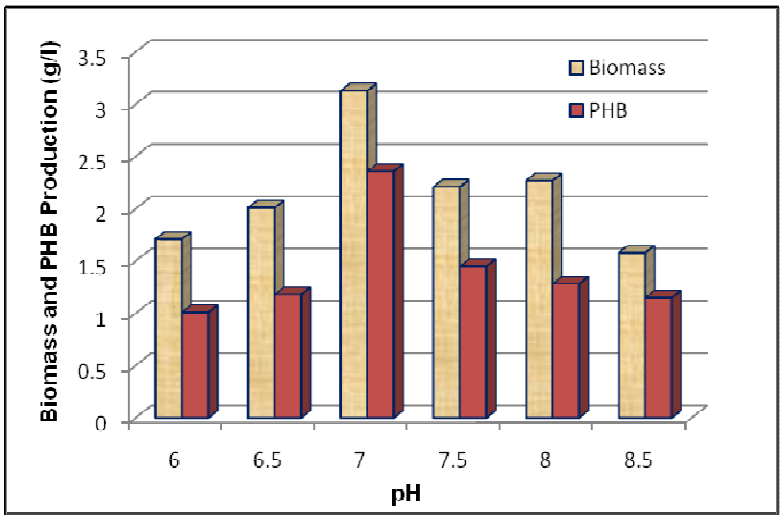
between the variables. This was done by Software - MINITAM Release 12.2.

RESULTS AND DISCUSSION

Effect of different initial pH on production of PHB

The effect of initial pH on PHB production using *A.vinelandii* KDP was studied by conducting the experiments at various initial pH 6.0, 6.5, 7.0, 7.5 and 8.0 of Burk's mineral salts medium with other parameters constant. As the initial pH increased from 6.5 to 7.0 the biomass and the PHB concentration also increased. The maximum PHB production was found to be at pH 7.0 (Fig.1). The PHB concentration decreases for further increase in the initial pH values. Similarly, Thirumal et al., (2010) reported that the optimum pH for PHB production by *Bacillus* sp. 87I and *Bacillus* sp as 7.0. Majority of the gram negative and positive bacteria showed their maximum PHB production at pH 7.0. In this study we found that the pH and PHB production are interdependent because they showed high degree of positive correlation (γ = +1) in the statistic analysis.

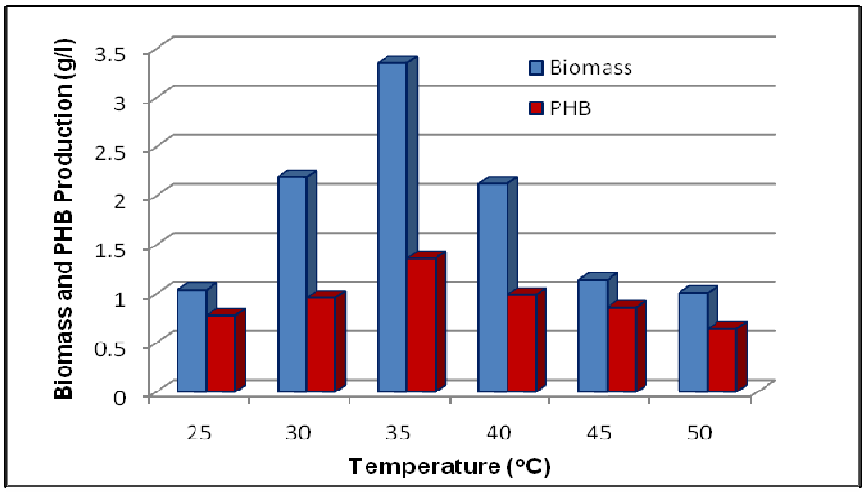
Figure 1
Effect of different initial pH on biomass and PHB production by A.vinelandii KDP



Effect of different initial temperature on production of PHB

To study the effect of initial temperature on PHB production by *A.vinelandii* KDP, the cultures were incubated at various initial temperatures 25, 30, 35, 40, 45 and 50^{0}C. Maximum biomass and PHB production was found at 35^{0}C. Further increases in the temperature decreased both the biomass and PHB production. Ramadas et al. (2010) reported that the incubation temperature range of 30 – 35 °C is suitable for both biomass and PHB accumulation of *Bacillus sphaericus* NCIM 5149 under Submerged Fermentation. When subject these data to statistic analysis they showed high degree of positive correlation hence the physical parameter temperature and PHB production are interdependent. The optimum temperature require for cell division as well as enzyme activity of microorganisms.

