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Effects of Culture Conditions on PHB Synthesis by *Bacillus subtilis*

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Poly-beta-hydroxyl butyrate (PHB) is a biodegradable thermoplastic which can be extracted from a wide range of bacteria as intracellular granules. They are used in packaging, medicine and agriculture for a wide range of applications. The aim of the present work was selection of *Bacillus subtilis* PHB producers. In this study, the *Bacillus subtilis* from soil were isolated and identified, and their PHB production was determined under different conditions such as incubation time, carbon source and nitrogen source. The results showed that, starch was the best carbon sources with significant effect and was better in PHB production and known as low cost substrates to compare with the other sources .Amongest variety of nitrogen sources, glycine was the most suitable. At last, the best optimum incubation period and starch concentration to PHB production were 48 hours and 5% (v/v) and for glycine were 48 hours and 3% (v/v) respectively. The results showed that the best percent of carbon source in PHB production, was not the highest amount, but the best concentration was that do not lead to limitation of bacteria growth.

Key words: *Bacillus subtilis*, Poly-beta-hydroxyl butyrate, PHB, optimization.

Poly-β-hydroxyl-butyrate (PHB) is a biodegradable and biocompatible thermoplastic Produced by various microorganisms. It can be made into films, fibres, sheets even molded to the shape of a bag and bottle. PHB and poly hydroxyvaleric acid (PHV) are being developed for a variety of applications[1]. PHB is a widely distributed intracellular reserve substance typical of prokaryotes. PHB exists in the cytoplasmic fluid in the form of crystalline granules about 0.5 μm in diameter and can be isolated as native granules or by solvent extraction[2,3]. Various researches have explained that soil bacteria generally produce PHB. PHB production increases if convenient condition is made available .Besides these biopolymers increase the resistance of bacteria[4].

Synthetic polymers (known as plastics) have become significant since the1940s, and since then they are replacing glass, wood and other constructional materials, and even metals in many industrial, domestic and environmental applications[5-8]. These widespread applications are not only due to their favorable mechanical and thermal properties but mainly due to the stability and durability[9] . On the other hand, plastic also play important role for many "short live" applications such as packaging and these represent

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the major part of plastic waste[5,9-11]. Because of their persistence in our environment, several communities are now more sensitive to the impact of discarded plastic on the environment, including deleterious effects on wildlife and on the aesthetic qualities of cities and forest. The increased cost of solid waste disposal as well as the potential hazards from waste incineration such as dioxin emission from PVC makes synthetic plastic waste management problems. Consequently, for the past two decades, there have been growing public and scientific interests regarding the use and development of biopolymer (biodegradable polymers) materials as an ecologically useful alternative to plastics, which must still retain the desired physical and chemical properties of problem of plastic waste[7,11-13]. Biodegradable plastics are made from renewable resources and do not lead to the depletion of finite resources.

MATERIALS AND METHODS

Isolation and identification

Different soil samples were taken from different regions Lahijan in Iran. About 15–20 g of soil samples scraped within 5–8 cm depth with a sterile spatula was collected from native grass lands in different areas Lahijan, Iran. The samples were placed in sterile plastic bags and stored at 4 °C. Each gram of the sample was suspended in 9 ml sterile distilled water and shaken vigorously for 2 min. The samples we reheated at 60°C for 60 min in water bath. Then the liquid was serially diluted in sterile distilled water, and dilutions from 10^{-1} to 10^{-6} were plated on nutrient agar medium. Plates were incubated at 30-35°C for 24–48 h. In the identification process, Bacillus subtilis was initially selected based on the Gram reaction, spore morphology ,catalase test , starch hydrolysis, utilization of citrate,Voges- proskauer , Methyl red test , Motility, producing H_2S and acid from TSI ,utilization of D-glucose, D-mannitol, D-xylose, L-arabinose(1 g/100 ml). The isolates were then characterized by their growth at various temperatures (30, 40, 50, 55 and 65°C) and at different pH values, tolerance of different salt levels (2, 5, 7 and 10 g NaCl/100 ml), and reduction of nitrate. According to the results obtained from the tests above the *Bacillus subtilis* were determined.

Media and growth conditions

Optimum temperature is 30-35 °C and optimum pH for PHB synthesis is determined as 6.8. The strains were grown in nutrient broth culture medium contained (g L^{-1}) peptone, 2.5; NaCL, 2.5; yeastextract 1.0; beef extract 0.5.

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

The ratio 2% glucose, sucrose, mannitol, arabinose and starch were added into nutrient broth medium (100 mL in 250 mL Erlenmeyer flasks) as carbon sources. Peptone was taken out, and the ratio 2% L-cysteine, L-glycine, $(NH4)_2 SO_4$ and potassium nitrate were added as nitrogen sources. Cultures were incubated at 30-35°C with vigorous orbital shaking at 225-250 rpm. Also it was determinate PHB production of *Bacillus subtilis* at different incubation times (24,48,72 h).

