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Full Length Research Paper

Effect of carbon and nitrogen sources and incubation times on poly-beta-hydroxybutyrate (PHB) synthesis by *Bacillus subtilis* 25 and *Bacillus megaterium* 12

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In this study, poly-beta-hydroxybutyrate (PHB) production by the *Bacillus subtilis* 25 and *Bacillus megaterium* 12 strains was investigated in nutrient broth medium at different incubation times (between 6 h and 48h). The best PHB production and all yields of these strains were determined. The productions were 0.101 g/L, 0.142 g/L and the percentage yields were 18.03%, 14.79% after 45h, respectively. At 48th h, there was a decrease in PHB yields. In our study, the effects of different carbon and nitrogen sources on PHB production in these strains were also tested. While the strains produced less PHB in nutrient broth medium with different carbon and nitrogen sources, the highest level of PHB accumulation of the strains was observed in the medium with protease peptone. In this nutrient broth medium with protease peptone the percentage PHB yield of *B. subtilis* 25 was determined as 78.69%, while in the same nitrogen sources this percentage in *B. megaterium* 12 was determined to be 77.00%.

Key words: Poly-β-hydroxybutyrate, *Bacillus subtilis, B. megaterium* , Different carbon and nitrogen sources, incubation time.

INTRODUCTION

Polyhydoxyalkonoates (PHAs) are polyesters of hydroxyacida naturally synthesized in bacteria as a carbon reserve. PHAs have properties of biodegradable thermoplastics and elastomers and their sythesis is seen as an attractive system for the sustained production of large amounts of polymers at low cost (Poirier, 2002). PHA is a polyester of repeating subunits (100-30000) with the structure, -[O-CH(R)(CH_2)$_x$CO]-. Polyhydroxybutyrate (PHB) is the most common form where x=1, and R=CH_3 (McCool et al., 1996).

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All bacteria capable of PHB syntesis accumulate PHB during the stationary phase of growth when the cells become limited for an essential nutrient but have excess of carbon source (Page, 1989). The industrial-scale production of PHB has begun by using *Alcaligenes eutrophus* and *A. latus* (Hiramitsu et al., 1993). During the initial balanced growth phase, cell mass is produced but not PHB. Phosphate limitation is imposed in the second phase and PHB accumulates. By necessity this process uses a defined medium containing glucose or sucrose because un refined feedstocks like molasses contain sufficient nitrogen and phosphate to interfere with the timing of the nutrient limitation used to induce PHB synthesis (Page, 1989).

In this study, we investigated the capabilities of some *Bacillus* species to produce PHB in various culture

conditions. By selecting the strain producing the highest PHB for use in industry, the appropriate cultivatium time and the appropriate source of nitrogen and carbon were tested for further PHB yields. The possibility of increasing of the PHB produced by *Bacillus subtilis* 25 and *Bacillus megaterium* 12 with different growth conditions were investigated.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

Bacillus subtilis 25 and *Bacillus megaterium* 12 were used this study. Both the strains were obtained from the culture colletcion of the Section of Biotechnology (Gazi University, Faculty of Sciences and Arts, Department of Biology).

The strains were grown in nutrient broth culture medium (Atlas, 1997) contained (g L^{-1}) peptone, 2.5; NaCl, 2.5; yeast extract 1.0; beef extract 0.5. Cultures (100 mL in 250 mL erlenmayer flaks) were inoculated with a 2% (v/v) inoculum and incubated 37°-30°C with vigorous orbital shaking at 225-250 rpm.

Analytical Procedures

Determination of the amount of PHB was performed chemically. The samples were centrifuged for 45 min at 6000 rpm. Then the pellets were incubated at 60°C for 1 h with sodium hypochlorite to break the cell walls of bacteria. Supernatant was obtained by centrifugation at 6000 rpm was transferred to a Soxhlet system. Cell lipids and other molecules (except PHB) were extracted by adding 5 mL 96% (1:1 v/v) ethanol and acetone. PHB was extracted by chloroform. Chloroform extract was dried at 40ºC and 10 mL of concentrated sulfuric acid was added. They were heated at 100°C in a water bath for 20 min. After cooling, the amount of PHB was determined on a spectrophotometer, at wavelength of 235nm (Kuniko et al., 1989; Bowker, 1981; Ishizaki and Tanaka, 1991).