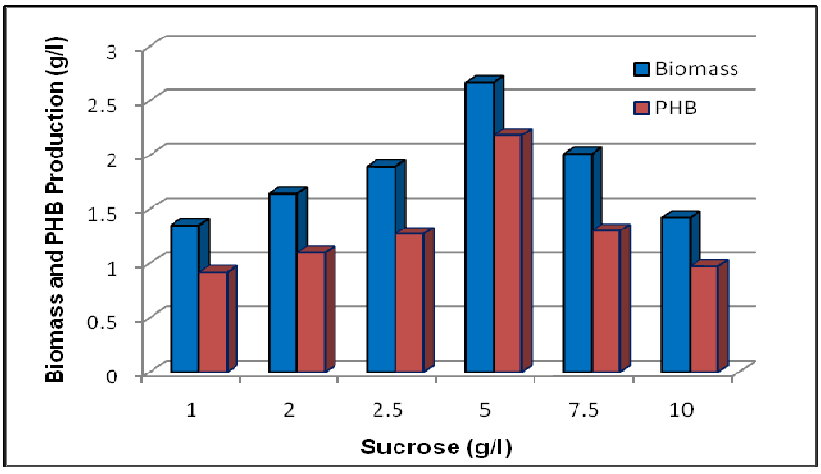
Figure 2
Effect of different initial temperature on biomass and PHB production by A.vinelandii KDP



Effect of different concentration of sucrose on production of PHB

Sucrose is one of the most suitable carbon sources for large-scale PHB production. The greatest success with PHA production from sugar has been reported by Kim et al. (2004). In the present study *A.vinelandii* KDP was grown in Burk's mineral salts medium amended with different concentration of sucrose (1, 2, 2.5, 5, 7.5, and 10g/l), the maximum production of biomass and PHB are obtained at 5 g/l sucrose (Fig.3). Numerous studies have been carried out on the feeding of glucose and an organic acid to achieve a high cell density and high productivity. After testing, several glucose feeding conditions have pointed out that the concentration of glucose from 10 to 25 g L^{-1} was important for high productivities. However, in all of them, the controlled glucose concentrations have still varied widely and needed more supporting of knowledge from the research for development further (Luengo *et al*., 2003). The present study shows that the PHB production was sucrose dependent because positive correlation obtained by statistic analysis.

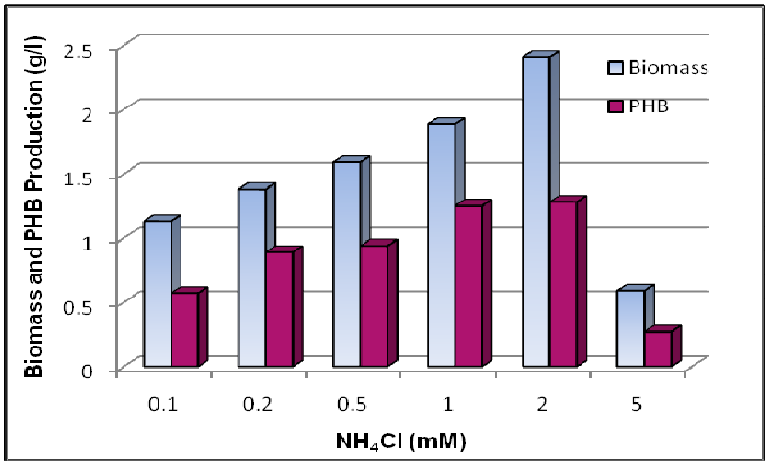
Figure 3
Effect of different concentration of sucrose on biomass and PHB production by A.vinelandii KDP



Effect of different concentration of NH_4Cl on production of PHB

Effect of various concentration of NH_4Cl (0.1, 0.2, 0.5, 1.0, 2.0 and 5.0mM/L) on the growth and PHB production from *A.vinelandii* KDP was investigated. When increase the concentration form 0.1 to 2.0 mM/l, both the biomass and PHB production increased significantly but above the 2.0mM/l showed decrease in the biomass and PHB production (Fig.4). Sangkharak and Prasertsan (2008) reported that the $(NH_4)_2SO_4$ (0.02g/l) was optimal nitrogen source for PHB production from halotolerant photosynthetic bacteria compared to NH_4NO_3 and NH_4Cl and also for other microorganisms such as *Alcaligenes eutrophus* (Grothe et al. 1999; Koutinas et al. 2007), *Methylobacterium* sp. (Kim et al. 2006) and *Sinorhizobium fredii* (Liangqi et al. 2006). Statistic analysis shows the positive correlation.

