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Optimization of Poly - Hydroxyl Butyrate (PHB) Production by *Bacillus megaterium* PTCC 1656

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Poly –hydroxyl butyrate(PHB) is a natural,biodegradable polymer accumulated in the form of intracellular granules by a large variety of bacteria. It is among the most investigated biodegradable polymer in recent years. The PHB$_s$ are non-toxic, biocompatible and biodegradable thermoplastics that can be produced from renewable resources. The study aimed at screening and identifying a potential PHB accumulating *B.megaterium* PTCC 1656 and optimization of media parameters for increased PHB production by this strain bioplastics. For this study *B.megaterium* PTCC 1656 was purchased from the fungi and bacteria collection center of the Iranian science and technology research organization. An their PHB production was determined under different conditions such as incubation time, Carbon and Nitrogen source. The result showed that , glucose was the best carbon source and in this conditions the maximum production of PHB was 0.42 g/l at 45h. PHB production starts in response to stress imposed on cell usually by nitrogen limitation,although in the presence of abundant carbon source. Actually PHB production was dependent on nutrient limitation.

Key words: *Bacillus megaterium*, poly-hydroxyl butyrate, optimization.

Plastic chemicals can hardly be decomposed in nature. They are harmful to human health and environment. Now, people have become more concerned about protecting the environment to reduce white pollution , biodegradable plastic is an inspiring measure to solve the problem and more scientists are engaged in related research. PHB have received increased attention because of their thermoplastic or elastomeric properties, which resemble those of petroleum based plastics ,yet they're completely biodegradable. There are many natural bacteria or even plants that can directly produce PHB(1). PHB is a biodegradable thermoplastic which can be extracted from a wide range of bacteria(2.3).It can be made into films,fibers,sheets even molded to the shape of a bag and bottle(4,5). The main factor preventing large scale production and commercialization of PHB is its high cost of production(4). Use of less expensive substrates, improved cultivation strategies and easier downstream processing methods are required of reducing the cost(4,6,7). Reducing the cost of PHB production by optimizing fermentation process in the basic research objective for industrial application(8). Medium optimization by application of statistical optimization method , compared to the common "one – factor – at – a – time" method, proved to be powerful and useful tool(8). PHB can be produced from renewable resource. They have a high degree of polymerization, are highly

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crystalline, optically active, isotactic, piezoelectric and insoluble in water. These features make them highly competitive with polypropylene, the petro chemically derived plastics (4, 9). PHB is being developed for a variety of applications(5,6).The polymer which provides a reserve of carbon and energy accumulates as intracellular granules(2). Research have shown that PHB is an important molecule on cytoplasm and cell walls. *Bacillus* have been shown to accumulated PHB during the sporulation of bacterial growth(2). The aim of this study was: 1- Optimization of culture parameters for maximum PHB yields 2- Extraction PHB yields produced by *B.megaterium*1656.

MATERIALS AND METHODS

Preparation of standard strain

*B.megaterium*PTCC 1656 was purchased from the fungi and bacteria collection center of the Iranian science and technology research organization. They were cultured in Nutrient broth and then incubated at 35°C for 24h. The resulting bacterial colonies were tested for PHB accumulation by staining with Sudan black. In the identification process ,*B.megaterium* was initially selected based on the Gram reaction, spore morphology catalase test, starch hydrolysis, methyl red test,motility,producing H_2O and utilization of citrate,D – glucose,Mannitol ,xylose ,arabinose.The isolates were then characterized by their growth at various temperatures and at different pH values and reduction of nitrate. According to the results obtained from the tests above the *B.megaterium* were determined optimization of cultural medium by "one – factor – at – a – time" method.The resulting bacterial colonies were inoculated to basic culture medium . Containing 2.5 g/l peptone,2.5 g/l NaCl, 1.0 g/l yeast extract and 0.5 g/l beef extract were mixed in a rotary shaker at 30 for 24h. Then 100ml inocula was transferred into 250ml conical flask and the ratio 2% nitrogen and carbon source were added and incubated in a rotary shaker at 225-250 rpm at 30-35. Also for the checking nitrogen sources, at first peptone was taken out, and the ratio 2% L-cysteine, L-glycine $(NH_4)_2 SO_4$ and potassium nitrate were added as nitrogen sources. That was determinate PHB production of *B.megaterium* PTCC 1656 at different incubation time (6, 21, 25, 30, 45, 48, 50, and 60 h).

Determination of cell dry weight

After centrifugation of the culture medium , the supernatant was discarded and the cell pellet was washed with distilled water. The washed pellet was resuspended in 1ml of distilled water. Transferred to pre-weighed boats, and dried to constant weight at 50 for 72h. The dry weight of the cells was determined by during the washed cell to constant dry weight.

Extraction and determination of PHB

Determination of PHB was performed chemically.The samples were incubated at 60 for 1h with 1 ml of sodium hypochlorite to break the cell walls of bacteria than centrifuged for 45 min at 6000 rpm. Supernatant was transferred to a soxhlet system. Cell lipids and other molecules (except PHB) were extracted by adding 5ml 96% (1:1 v/v) ethanol and acetone. PHB was extracted by chloroform .Then 15 ml of concentrated sulfuric acid was added. They were heated at 100 in a water bath for 20min. After cooling, the amount of PHB was determined on a spectrophotometer, at wave length of 235nm.

