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PRODUCTION AND RECOVERY OF POLY-B-HYDROXYBUTYRATE FROM WHEY DEGRADATION BY *AZOTOBACTER*

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ABSTRACT

Three strains of *Azotobacter chroococcum* were studied to produce poly-β hydroxybutyrate as a inclusion body by whey degradation. Optimum degradation whey results were obtained when using whey broth as a fermentation medium without extra salt, temperature at 35 °C and pH 7 ($P<0.05$). Lambda max for whey broth medium was determined probably about 400 nm. The effect of different nitrogenous rich compounds (NH_4NO_3, Bactopeptone, Casein, Yeast extract, Meat extract, Protease peptone and Tryptone) on whey degradation showed that incorporation of nitrogenous compounds into the medium did not increase whey degradation by *Azotobacter chroococcum* 1723 ($P<0.05$). But poly-β hydroxyl-butyrate production was increased in presence Meat extract up to 75% of the cell dry weight after 48h. The addition of nitrogenous sourced (except ammonium nitrate) had a positive effect on poly-β hydroxyl-butyrate production as it peaked in the presence of Meat extract and 4.43 g/L was accumulated in comparison to 0.5g at diazotrophically growing cells. Increasing the O_2 values resulted by shaking at 122 rpm in decreased poly-β hydroxyl-butyrate yield form 4.43 to 0.04 g/L. The results show that this medium supports the growth of strain 1735 and also that this waste could be utilized as a carbon and nitrogen source. Production of poly-β hydroxyl-butyrate by using whey as a medium looks promising, since the use of inexpensive feed-stocks for poly-β hydroxyl-butyrate is essential if bioplastics are to become competitive products.

Key words: *Azotobacter chroococcum*, whey broth, poly-β hydroxyl-butyrate and nitrogen source

INTRODUCTION

Poly-β hydroxyl-butyrate, (PHB) is a biodegradable thermoplastic which can be extracted from a wide range of bacteria. PHB belongs to the class of bacterial polyesters collectively called polyhydroxyalkanoates, (PHAs). PHAs have properties similar to polypropylene and are important due to their complete biodegradability, with recognised potential applications in reducing disposable waste problems and in certain medical applications (Gostomski, and Bungay, 1996). These biodegradable thermoplastics can be used as packaging material and can be utilized as drug delivery systems, since these polymers are immunologically inert. Biodegradable polymers would help to reduce solid waste disposal problems associated with most plastics. The polymer which provides a reserve of carbon and energy, accumulates as intracellular granules. Several factors influence the economics of biodegradable polymer production. One factor is the cost of the substrate. The ability to produce biodegradable polymers from inexpensive and renewable carbon sources, such as xylose and lactose, may help to improve the economics of the process.

Whey is the liquid part of milk that separates from the curd at the beginning of the cheese making process. In the past whey was considered waste. It was difficult to dispose of and sometimes contributed to environmental damage, for example by adding salt to soil or waterways. It is rich in nutrients and contains at least half the solids found in whole milk (Horton, 1993). Whey has a very strong polluting capacity, with a biological oxygen demand (BOD) of 40,000 to 45,000 mg/L (Hacking, 1988; Kemp and Quickenden, 1989). In recent years, value added products form whey have attracted much interest. One such area is the production of microbial biopolymer (Schwartz

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and Bodie, 1985; Fu and Tseng, 1990; Flatt *et al.*, 1992; Konicek *et al.*, 1993). *Azotobacter sp* capable of accumulating PHAs (homo- and copolymers) in chemically defined media during growth without a nutrient limitation (Gonzalez-Lopez *et al.*, 1997). *Azotobacter sp.* is Gram negative bacteria, polymorphic i.e. they are of different sizes and shapes. Old population of bacteria includes encapsulated forms and have enhanced resistant to heat, desication and adverse conditions. The cyst germinates under favourable conditions to give vegetative cells. They also produce polysachharides. These are free living bacteria which grow well on a nitrogen free medium. These bacteria utilize atmospheric nitrogen gas for their cell protein synthesis (Gonzalez-Lopez *et al.*, 1997). The general purpose of the present study was to develop a model system for degradation of whey by *Azotobacter* sp, optimize condition and the possibility of producing a known commercial biopolymer PHB.

