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SHORT COMMUNICATION

Investigation of Poly-β-Hydroxybutyrate (PHB) Production by *Bacillus subtilis* ATCC 6633 Under Different Conditions [1]

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Summary

The aim of our study was to assess the PHB productivity of *Bacillus subtilis* ATCC 6633 strain under different conditions. PHB amount was measured by UV-VIS spectrophotometer at 235 nm. The effects of the incubation time, temperature, pH, carbon sources, nitrogen sources and the rate of carbon/nitrogen ratio on PHB production rate were investigated. The results demonstrated that the highest PHB production was obtained after 24 h of incubation (10.4981 µg/ml), at 7.0 of pH (10.4981 µg/ml), with D-mannitole (23.6623 µg/ml) as carbon source, L-glycine (14.6217 µg/ml) as nitrogen source, C/N ratio of 2.5 (3.2481 µg/ml) and with a temperature of 30°C in culture media (10.4981 µg/ml).

Keywords: *Bacillus subtilis, Carbon and nitrogen sources, Temperature, pH, Poly-β-hydroxybutyric asid (PHB), Bioplastic production*

Farklı Koşullar Altında *Bacillus subtilis* ATCC 6633 Tarafından Poli-β-Hidroksibütirat (PHB) Üretiminin Araştırılması

Özet

Çalışmamızın amacı *Bacillus subtilis* ATCC 6633 suşunun farklı koşullar altında PHB verimliliğinin tayin edilmesidir. PHB miktarı 235 nm'ye ayarlanan UV spektrofotometre kullanılarak belirlenmiştir. İnkübasyon süresi, sıcaklık, pH, farklı karbon kaynakları, azot kaynakları ve karbon/azot oranının PHB sentezine etkisi araştırılmıştır. Çalışmanın sonuçları *Bacillus subtilis* ATCC 6633 suşunda en yüksek PHB birikiminin, 24 saat inkübasyonda (10.4981 µg/ml), pH; 7'de (10.4981 µg/ml), karbon kaynağı olarak D-mannitol içeren besiyerinde (23.6623 µg/ml), azot kaynağı olarak L-glisin içeren besiyerinde (14.6217 µg/ml), karbon/azot oranı 2.5 olan besiyerinde (3.2481 µg/ml) ve 30°C sıcaklıktaki kültür ortamında (10.4981 µg/ml) elde edildiğini göstermiştir.

Anahtar sözcükler: *Bacillus subtilis, Karbon ve azot kaynakları, Sıcaklık, pH, Poly-β-hidroksibutirat (PHB), Biyoplastik üretimi*

INTRODUCTION

There is an increasing awareness about the impact of non-biodegradable plastics on the environment with the increase both in population and industrialization [1]. PHB and other copolymers are synthesized under limited culture conditions and are used as carbon and energy reserve [2].

PHB is accumulated as intracellular granules by many prokaryotic organisms (including *Bacillus* spp., *Azotobacter* spp., *Pseudomonas* spp.) as they enter the stationary phase of growth, to be used later as an internal reserve of carbon and energy [3,4].

The aim of the present study is to assess the ability of *Bacillus subtilis* ATCC 6633 strain to accumulate PHB under different conditions such as incubation time, temperature, pH, carbon source, nitrogen source and the rate of carbon/nitrogen ratio.

MATERIAL and METHODS

Bacillus subtilis ATCC 6633 strain was obtained from the culture collection of Ege University (Izmir - Turkey).