RESULTS AND DISCUSSION

The result showed that, glucose was the best carbon source and in this study and the maximum production of PHB was 0.42 g/l at 45h. The rate of cell dry weight, when was used of glucose as the carbon source was 1.70 g/l and PHB yield was 24.70% . After glucose ,mannitol was the best carbon source, and after that, sucrose and arabinose were the best carbon sources and PHB yield for these carbon sources were respectively 12.8%,5.29% and 4.0%.

PHB is a biodegradable material that produced by some bacteria in culture media under unbalanced growth condition, such as nitrogen , phosphorous or magnesium deficiency or the presence of excess carbon(10). PHB has been identified in more than 20 bacterial genera, including *Azotobacter, Bacillus, (11, 12)*. The aim of this present work was selection of *Bacillus megaterium* PHB produces. In this study *B. megaterium* PTCC 1656 was purchased from the fungi and bacteria collection center of the Iranian science and technology

Research organization, and their PHB production was determined under different conditions such as incubation time, carbon and nitrogen source. The first effects of various carbon sources (glucose , sucrose , arabinose and mannitol) were studied. Also the various nitrogen sources (glycine – cysteine , ammonium sulfate and potassium nitrate)were studied on PHB production by *B.megaterium* PTCC 1656. Although some studies report that the incubation time for PHB synthesis is 45h, the other reported that the peak levels of PHB synthesis are at 24th, 48th, 72nd and 120th h(10,13). In this study production of PHB by *B. megaterium* PTCC 1656 was detected between 24h and 48 in nutrient broth medium. It was determinate that the PHB yield increased between 24 h and 48h and decreased between 48 h and 72h with 2% carbon sources. It can be thought that until be sporulation time it produced PHB and then used PHB.Spores were produced during the stationary phase of Bacillus. culture and at a time when PHB was being produced(14,15). The result showed that glucose was the best carbon source and maximum PHB synthesis(0.42 g/l) was found in *B.megaterium* PTCC1656 when glucose was used as the carbon source. The production of PHB in *B.megaterium* was studied by Hori et al .and found the highest value of PHB contents when glucose was used(16).and in our study maximum PHB synthesis was detected when glucose was used as the carbon source at 45 with concentration 2%. One of the objective of this study was to determine the effects of nitrogen sources on PHB accumulation. We found that the maximum PHB synthesis in *B.megaterium* PTCC 1656 was obtained when glycine was used as the nitrogen source at 48h with concentration 2%. Page (1992) tested PHB production in a variety of commercially available complex nitrogen source increased the yield of PHB produced by *A.vinelandii*UWD strain (17).Mercan et al. also reported that PHB accumulation was high in two strains of *Rhizobium sp*. When L- cysteine and glycine were used as the nitrogen source(18). The highest amount of cell dry weight could be obtained in *B.megaterium* grown in mannitol and the other hand in cysteine. So, effect of different C:N ratios is very important on yield of PHB production. Hikmet et al.reported an increase in the PHB yield of *B.megaterium*Y6, *B. subtilis*K8, and *B. firmus*G2 via mutation(6).Dave et al. reported DCWof 70% PHB in optimum culture conditions for *Bacillus* sp. IPCB-403,while Findlay and White(1983) reported the presence of PHB in *B. megaterium* chromatographically(6).Chen et al. also studied D(-)-3-hydroxyalkanoate in 11 different *Bacillus* spp.and observed that PHB accounted for 50% of DCW in the bacteria(6).Other researchers have reported PHB production changes in *Bacillus* mutant strains(6). Interestingly, most of *Bacillus*

Table 1. The production of PHB by *B.megaterium* PTCC 1656 with different carbon sources

Carbon sources	Dry cell weight (g/l)	PHB (g/l)	Yield of PHB
Glucose	1.70	0.42	24.70%
Mannitol	2.50	0.32	12.80%
Arabinose	1.75	0.07	4.0%
sources	1.70	0.09	5.29%

Table 2. The production of PHB by B.megaterium PTCC 1656 with different nitrogen sources

Nitrogen sources	Dry cell weight (g/l)	PHB (g/l)	Yield of PHB
Ammonium sulfate	1.30	0.09	6.92%
potassium nitrate	2.8	0.04	1.92%
L- Cysteine	3.14	0.01	3.18%
L- Glycine	0.35	0.08	22.85%

Table 3. The production of PHB by *B.megaterium* PTCC 1656 with different incubation time

Time (h)	Dry cell weight(g/l)	PHB (g/l)	Yield of PHB
6	0.52	0/01	1.92%
21	0.62	0/03	4.83%
25	0.80	0/04	75.0%
30	0.95	0/05	5.26%
45	1.32	0/07	5.30%
48	1.38	0/03	2.17%
50	1.49	0/02	1.34%
60	1.50	0/01	75.0%

strains such as *B. megaterium*OU303A,*B. cereus* SPV and *B. thuringienesis*IAM 12077 showed maximum PHB production in presence of glucose and under limitation of ammonium sulfate concentration(8).

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