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NAZİME MERCAN

BELMA ASLIM

ZEHRA NUR YÜKSEKDAĞ

YAVUZ BEYATLI

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Production of Poly-β-Hydroxybutyrate (PHB) by Some *Rhizobium* Bacteria

Nazime MERCAN
Pamukkale University, Faculty of Science and Arts, Department of Biology, Denizli - TURKEY
Belma ASLIM, Z. Nur YÜKSEKDAĞ, Yavuz BEYATLI
Gazi University, Faculty of Science and Arts, Department of Biology, Teknikokullar, Ankara - TURKEY

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Abstract: In this study, the production of Poly-β-hydroxybutyrate (PHB) was determined in 1 *Rhizobium japonicum*, 6 *Rhizobium cicer*, 8 *Rhizobium* spp. and *Bradyrhizobium japonicum* USDA 110. The content of according to dry cell weight was determined to be 1.38-40.0%. In our study, *Rhizobium* spp. 2426, which produced the highest percentage yield of PHB, and *Rhizobium* spp. 640, which produced the intermediate percentage yield of PHB, were first selected among all the strains, and then the effect of different carbon and nitrogen sources on PHB production in these strains was tested. While the strains produced less PHB in yeast extract mannitol (YEM) broth media with different carbon and nitrogen sources, the highest level of PHB accumulation was observed in the media with L-cysteine and glycine. In this YEM medium with L-cysteine and glycine the percentage of PHB yield of *Rhizobium* spp. 640 was respectively determined to be 13.40% and 56.67%, while in the same nitrogen sources this percentage in *Rhizobium* spp. 2426 was determined to be 70% and 61.43%. The *Rhizobium* spp. 2426 strain capable of PHB accumulation was investigated in YEM with L-cystein at different incubation times (between 24 h and 120 h). The best PHB production and percentage yield of this strain was determined. The PHB production was 0.285 g/l and the percentage yield was 74.03% after 48 h. After this time there was a decrease in PHB yield.

Key Words: Poly-β-hydroxybutyrate, Biopolymer, *Rhizobium*, Different carbon and nitrogen sources

Bazı *Rhizobium* Bakterilerin Poli-β-Hidroksibütirat (PHB) Üretimleri

Özet: Bu çalışmada, 1 adet *Rhizobium japonicum*, 6 adet *Rhizobium cicer*, 8 adet *Rhizobium* spp. ve *Bradyrhizobium japonicum* USDA 110 suşunda PHB üretimi tespit edilmiştir. Suşların PHB içerikleri 0.01-0.5 g/l ve PHB verimleri de hücre kuru ağırlığına gore %1.36-40.0 arasında bulunmuştur. Çalışmamızda, suşlar arasından PHB üretimi en yüksek olan *Rhizobium* spp. 2426 ile orta verimliliğe sahip olan *Rhizobium* spp. 640 suşları seçilerek, farklı karbon ve azot kaynaklarının PHB üretimine etkisi test edilmiştir. Suşlar farklı karbon ve azot kaynağı içeren YEM sıvı besiyerinde düşük miktarda PHB üretirken, yüksek PHB üretimi L-Sistein ve Glisin içeren besi ortamında elde edilmiştir. Bu besi ortamında (L-Sistein ve Glisin) %PHB verimi *Rhizobium* spp. 640 suşunun sırasıyla %13.40 ve %56.67 olarak tespit edilirken, aynı azot kaynaklarında bu oran *Rhizobium* spp. 2426 suşunda sırasıyla %70.0 ve %61.43 olarak tespit edilmiştir. *Rhizobium* spp. 2426'nın, L-Sisteinli YEM sıvı besiyerinde farklı zamanlardaki PHB üretme yeteneği araştırılmıştır. Bu suşun en iyi PHB üretim ve yüzde verimi 48. saatte belirlenmiş, PHB üretimi 0.285 g/l ve yüzde verimi ise %74.03 olarak tespit edilmiştir. Bu saatten sonra PHB üretiminde düşüş olmuştur.

Anahtar Sözcükler: *Rhizobium*, PHB, farklı karbon ve azot kaynağı, biyopolimer

Introduction

Plastic materials have become an integral part of contemporary life because of their many desirable properties including durability and resistance to degradation. These nondegradable plastics accumulate in the environment at a rate of millions of ton per year. Recently, problems concerning the global environment and solid waste management have created much interest in the development of biodegradable plastics that still retain the desired physical and chemical properties of conventional synthetic plastics (1). Poly-β-hydroxybutyrate (PHB) is the best known polyhydroxyalkanoate (PHA). It is generally accepted that microorganisms isolated from a natural environment poor in nutrient sources (from soil or spring water) exhibit a higher survival ability than those living in the alimentary tract of higher organisms (2). It is now well recognized that this lipid inclusion is accumulated by many bacteria as they enter the stationary phase of growth to be used later as an internal reserve of carbon

and energy (3,4). Under limited nitrogen and in the presence of an abundant source of carbon, some bacteria can accumulate up to 60-80% of their weight as PHB (1). *Alcaligenes eutrophus* is the most widely used organism for the production of PHB because it is easy to grow, it accumulates large amounts of PHB (up to 80% of dry cell weight) in a simple medium, and its physiology and biochemistry leading to PHB synthesis are best understood (5).