Effect of Production of PHB In Different Carbon, Nitrogen Sources and at Different Incubation Times

The ratio 2% glucose, sucrose, mannitol and arabinose were added into nutrient broth medium as carbon sources. Peptone was taken out, and the ratio 2% L-cysteine, L-glycine, protease peptone, $(NH_4)_2SO_4$ and potassium nitrate were added as nitrogen sources. Nitrogen and carbon sources were sterilized by Millipore filter with a por size of 0.45 μm. Also, ıt was determinated PHB production of *Bacillus subtilis* 25 and *Bacillus megaterium* 12 at different incubation times (6,21,25,30,45,48 h).

RESULTS AND DISCUSSION

In this study, production of the *B. subtilis* 25 and *B. megaterium* 12 strais were detected between 6h and 48h in nutrient broth medium (Figures 1 to 4). It was determinated that the PHB yield of the both strains increased (18.03%, 14.79%) until 45 th hours and decreased (7.98%, 6.55%) in 48 th hours. It can be thought that until the sporulation time it produced PHB and then used PHB. Spores were produced during the stationary phase of *Bacillus* cultures and at a time when

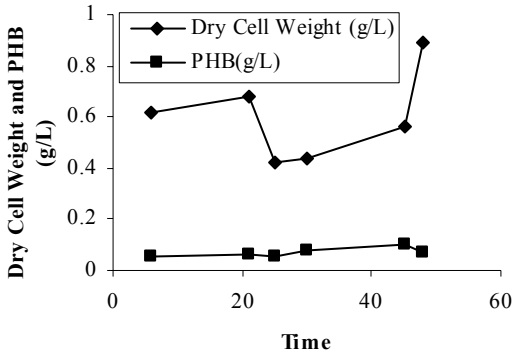

Figure 1. The content of PHB and dry cell weight of the *B. subtilis* 25 strains media as a funtion of incubation time.

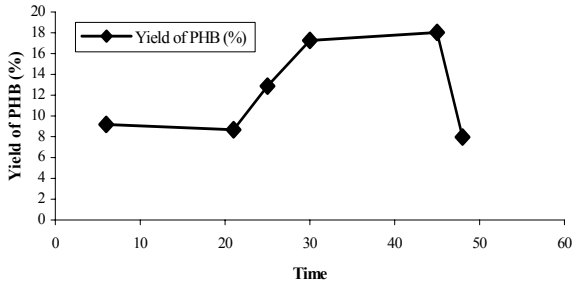

Figure 2. The content of yield of PHB (%) of the *B. subtilis* 25 strains media as a function of incubation time.

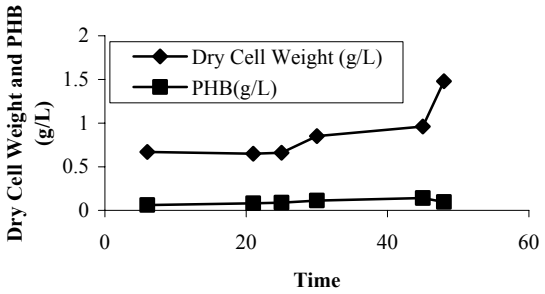

Figure 3. The content of PHB and dry cell weight of the *B. megaterium* 12 strains media as a function of incubation time.

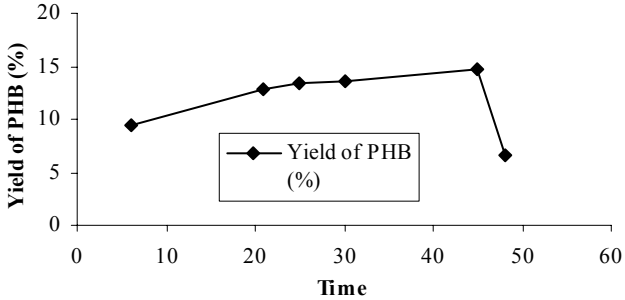

Figure 4. The content of yield of PHB (%) of the *B. megaterium* 12 strains media as a function of incubation time.

Table 1. The production of PHB of the *B. megaterium* 12 on media with different carbon and nitrogen sources.