MATERIALS AND METHODS

Strain and media

The bacteria used in the experiments were three strains of *Azotobacter chroococcum* with DSM No. 281, 398 and 1723. The strains were originally obtained from Microbiology Department, Faculty of Science, Islamic Azad University, North of Tehran-Iran. The colonies of strain on Manitol agar medium (Oxoid) and Whey agar [0.1% (w/v) yeast extract, 0.1% (w/v) yeast extract (Oxoid), 0.05% (w/v) K_2HPO_4, 0.01% (w/v) NH_4NO_2, 100% (w/v) whey broth, 1.5% Bacto agar (Merk), pH was adjusted to 7 using 5 M NaOH] and incubated at 30 °C for 24-48 hours. This medium was prepared with different inorganic and organic nitrogen sources (NH_4NO_3, Bactopeptone, Casein, Yeast extract Meat extract Protease peptone and Tryptone 1%), and kept at different temperature (25, 30, 35, 37ºC) to optimize PHB production. Gram and capsules stains were used for staining bacteria. Isolation broth [0.1% (w/v) yeast extract, 0.05% (w/v) malt extract (Oxoid), 0.05% (w/v) K_2HPO_4, 0.01% (w/v) NH_4Cl, 0.01% (w/v) $MgSO_4$] used for screening of whey utilizing commercial biopolymer producer. Samples were taken every 24 h to measure optical density (OD_{400}) by spectrophotometer assay.

Determination of λ max

Whey broth concentration was estimated by measuring the absorbance at 200-400 nm by UV spectrophotometer assay to determine λ max (Andrewes *et al.*, 1976).

Staining of intracellular lipid

Sudan black stain (sudan black B powder 0.3 g, 70% ethyl alcohol 100 mL) was used to show microbial intracellular lipid. In this staning, lipid inclusion granules are stained blue-black or blue-grey, whilst the bacterial cytoplasm is stained light pink (Collee *et al.*, 1989).

Determination of PHB

Determination of the amount of PHB was performed chemically. Bacteria were grown on Manitol broth and Whey broth at 30 ºC for 48 h on a shaker and without shaker. A sample of culture solution (0.1-0.2 ml) was added to 4 ml of the sample were centrifuged for 30 min to precipitate solution and then left for 1 day. 0.4ml of the sample was centrifuged for 30 min to precipitate the PHB. The solid pellet was resuspened and washed with 1ml portions of water Acetone and ether. Chloroform was added and allowed to boil in a water bath at 100°C. The settled material after evaporation of chloroform was dried at 40°C for 30min. The white powder was dissolved in concentrated H_2SO_4 (2.5ml) and heated for 10min at 100°C. After cooling, the solution was measured photo metrically at 235 nm against H_2SO_4 blank (Dekwer and Hample, 1999).

PHB spectrophotometer assay

For the spectrophotometer assay of polymer a sample containing 5 to 50 µg polymer in chloroform is transferred to a clean test tube the chloroform is evaporated and 10 ml of concentrated H_2SO_4 are added the tube is capped with a glass marble and heated for 10 min at 100 °C in a water bath the solution is cooled and after thorough mixing a sample is transferred to a silica cuvette and the absorbance at 235 nm is measured against a sulfuric acid blank. A standard curve was established with PHB concentrations ranging from 2-20µ g/ml PHB (Dekwer and Hample, 1999).

Optimize of PHB production in different nitrogenous sources

Different nitrogenous sources with ratio 1% such as NH_4NO_3, Bactopeptone, Casein, Yeast extract Meat extract Protease peptone and Tryptone were added to Whey broth medium. After bacteria cultured and incubated at 30 °C for 48 h, PHB extracted and it was measured photo metrically at 235 nm.

Statistical analysis

Significant differences between bacterial treatments were tested by analysis of variance using Minitab. Data were normalized, and Tukey tests were performed. The results of different incubated time experiments were subjected to Student's *t* test to identify significant differences between bacterial treatments. Probability (*P*) values of <0.05 were considered significant.

RESULTS

All three strains were studied could ability to use whey as fermentation substrate. *Azotobacter chroococum* (1723 and 398) were produced colonies that were mucoid, ropy and capsule positive with yellow pigment in 24 h at 30 °C in whey agar than Manitol agar media**.** The bacteria were Gram-negative rods with rounded ends, formed coccoid cells, even at a very early stage. These coccoid cells showed high content of PHAs granules. Capsule material was observed when strains (1723 and 398) were grown on Whey agar medium than Manitol agar. These strains were regarded as efficient producer and were subjected to further study. Optimum degradation whey results were obtained when using whey broth as a fermentation medium without extra salt, temperature at 35 °C and pH 7 ($P<0.05$). Since the solution absorbs in 200 to 400 nm, showed there will be a peak in the graph of absorbance versus wavelength in 400 nm region. Lambda max for whey broth medium was determined probably about 400 nm. A standard curve was established with PHB concentrations ranging from 2-20µg/mL PHB (Fig. 1).