Determination of PHB content

Determination of the amount of PHB was performed chemically. The samples were centrifuged for 2h at 3000 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 3000 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 40 °C for 30 min and 10 mL of concentrated sulfuric acid was added. They were heated at 100°C in a water bath for 10 min. After cooling, the amount of PHB was determined on a spectrophotometer, at wavelength of 235nm.

RESULTS

PHB synthesis in *Bacillus subtilis* isolated from soil was constantly reaching its peak level at 48th h.

Maximum PHB synthesis was detected when starch was used as the carbon source and maximum PHB synthesis in *Bacillus subtilis* was obtained when glycine was used as the nitrogen source (Table 1).

The highest PHB synthesis in *Bacillus subtilis* with starch as carbon source (251µg/mL) was observed when concentration of starch was 5% (Table 2).

And the highest PHB synthesis in *Bacillus subtilis* with glycine as nitrogen source (317µg/mL) was observed when concentration of glycine was 3% (Table 3).

These results of different carbon and nitrogen sources were obtained at optimum time (48th h) for *B.subtilis* .

Table 1. The production of PHB by *B.subtilis* isolates on media with different carbon and nitrogen sources at 48th h (concentration : 2%)

Carbon and Nitrogen Sources	Dry Cell Weight (µg/mL)	PHB (µg/mL)	Yield of PHB (%)
Glucose *	223	137	61
Sucrose*	172	39	23
Arabinose*	224	89	40
Mannitole*	166	103	62
Starch*	224	146	65
Glycine**	403	196	49
Amonium Sulfate**	409	151	37
Potassium Nitrate**	402	119	30
Cysteine**	458	81	18

*Carbon Sources, **Nitrogen Sources

Table 2. Effects of different concentrations of starch (1-8%) on PHB production in *B.subtilis* isolates at 48th h

Concentration of Starch (%)	Dry Cell Weight (µg/mL)	PHB (µg/mL)	Yield of PHB (%)
1	183	85	46
2	219	140	64
3	263	171	65
4	318	211	66
5	367	251	68
6	341	209	61
7	276	137	46
8	254	99	39

Table 3. Effects of different concentrations of glycine (1-8%) on PHB production in *B.subtilis* isolates at 48th h

Concentration of Glycine (%)	Dry Cell Weight (µg/mL)	PHB (µg/mL)	Yield of PHB (%)
1	373	124	33
2	415	199	49
3	575	317	55
4	555	286	51
5	498	237	47
6	456	186	40
7	413	113	27
8	373	93	24

DISCUSSION

The industrial-scale production of PHB has begun by using *Alcaligenes eutrophus* and *A. latus* [14]. PHB has been identified in more than 20 bacterial genera, including *Azotobacter*, *Bacillus*, *Beijerinckia*, *Alcaligenes*, *Pseudomonas*, *Rhizobium* and *Rhodospirillium* [15]. The aim of the

present work was selection of *Bacillus subtilis* PHB producers. In this study, the *Bacillus subtilis* from soil were isolated and identified, and their PHB production was determined under different conditions such as incubation time, carbon source and nitrogen source. At first, the effects of different carbon sources and culture conditions were optimized by one- factor- at- a- time method. The first effects of various carbon sources (glucose, sucrose, arabinose, mannitol and starch) were studied. Also the various nitrogen sources (glycine, cysteine, ammonium sulphate and potassium nitrate) were studied on PHB production by *Bacillus subtilis* from soil were isolated. Although some studies report that the incubation time for PHB synthesis is 45 h, the others reported that the peak levels of PHB synthesis are at 24th, 48th, 72nd and 120th h[16,17] . In this study, production of PHB by *B. subtilis* was detected between 24h and 72h in nutrient broth medium. It was determinated that the PHB yield of the both strains increased between 24h and 48h and decreased between 48h and 72h with 2% carbon sources. 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 PHB was being produced and consumed[18,19]. Maximum PHB synthesis (23.6623 µg/ml) was found in *B. subtilis* when mannitol was used as the carbon source[20.] Yüksekdað *et al.,* reported that the highest PHB synthesis was found in *B. subtilis* 25 strain and *B. megaterium* 12 strains when glucose was used as the carbon source[17] . 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 (21). But in our study maximum PHB synthesis was detected when starch was used as the carbon source at 48th h with concentration 5%. One of the objectives of this study was to determine the effects of nitrogen sources on PHB accumulation; we found that the maximum PHB synthesis in *Bacillus subtilis* isolated from soil was obtained when glycine was used as the nitrogen source at 48th h with concentration 3%. Yüksekdað et al. similarly reported with our study[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[22] . The highest amount of cell dry weight could be obtained in *B.subtilis* grown in starch (5%) and on the other hands in glycine (3%).