Carbon and Nitrogen Sources	Dry Cell Weight (g/L)	PHB (g/L)	Yield of PHB (%)
Glucose	1.23±0.43	0.240±0.05	19.51
Sucrose	0.95±0.04	0.070±0.01	7.37
Arabinose	2.47±0.05	0.050±0.02	2.02
Mannitole	3.94±1.01	0.160±0.06	4.06
L-Cysteine	2.36±0.70	0.198±0.03	8.39
L-Glycine	0.68±0.20	0.113±0.06	16.60
$(NH4)_2SO_4$	4.88±1.47	0.050±0.01	1.02
Protease Peptone	1.30±0.60	1.023±0.30	78.69
Potassium Nitrate	2.59±0.10	0.061±0.00	2.36
Control (NB)	0.89±0.06	0.071±0.00	7.98

NB: Nutrient Broth

Table 2. The production of PHB of the *B. megaterium* 12 on media with different carbon and nitrogen sources.

Carbon and Nitrogen Sources	Dry Cell Weight (g/L)	PHB (g/L)	Yield of PHB (%)
Glucose	1.59±0.41	0.310±0.01	19.49
Sucrose	1.26±0.72	0.054±0.01	4.29
Arabinose	1.52±0.01	0.033±0.01	2.17
Mannitole	2.72±0.31	0.170±0.02	6.25
L-Cysteine	3.15±0.10	0.112±0.01	3.56
L-Glycine	0.33±0.02	0.069±0.00	20.91
$(NH4)_2SO_4$	1.35±0.19	0.108±0.00	8.00
Protease Peptone	0.76±0.04	0.059±0.09	77.00
Potassium Nitrate	2.77±0.82	0.035±0.00	1.26
Control (NB)	1.48±0.03	0.097±0.03	6.55

NB: Nutrient Broth

PHB was being produced and consumed (Benoit, 1990; Nam and Ryu, 1985). *Streptomyces griseorubiginosus* DBCC-719 isolated was accumulated PHB amounting to 9.5% of the mycelial dry mass in the early stationary phase when grown in chemically defined medium with 2% (wt/vol) glucose as the sole source of carbon (Manna et al., 1999). Klüttermann et al. (2002), reported that, the highest PHB level (60% PHB of cell dry weight) in *Agrobacterium radiobacter* was achieved in the stationary growth phase (after 96 h). After this time, the PHB content was decreased.

Except 45th h, others time was a decrease in PHB yield. For both strains, although dry cell weight increased at 48th h, the decrease of PHB might indicate that the bacteria used PHB as a sources of carbon and nitrogen, caussing an unsuitable condition due to inadequate nitrogen and carbon sources in the medium.

The highest level of PHB accumulation was observed in the medium with glucose as carbon sources in *B. subtilis* 25 (19.51%), *B. megaterium* 12 (19.49%) (Tables 1 and 2).

Hori et al. (2002), in *B. megaterium* PHB content in the cell was reached a maximum level after growth with glucose. In one of the studies conducted by Wu et al. (2001), it was reported that *Bacillus* sp. JMa5 strain accumulated 25-35%, (w/w) PHB during sucrose fermentation.

The highest level of PHB accumulation was observed in the media with proteaz peptone as nitrogen sources in *B. subtilis* 25 (78.69%) and in *B. megaterium* 12 (77.00%) (Tables 1 and 2).

Page (1992) tested PHB production in a variety of commercially available complex nitrogen sources (fish peptone, protease peptone, yeast extract, casitone, phytone and tryptone). It was found that complex nitrogen sources increased the yield of PHB produced by *Azotobacter vinelandii* UWD strain. Mercan et al. (2002) investigated the effect of different nitrogen and carbon

sources and PHB production in two strains of *Rhizobium* sp. They noted that the strains produced less PHB in yeast exract mannitol (YEM) broth media with different carbon (glucose, sucrose, arabinose) and nitrogen (L-cysteine, L-glycine, DL-tryptophan, protease peptone, potassium nitrate) sources, while the highest level of PHB accumulation was observed in the media with L-cysteine, L-glycine.

Thus, we have shown that, depending upon the utilized sources of carbon and nitrogen, PHB synthesis may be selectively induced in *Bacillus* strains.

In the medium with protease peptone, both strains showed the higest PHB yield (78.69%, 77.00%). Accordingly, it was interesting that they showed high production as much as the others used as industrial. On the basis of data obtained in the present work, *B. subtilis* 25 strain capable of PHB accumulation up to 78.69% of dry cell weight was selected, as a candidate for industrial production after the optimization of the conditions of PHB synthesis. If bacilli are used for industrial PHB production, the optimum incubation time must be determinated regarding onset of sporulation.

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