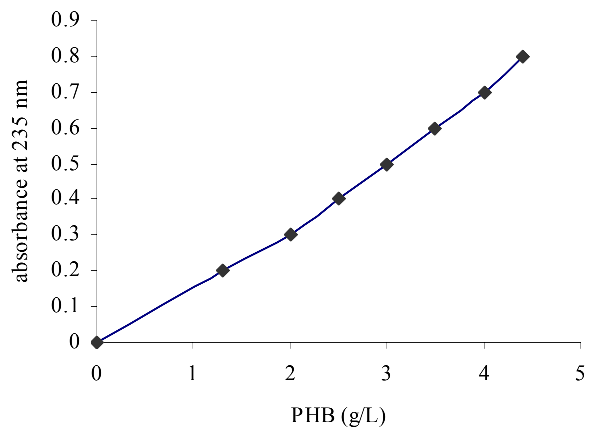

Fig. 1: Relationshipe between poly hydroxybutyric acid concentration and absorbance at 232 mµ

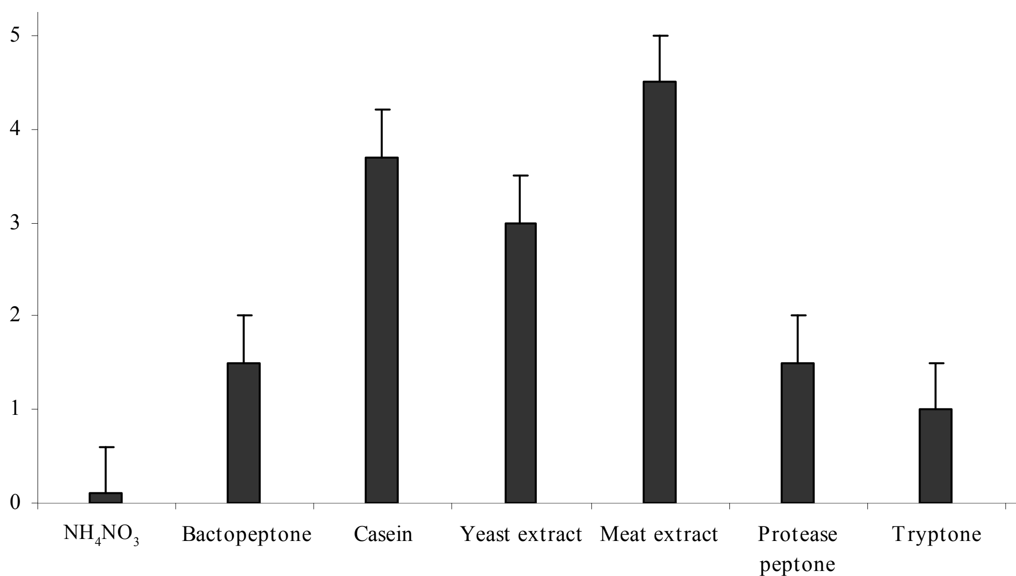

Fig. 2: PHB production as a function of different nitrogenous sources

The effect of different nitrogenous rich compounds (NH_4NO_3, Bactopeptone, Casein, Yeast extract, Meat extract, Protease peptone and Tryptone) on whey degradation showed that incorporation of nitrogenous compounds into the medium did not increase whey degradation by *Azotobacter chroococcum* 1723($P<0.05$). But PHB production was increased in presence Meat extract (Fig. 2). The production of PHB in Manitol broth medium supplemented with 1% Meat extract was considerably lower than cells grown in Whey broth medium with 1% Meat extract (Fig. 2). The results showed that maximum production of PHB was obtained by *A. chroococcum* 1735 (Table 1). The effect of oxygen limitation on PHB production was tested by agitation rate (122rpm) and without it. The result showed that maximum production of polymer was obtained in culture cells grown in Meat extract Whey -culture medium without shaking. Increasing the O_2 values resulted by shaking at 122 rpm in decreased PHB yield form 4.43 to 0.04 g/L. The addition of nitrogenous sourced (except ammonium nitrate) had a positive effect on PHB production as it peaked in the presence of meat extract and 4.43 g/L was accumulated in comparison to 0.5 g at diazotrophically growing cells. *A. chroococcum* 1735 was sink reducing power for nitrogen fixation.