In this study, we investigated the abilities of some *Rhizobium* species to produce PHB in various culture conditions. By selecting the strain producing the highest PHB for use in industry, the appropriate growing time and the appropriate source of nitrogen and carbon were tested for further PHB yields.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used in this study obtained from Prof. Dr. Kamuran Ayhan (Ankara University, Faculty of Agriculture) are listed in Table 1. Each liter of the yeast extract mannitol (YEM) broth culture medium used for PHB production contained 10 g mannitol, 0.5 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, 2.5 g tryptone, 2.5 g peptone and 2.5 g yeast extract (6). The pH was adjusted to 7.0 with HCl. Fermentations were carried out in 250–ml Erlenmeyer flasks containing 100 ml of culture medium. The temperature was maintained at 30 ºC, and the agitation was maintained at 100 rpm. The cultures were inoculated with a 4% (v/v) inoculum.

Determination of PHB

Determination of the amount of PHB was performed chemically. Bacteria were grown on YEM broth at 30 ºC for 48 h on a shaker. Suspensions of cultures were centrifuged at 6000 xg 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 we added 2 ml of 2 N HCl and heated it at boiling temperature for 2 h in a water bath; then the tubes were centrifuged at 6000 xg for 20 min. To obtain precipitate we added 5 ml of chloroform. The test tubes were left overnight at 28 ºC on a shaker at 150 rpm. Then the contents of the test tubes were centrifuged at 6000 xg for 20 min, 0.1 ml of chloroform extract was dried at 40 ºC and 5 ml of concentrated sulfuric acid was added. They were heated at 100 ºC in a water bath for 20 min. After cooling to 25 ºC, the amount of PHB was determined on a spectrophotometer, wavelength 235nm (7). The correlation between production of PHB and dry cell weight was determined by Spearman's test (8).

The effect of production of PHB in different carbon and nitrogen sources

After mannitol in YEM medium broth was taken out, the ratio 1% glucose, sucrose and arabinose were added to the medium as carbon sources. Peptone and tryptone were taken out, and the ratio 1% L-cysteine, L-glycine, DL-tryptophan, protease peptone and potassium nitrate were added as nitrogen sources. Nitrogen and carbon sources were sterilized by Millipore filter with a por size of 0.45 mm.

Results and Discussion

PHB is a carbon storage polymer widely distributed among prokaryotes including *Rhizobium*, Brady*rhizobium* and nodule bacteroides (9). In recent years, PHB and other PHAs have been considered commercially important because of their possible use as biodegradable thermoplastics (4).

Table 1 shows the content of PHB in *Rhizobium* strains.The amount of PHB in strains was 0.01-0.5 g/l, and the percentage of PHB in these cells was between 1.38 and 40.0% of dry cell weight. While the PHB productivity percentage in *Rhizobium* spp. 2426 was the highest (40.0%), the lowest PHB productivity was found in *Rhizobium* spp. 3173 (1.38%). The relationship between the dry cell weight of 16 *Rhizobium* species and PHB production was tested and found to be r = 0.541. After comparing our results with a statistical table (r = 0.541 > 0.4265) it was found that a significant relationship existed between dry cell weight and PHB production.

Many nitrogen-fixing microorganisms synthesize PHB. According to Tombolini and Nuti (10), the content of this polymer in rhizobia ranges from 30 to 55% of dry cell weight. Bonartseva et al. (11) 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*

Table 1. PHB content of some *Rhizobium* species on YEM medium.

Strains	Dry Cell Weight (g/l)	[a]PHB (g/l)	[b]Yield of PHB (%)
Rhizobium spp 3173	4.35 ± 2.84	0.06 ± 0.02	1.38
Rhizobium spp 1aa2	0.13 ± 0.03	0.01 ±0.00	7.69
Rhizobium japonicum 620	0.69 ± 0.19	0.04 ± 0.02	5.79
Rhizobium cicer N2	1.76 ± 0.72	0.50 ± 0.07	28.41
Rhizobium cicer 7aa2	0.17 ± 0.00	0.05 ± 0.01	29.41
Rhizobium spp K36	0.81 ± 0.25	0.02 ± 0.01	2.47
Rhizobium cicer Y16	2.08 ± 0.05	0.07 ± 0.05	3.37
Rhizobium cicer 2aa1	0.26 ± 0.02	0.08 ± 0.03	30.77
Bradyrhizobium japonicum USDA 110	0.43 ± 0.20	0.06 ± 0.05	13.95
Rhizobium cicer 45	3.96 ± 0.17	0.29 ± 0.07	7.32
Rhizobium spp. G49	0.36 ± 0.02	0.02 ± 0.01	5.56
Rhizobium spp 1402	0.15 ± 0.02	0.04 ± 0.01	26.66
Rhizobium cicer 13a	2.20 ± 0.90	0.12 ± 0.09	5.45
Rhizobium spp. 2357	0.58 ± 0.25	0.04 ± 0.01	6.90
Rhizobium spp. 2426	0.20 ± 0.01	0.08 ± 0.05	40.0
Rhizobium spp. 640	0.11 ± 0.01	0.01 ± 0.00	9.09

[a] Determined cell dry weight.
[b] According to cell dry weight.

