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Production of poly-β-hydroxybutyrate (PHB) and differentiation of putative *Bacillus* mutant strains by SDS-PAGE of total cell protein

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In this study, the putative mutant strains of *Bacillus megaterium* Y6, *B. subtilis* K8, *B. sphaericus* X3 and *B. firmus* G2 were studied for their poly-β-hydroxybutyrate (PHB) production capacities. Mutations were induced by using UV light, acriflavin and 5-bromourasil. Total cell proteins were extracted from 59 strains and compared using SDS-PAGE. For each strain, percentage yield of PHB according to cell dry weight was determined in a range of 1.46-63.45%. PHB production of 8 mutant strains were found to increase in comparison with parental strains. However, no increase in PHB production of mutant strains of *B. sphaericus* X3 was found. It was also determined that the protein profiles of the mutant strains with high PHB yield generally differed from the protein profiles of parental strains.

Key words: *Bacillus*, poly-β-hydroxybutyrate, PHB, total cell protein.

INTRODUCTION

The observation of Findlay and White (1983) that a strain of *Bacillus megaterium* accumulates a polymer called poly-beta-hydroxybutyrate (PHB) under nutrient stress led to investigations of the role of these polymers in the physiology of bacteria. The polymer was thought to be a simple polyester of beta-hydroxybutyrate monomers. A wide variety of prokaryotic organisms have been shown to accumulate this polymer, including numerous heterotrophic and autotrophic aerobic bacteria, photosynthetic anaerobic bacteria, gliding bacteria, *Actinomycetes* spp., cyanobacteria and recently, an anaerobic, fatty acid-oxidizing, gram-negative bacterium (Anderson and Dawes, 1990).

PHB is a biodegradable thermoplastic which can be extracted from a wide range of bacteria. The polymer which provides a reserve of carbon and energy, accumulates as intracellular granules. Reusch and Sadoff (1983) have shown that PHB is an important molecule on cytoplasm and cell walls. *Bacillus* species have been shown to accumulate PHB during the sporulation of bacterial growth. The PHB production capacities of bacteria have been investigated for possible application in industry (Lee, 1996; Hanzlikova et al., 1985; Nickerson et al., 1981; Lach et al., 1990). During the 1970's, in the aftermath of the first oil crisis, that the British chemical giant, Imperial Chemical Industries (ICI) began investigating the polymer-forming properties of bacteria. *Alcaligenes lotus* and *A. eutrophus* are presently utilized by ICI to produce a PHB-PHV copolymer under the trade name "Biopol" (Fiechter, 1990).

The presence of PHB has also been used in bacterial taxonomy for classification and identification (Dave et al., 1996). SDS-Page, based on total protein profiles, is used in bacterial taxonomy to the levels of the species and subspecies (Qhobela et al. 1991, Costas, 1990). Therefore, it can used to distinguish between mutants. The present study attempts to obtain mutant strains of *Bacillus* with high PHB yield and then compare the PHB production capacities with the total protein profiles.

MATERIALS AND METHODS

Reference organisms

B. megaterium Y6, *B. subtilis* K8, *B. sphaericus* X3 and *B. firmus* G2 strains were obtained from the stock collection of Biotechnology Laboratory at Gazi University. They were cultured in nutrient broth at 30°C for 24 h on a rotary shaker (100 rpm).

Mutant isolation

Acriflavin (80-100 μg ml^{-1}) and 5-bromourasil (80-180 μg ml^{-1}) were employed as chemical mutagens, while UV light (254 nm) was use as physical mutagen. In mutant isolations, concentrations which

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Table 1. PHB accumulation of parental and possible mutant *Bacillus* strains.

Strain	PHB ($g.l^{-1}$)	Yields* %
***Bacillus megaterium* Y6 (parental)**	**0.21 ± 0.02**	**48.13**
Bacillus megaterium A4	0.13 ± 0.06	63.02
Bacillus megaterium A13	0.09 ± 0.02	63.45
Bacillus megaterium A16	0.07 ± 0.00	54.62
***Bacillus subtilis* K8 (parental)**	**0.13 ± 0.01**	**32.50**
Bacillus subtilis B3	0.10 ± 0.04	33.71
Bacillus subtilis B4	0.09 ± 0.01	41.86
Bacillus subtilis B8	0.12 ± 0.00	61.32
***Bacillus firmus* G2 (parental)**	**0.10 ± 0.01**	**15.62**
Bacillus firmus D7	0.06 ± 0.01	34.12
Bacillus firmus D11	0.03 ± 0.01	17.37

*Calculated based on dry cell weight.

showed 99.99% lethality ratio were used. The possible mutants were isolated on nutrient agar with 1% Nile blue solution. Bacterial colonies then were stained by the Nile blue and examined under ultraviolet light (235 nm). PHB-positive colonies appeared orange wherease PHB negative colonies were white (Ostle and Holt, 1982).