Table 1: PHB Production by *A. chroococcum* strains in Whey broth medium with 1% Meat extract

Bacterial strains	Total dry wt (g/L)	PHB (g/L)	% PHB (dry wt)
A. chroococcum 281	5.38±0.31	1.95±0.16	36.3
A. chroococcum 1735	5.86±0.24	4.43±0.41	75.7
A. chroococcum 398	5.71±0.46	2.88±0.1	50.6

Values are means and SE of five replicates. All cultures were incubated for 48 h at 30 °C without shaking

DISCUSSION

PHB is a carbon storage polymer widely distributed among prokaryotes including *Azotobacter sp.* (Nair *et al.*, 1993). PHB and other PHAs have been considered commercially important because of their possible use as biodegradable thermoplastics (Lee, 1996). Whey is a by-product of the dairy industry that has presented many disposal problems (Horton, 1993). Lactose is the most abundant constituent of whey and it forms at least 78% (w/w) of the whey's total solids. This indicates that for an organism to efficiently use whey as substrate, it must be able to metabolize the lactose as its carbon source.

Such organisms should produce sufficient lactose hydrolyzing enzymes (β- galactosidase) to hydrolyze the lactose to its monomers. The media used for the production of commercial microbial polysaccharides usually contains glucose or sucrose as carbon source (Belder, 1993, Kang *et al.*, 1993). This increases the production costs of microbial biopolymer (Hansen, 1993). According to Bonartseva *et al.*, (1994). observed maximal PHB accumulation in Rhizobium lupiniafter growth with mannitol and glutamate. Increasing the O_2 values resulted by shaking at 122 rpm in decreased PHB yield form 4.43 to 0.04 g/L respectively, these results were in agreement with those obtained by Page *et al.*, (1997) using different *Azotobacter sp.* Thus N2 – fixing cells have a very low PHB content and the addition of organic nitrogen spares the need for nitrogen fixation and the respiratory protection of the oxygen labile nitrogenase complex and thus allows the reducing power and Acetyl Co A derived from active sugar metabolism to be used for PHB production (Page *et al.*, 1997) Similar result were obtained by present research.

Many nitrogen-fixing microorganisms synthesize PHB. According to Tombolini and Nuti (1989), the content of this polymer in rhizobia ranges from 30 to 55% of dry cell weight. Bonartseva *et al.*, (1994) tested the capacity for PHB production in active and less active strains of *Rhizobium phaseoli*, *R. meliloti* and *R. trifolii* during growth on media with different carbon and nitrogen sources. It was found that PHB synthesis can be selectively induced either in active or less active *Rhizobium* strains by sources of carbon and nitrogen. They reported that the less active strain of *R. phaseoli* 680 was a promising producer of

PHB, and the PHB content in cells of this strain was up to 65% of dry cell weight during growth on a medium with sucrose and nitrate; the PHB content was much lower when organic acids were used. Tavernier *et al*., (1997) investigated the effects of different nitrogen and carbon sources and pH on exopolysaccharide (EPS) and PHB production in two strains of *R. meliloti*. They reported that these two strains showed different growth rates in the medium. They also noted that there was a decrease in PHB content in the medium with an acidic pH. In the medium with fructose and yeast extract, the PHB yield was 85%. In our study, the production of PHB in *A. chroococcum* 1735 which produced the maximum PHB percentage was determined in Meat extract as a nitrogen source. While the percentage yield of PHB in this strain was lower with different nitrogen sources in Monitol broth, the highest level of PHB accumulation was observed in the media with Lactose as a carbon source and Meat extract as nitrogen source. This interpretation could be supported by the fact that the energy charge of the cells grown on Whey broth -medium was higher than that of cells grown in other medium. Beom Soo Kim and Ho Nam Chang (1998) reported the ability of *Azotobacter chroococcum* to production of PHB from starch. In flask culture, PHB content increased up to 74% of dry cell wt with increasing culture volume. In batch culture, PHB content increased to 44% with O_2 limitation. In fed-batch culture, cell concentration of 71 g/L with 20% PHB was obtained without O_2 limitation, whereas cell concentration of 54 g/L with 46% PHB was obtained with O_2 limitation.

However, the possibility that Whey medium could be utilized as an inexpensive substrate for the industrial application of these microorganisms could be of considerable importance. In this context, the production of PHB by using Whey medium looks promising, since the use of inexpensive feed-stocks for bioplastic production.

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