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REFERENCES

1. Barnard, G.N., Sanders, J.K.M. The Poly β-hydroxybutyrate granules in in-vivo, *J Biol Chem*., 1989; **264**: 3286-3291.
2. Anderson, A.J., Dawes, E. Occurrence, metabolic role and industrial uses of bacterial polyhydroxyalkanolates .*Microbial. Rev*, 1, Vol., 1990; **54**: 450-472.
3. Page, W.J. Production of poly-beta-hydroxybutyrate by Azotobacter vinelandii UWD in media containing sugars and complex nitrogen sources. *Appl. Microbial and Biotechnol*. 1992 ; **38** : 117-121.
4. Hanzlikova, A., Jandera, A., Kunc, F. Formation of poly-3-hydroxybutyrates by a soil microbial community in the soil.*Folia Microbiologica.* 1985; **30**: 58-64.
5. Poirier, Y., Nawrath, C., Somerville, C. Production of polyhydroxyalkanoates, a family of Biodegradable plastics andelastomers, in bacterial and plant. *Biotechnol.,* 1995; **13**: 142-150.
7. Lee, S.Y. Bacterial polyhydroxyalkanoates. *Biotechnol. Bioeng.,* 1996; **49**: 1-14.
6. Cain, R.B. Microbial degradation of synthetic polymers. In: Frey et al. (eds) Microbial Control of Pollution. 48th Symposium of the Society for general microbiology at University of Cardiff., 1992; 293-338.
8. Lee, B., Pometto , A.L., Fratzke, A., Bailey, T.B. Biodegradation of degradable plastic polyethylene by *Phanerochaete* and *Streptomyces* species. *Appl. Environ. Microbiol.,* 1991; **57**: 678-685.
9. Rivard, C., Moens, L., Roberts, K., Brigham, J., Kelley, S. Starch esters as biodegradable plastics: Effects of ester group chain length and degree of substitution on anaerobic biodegradation. *Enzyme and Microbial Tech.*, 1995; **17**: 848-852.
10. Witt, U., Muller, R.J., Deckwer, W.D. Biodegradation behaviour and material properties of aliphatic/aromatic polyesters of commercial importance. *J. Environ. Polymer. Degrad*., 1997;

l5: 81-89.

11. Muller, R.J., Kleeberg, I., Deckwer, W.D. Biodegradation of polyesters containing aromatic constituents. *J. Biotechnol.*, 2001; **86**: 87-95.
12. Bichler, C., Drittler, R., Langowski, H.C., Starnecker, A., Utz, H. Biologisch abbaubare Verpackungsmaterialien-Die Losun des Mullproblems ? *Bioengineering*., 1993; **19**: 9-17.
13. Amass, W., Amass, A., Tighe, B. A review of biodegradable polymers: use, current developments in the synthesis and characterization of biodegradable polyesters, blends of biodegradable polymers and recent advances in biodegradable studies. *Polymer Int.,* 1998; **47**: 89-144.
14. Hiramitsu, M., Koyama, N., Doi, Y. Production of poly (3-hydroxybutyrate-Co-4-hydroxybutyrate) by *Alcaligenes latus*. Biotechnol. Letters., 1993; **15**: 461-464.
15. Kato, N., Konishi, H., Shimao, M., Sakazawa, C. Production of 3hydroxybutyric acid trimer by Bacillus megaterium B-124. *J. Ferm. And Bioeng.* 1992; **73**(3): 246-247.
16. Yüksekdað, Z.N., Aslým, B., Beyatlý, Y., Mercan, N. Effect of carbon and nitrogen sources and incubation times on poly-beta-hydroxybutyrate (PHB) synthesis by *Bacillus subtilis* 25 and *Bacillus megaterium* 12. *Afr J* Biotechnol., 2004; **3**(1): 63-66, 2004.
17. Klütterman, K., Tauchert, .H, Kleber, H.P. Synthesis of poly-β-hydroxy-butyrate by *Agrobacterium radiobacter* after growth an D-Carnitine. *Acta Biotech.,*2002; **22**: 261-269.
18. Hori, K., Kaneko, M., Tanji. Y., Xing, X., Unnu, H. Construction of self-disruptive *Bacillus megaterium* in response to substrate exhaustion for polyhydroxybutyrate production. *Appl Microbiol Biotechnol.,*2002; **59** (2-3): 211-216.
19. Benoit, T.G., Wilson, G.R., Baygh, C.L. Fermentation during growth and sporulation of *Bacillus thuringiensis* HD-1. *Lett. in Appl. Microbiol*., 1990; **10**: 15-18.
20. Nam, D.H., Ryu, D.D.Y. Relationship between butirosin biosynthesis and sporulation in *Bacillus circulans*. Antimicrob. *Agents and Chemotherapy*., 1985; **27**: 789-801.
21. Tamdogan, N. Investigation of Poly-â-Hydroxybutyrate (PHB) Production by *Bacillus subtilis* ATCC 6633 Under Different Conditions. Kafkas. *UniV. Vet. Fak. Derg* ., 2011; 173-176.
22. Mercan, N., Aslým, B., Yüksekdað, Z.N., Beyatlý, Y. Production of poly-β-hydroxybutyrate (PHB) by some *Rhizobium* bacteria. *Turk. J. Biol*.,2002; **26**: 215-219.