Table 2. The production of PHB of the *Rhizobium* spp. 2426 strain on media with different carbon and nitrogen sources.

Carbon and Nitrogen Sources	Dry Cell Weight (g/l)	[a]PHB (g/l)	[b]Yield of PHB (%)
Glucose	0.22 ± 0.10	0.026 ± 0.02	11.82
Sucrose	0.23 ± 0.04	0.049 ± 0.01	21.30
Arabinose	1.32 ± 0.10	0.072 ± 0.02	5.45
L-Cysteine	0.21 ± 0.11	0.147 ± 0.02	70.0
L-Glycine	0.07 ± 0.00	0.043 ± 0.01	61.43
DL-Tryptophan	0.67 ± 0.16	0.085 ± 0.01	12.69
Protease Peptone	1.07 ± 0.44	0.039 ± 0.01	3.64
Potassium Nitrate	0.31 ± 0.01	0.054 ± 0.02	17.42
Control (YEM broth)	0.28 ± 0.11	0.060 ± 0.01	21.43

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. (12) 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 *Rhizobium* spp. 2426, which produced the maximum PHB percentage, and *Rhizobium* spp. 640, which produced the intermediate percentage, was determined in different carbon and nitrogen sources. While the percentage yield of PHB in these strains was lower with different carbon sources in YEM broth, the highest level of PHB accumulation was observed in the media with L-cysteine and glycine as nitrogen sources in *Rhizobium* spp. 2426 (respectively, 70.0% and 61.43%) and *Rhizobium* spp. 640 (respectively, 13.40% and 56.67%) (Tables 2 and 3).

Table 3. The production of PHB of *Rhizobium* spp. 640 strain on media with different carbon and nitrogen sources.

Carbon and Nitrogen Sources	Dry Cell Weight (g/l)	[a]PHB (g/l)	[b]Yield of PHB (%)
Glucose	0.41 ± 0.01	0.030 ± 0.01	7.32
Sucrose	1.48 ± 0.06	0.125 ± 0.02	8.45
Arabinose	1.61 ± 0.53	0.025 ± 0.02	1.55
L-Cysteine	1.06 ± 0.70	0.142 ± 0.01	13.40
L-Glycine	0.09 ± 0.02	0.051 ± 0.01	56.67
DL-Tryptophan	0.67 ± 0.01	0.069 ± 0.00	10.30
Protease Peptone	0.74 ± 0.18	0.053 ± 0.01	7.16
Potassium Nitrate	0.65 ± 0.05	0.034 ± 0.01	5.23
Control (YEM broth)	0.22 ± 0.06	0.023 ± 0.01	10.45

Table 4. The content of PHB and dry cell weight of the 2426 strain in YEM with L-cysteine medium.

[c]Time (h)	600 nm (OD)	Dry cell weight (g/l)	[a]PHB (g/l)	[b]Yield of PHB (%)
24	0.73	0.420 ± 0.010	0.159 ± 0.005	37.86
48	1.8	0.385 ± 0.035	0.285 ± 0.001	74.03
72	1.83	0.385 ± 0.005	0.139 ± 0.007	36.10
96	2.0	0.465 ± 0.005	0.101 ± 0.038	21.72
120	2.05	0.925 ± 0.235	0.203 ± 0.024	21.95

[a] Determined dry cell weight.
[b] According to dry cell weight.
[c] Between 24 h and 120 h of culture.

According to Bonartseva et al. (11), Yushkova et al. observed maximal PHB accumulation in *Rhizobium* lupini after growth with mannitol and glutamate. In our study, production of the 2426 strain was detected between 24 h and 120 h in YEM medium with L-cysteine (Table 4).

As a result of this study, in YEM medium with L-cysteine, and at the end of 48 h, *Rhizobium* spp. 2426 produced very satisfying results in terms of PHB yield (74.03%, 0.285 g/l). After 48 h there was a decrease in PHB yield and an increase in the viscosity of the medium at 72, 96 and 120 h). After 48 h, the unfavorable conditions of the medium caused the decrease in PHB yield, because the increase in medium viscosity accompanying EPS production, resulting in oxygen transfer limitation, caused the decrease in PHB synthesis. The yield decreased to 36.10% at 72 h and 21.72% at 120 h. Although dry cell weight increased at 120 h, the decrease of PHB indicates that the bacteria used PHB as a source of carbon and nitrogen, caussing an unsuitable condition due to inadequate nitrogen and carbon sources in the medium.

On the basis of data obtained in the present work, *Rhizobium* spp. 2426 strain capable of PHB accumulation up to 70% of dry cell weight was selected, and it may be employed for industrial production after the optimization of the conditions of PHB synthesis. In YEM with L-cysteine medium after 48 h, the PHB yield obtained in *Rhizobium* spp. 2426 was determined to be higher than figures from the literature.

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