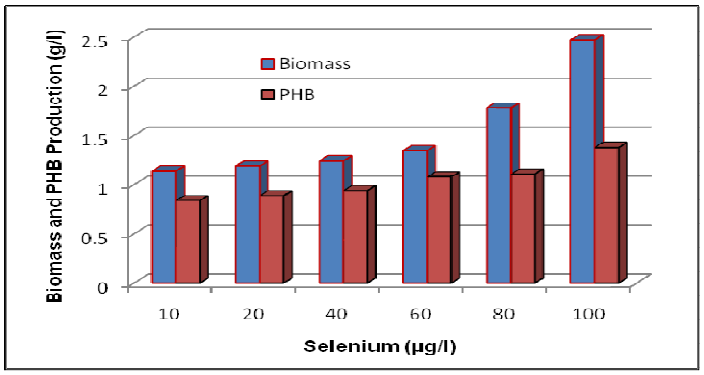
Figure 4
Effect of different concentration of NH_4Cl on biomass and PHB production by A.vinelandii KDP



Effect of different concentration of selenium on production of PHB

The effect of various concentration of selenium (10, 20, 40, 60, 80 and 100µg/L) on PHB production by *A.vinelandii KDP* was investigated by treating selenium as a media supplement. Figure 5 shows the effect of various concentration of selenium on PHB production by *A.vinelandii* KDP. In the present study it was observed that increasing the concentration of selenium in the growth media increased the PHB production. However, selenium at a concentration greater than 80 µg/l decreased the PHB production that was also accompanied with the decrease in the biomass. The reduction in biomass and PHB production may be due to the toxicity posed by the metal on the microorganism. This study suggests that the selenium may have the potential to enhance the biosynthesis of PHB in the cells (positive correlation). A suitable complement of trace elements is essential to attaining high PHB productivity and yield; however, excessively high or excessively low concentrations of trace elements reduce productivity (Grothe et al., 1999).

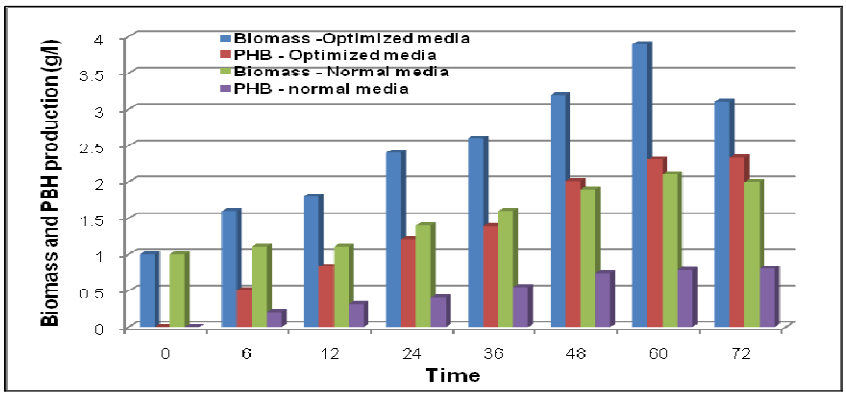
Figure 5
Effect of different concentration of selenium on biomass And PHB production by A.vinelandii KDP



The production of PHB by A. vinelandii KDP under optimal condition in batch culture

According to the results presented above, Batch fermentation was carried out in shake flasks with the optimal cultivation condition of *A.vinelandii* KDP using Burk's mineral salts medium with controlled pH at 7.0 and incubation at 35°C for 96 h at 150rpm. Besides the media was supplemented optimum concentration of sucrose 5g/l, NH_4Cl 2.0mM/l and selenium 80µg/l. Kinetic parameters were calculated and results are given in Fig.6. Maximum net specific growth rate (μ_{net}) of *A.vinelandii* KDP was 0.043 h-1 cultivated in optimized media. It is 2 fold higher than cultivated in normal Burk's mineral salts media (μ_{net} =0.020 h^{-1}). Yield coefficient ($Y_{p/x}$) was 1.11g PHB/g and 0.8 g PHB/g for optimized media and normal Burk's mineral salts media cultivation of *A.vinelandii* respectively. These data suggest that *A.vinalendaii* may be good potential strain for PHB production by this optimized media.

Figure 6
Growth and PHB production by A.vinalendaii cultivated in optimized media and normal media



CONCLUSION

The ain aim of this study was to optimize the media for PHB production procedures by selecting the prominent sources for reduce the production cost of PHB. Optimum pH and temperature for maximum PHB production by *A. vinelandii* was 7.0 and 35^0C respectively. The nutrient sources sucrose, NH_4Cl and selenium supported to the maximum PHB production at

5g/l, 2.0mM/l and 80µg/l respectively. Using both statistically and conventional method, the results indicated that the highest amount of biomass and PHB production was achieved with above controlled conditions. Tow fold biomass and PHB production was achieved when cultivated *A.vinelandii* Burk's mineral salts medium with the above controlled condition. These data suggest that *A.vinalendaii* may be good potential strain for PHB production by this optimized media.

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