Determination of PHB

Determination of the amount of PHB was performed chemically. Bacteria were grown on nutrient broth at 30°C for 48 h on a shaker. Suspensions of cultures were centrifuged at 6000 x g for 45 min. Then the pellets were suspended in 5 ml of sterile water and homogenized, using ultrasonic treatment (2 min). To 2 ml of the cell suspension, 2 ml of 2 N HCl was added and heated to boiling temperature for 2 h in a water bath and the tubes were centrifuged at 6000 x g for 20 min. To obtain precipitate, 5 ml of chloroform was added, and the tubes were left overnight at 28°C on a shaker at 150 rpm. Then the contents of the test tubes were centrifuged at 6000 x g for 20 min, extracted with 0.1 ml of chloroform, and was dried at 40°C. 5 ml of concentrated sulfuric acid was added, and the mixture was heated at 100°C in a water bath for 20 min. After cooling to 25°C, the amount of PHB was determined spectrophotometrically at 235 nm (Bonartseva and Myschkina, 1985; Kuniko et al., 1988).

SDS-PAGE of total cell proteins

Cell-wall proteins were extracted as described in the method of Kishore et al. (1996). Electrophoresis of protein extracts was carried out using the discontinuous buffer system of Laemmli (1970). Gels were stained with Comassie Blue R250. Relative distances and similarities of the mutant strains to the parental strain were determined by using SDS-PAGE gel banding profiles and analyzed with SPSS 9.0 (for Windows) (Wolf and Rijini, 1993).

RESULTS AND DISCUSSION

Petroleum derived plastics are widely used in our daily lives, but they cause environmental pollution because they are persistent for hundred of years. Because of this, biodegradable polymer production (microbial termoplastics) has gained importance. Furthermore the continuous depletion of petroleum sources has placed more emphasis on the need for biodegradable microbial plastics. PHB is an important raw material for microbial plastics. Today, most research efforts in this field concentrate on the isolation of PHB producing microorganisms from different sources and improvement of PHB production abilities of microorganisms.

The present study was under taken by applying various mutagens to PHB-producing *B. sphaericus* X3 (36.36%), *B. subtilis* K8 (32.50%), *B. megaterium* Y6 (48.13%) and *B. firmus* G2 (15.62%) with the aim of obtaining mutants with higher PHB percentage yield. Dave et al. (1996) has reported 70 percent PHB of dry cell weight in optimum culture conditions for *Bacillus* sp. IPCB-403, while Findlay and White (1983) showed presence of PHB in *B. megaterium* using chromatographic method. Chen et al. (1991) also studied D(-)-3-hydroxyalcanoate in 11 different *Bacillus* sp. and found PHB consisting 50 percent of cell dry weight of the bacteria. In addition these investigators reported that PHB content changed depending on growth culture conditions. Our study showed PHB percentage yield of parental strains in some cases similar to and in others different from those reported by other workers. This may be due to different growth and culture conditions.

Eight of the 59 putative mutant strains obtained this study were found to show significantly higher PHB yield percentages (Table 1). Other strains showed PHB yields equal to or less than the parent strains. Other researchers have also reported PHB production changes in *Bacillus* mutant strains (Lach et al., 1990). Therefore mutations are considered of having the potential to change bacterial PHB content. In this study, a yield of 63.45% was found in one mutant strain (*B. megaterium* A13) while two others (*B. subtilis* B8, *B. firmus* D7) have

increased yield to about double that of parental strain. Today, some of the *A. eutrophus* strains used for commercial PHB production have cell dry weight of approximately 96% PHB (Fiechter, 1990). Researchers have been focusing on increasing PHB percentage yields of other bacteria to this level using mutations.

Many researchers reported SDS-PAGE of total cell proteins as a powerful tool for the taxonomical discrimination of a great number of strains (Pot et al., 1992; Eaglesham et al., 1987). We have also employed total cell protein profile analysis (SDS-PAGE) of our parental and putative mutant strains to determine distances/similarities. The results are depicted in Table 2 as distance-similarity percent of mutant total protein profiles with parental proteins of *B. megaterium* Y6*, B. sphaericus* X3*, B. subtilis* K8 and *B. firmus* G2. In general, we found mutant strain protein profiles to closely resemble those of parental strains.

Table 2. Similarities and distances of mutant strains from parental strains.

Strains No	Similarities %	Distances %
Bacillus megaterium A4	78	22
Bacillus megaterium A13	40	60
Bacillus megaterium A16	86	14
Bacillus subtilis B3	88	12
Bacillus subtilis B4	93	7
Bacillus subtilis B8	67	33
Bacillus firmus D7	33	67
Bacillus firmus D11	73	27

In a similar experiment, Basha and Ulaganathan (2002) applied the chemical mutagen, nitrosoguanidine to *Bacillus* sp. BC121 and the probable mutant strain obtained *Bacillus* sp. BC121M protein extract to showed a missing parental 25 kD band when compared using SDS-PAGE. In addition they showed disappearance of a certain previously present band. Saxena et al. (2002), also obtained probable UV tolerant mutant from *B. thuringiensis* subsp. *kurstaki* and used total protein SDS-PAGE method to determine the differences in banding. They reported loss of 71 kD band in the mutant strain.

In this study, it was determined that protein profiles of the mutant strains with high PHB yield was generally different from the protein profiles of parental strains. Here we have shown that it is possible to increase PHB yield by using mutagenic treatments. Furthermore, it is also possible to determine the effect of mutation using the protein profiles.

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