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Determination of PHB amount was performed with the chemical method, and the samples were placed in sterile bags and stored at 4°C. Two milliliters of activated bacteria were inoculated in 98 ml of liquid nutrient broth and incubated 30°C for 48 h with vigorous orbital shaking at 225-250 rpm. Suspensions of cultures were centrifuged at 6.000 rpm for 20 min. Then the pellets were suspended in 5 ml of sterile distilled water and homogenized with using ultrasonic treatment for 10 min at 50 MHz. Five milliliters of 2N HCl was added to 5 ml of cell suspension and it was heated at 95°C for 2h in a water bath and tubes were centrifuged at 6.000 rpm for 20 min. Supernatant was discarded, 5 ml of chloroform was added on pellets and the tubes were left overnight at 28°C on a shaker at 150 rpm. Then the contents of the tubes were centrifuged at 6.000 rpm for 20 min, extracted with 0.1 ml of chloroform, and were dried at 40°C for 15 min. Five milliliters of pure sulphuric acid (95-98%) was added. The tubes were heated at 95°C in a water bath for 20 min. After cooling to 25°C, the amount of PHB was determined on a UV-VIS spectrophotometer (Perkin Elmer UV-VIS Spectrophotometer) at 235 nm [5]. Reproduction and density of bacteria in culture medium were established according to McFarland standard [6].

In this study, we used 2 g/ml carbon source (glucose, sucrose, D-mannitole, arabinose) and 0.2 g/ml nitrogen source (protease peptone, L-glycine, L-cysteine, ammonium sulfate and potassium nitrate). Glucose and glycine were used to assess the rate of carbon/nitrogen ratio on PHB synthesis. Reproduction of *Bacillus subtilis* ATCC 6633 strain and the cultural parameters effect on PHB accumulation were studied.

RESULTS

PHB synthesis in *Bacillus subtilis* ATCC 6633 strain was constantly reaching its peak level (10.4981 µg/ml) at 24th h *(Table 1)*. These results were obtained at optimum temperature (30°C) and optimum pH (7.0) for *Bacillus subtilis* ATCC 6633.

Optimum temperature was found to be 30°C and optimum pH for PHB synthesis was determined as 7.0 (10.4981 µg/ml), where both PHB synthesis (10.4981 µg/ml) and amount of the cells were determined at their highest values *(Table 2)*.

Maximum PHB synthesis (23.6623 µg/ml) was found in *B. subtilis* ATCC 6633 when mannitol was used as the carbon source and maximum PHB synthesis (14.6217 µg/ml) in *Bacillus subtilis* ATCC 6633 was obtained when glycine was used as the nitrogen source *(Table 3)*. These results of different carbon and nitrogen sources were obtained at optimum temperature (30°C) and optimum pH (7.0) for *Bacillus subtilis* ATCC 6633.

The highest PHB synthesis in *Bacillus subtilis* ATCC 6633 strain (3.2481 µg/ml) was observed when the ratio of C/N was 2.5 *(Table 4)*.

Table 1. *Time dependent cell reproduction, PHB production, and productivity in Bacillus subtilis ATCC 6633*

Tablo 1. *Bacillus subtilis ATCC 6633'de zamana bağlı olarak üreme, PHB üretimi ve verimlilik*

Time (Hour)	PHB (µg/ml)	Cell Count (× 10^6)	Yield of PHB (µg PHB/Total Cell × 10^{-9}
2	0.0175	55	0.319
4	2.1309	1151	1.851
6	6.7527	1906	3.542
8	7.6014	2115	3.594
10	9.7416	2139	4.554
12	9.0590	2333	3.882
14	9.1143	2443	3.730
16	10.1845	2402	4.240
18	8.3671	2262	3.698
20	10.2583	2482	4.133
22	9.4741	2432	3.895
24	10.4981	2489	4.217
26	9.7324	2506	3.883
28	9.2804	2466	3.763
30	9.9446	2517	3.950

Table 2. *Effects of different incubation temperature and pH on PHB production, and productivity in Bacillus subtilis ATCC 6633*

Tablo 2. *Bacillus subtilis ATCC 6633'de sıcaklığa ve pH'a bağlı olarak üreme, PHB üretimi ve verimlilik*

Temperature and pH	PHB (µg/ml)	Cell Count (× 10^6)	Yield of PHB (µg PHB / Total Cell × 10^{-9})
20°C	0.6088	1192	0.510
25°C	3.4963	1412	2.476
30°C	10.4981	2489	4.217
35°C	6.9372	1757	3.948
40°C	0.9778	1751	0.558
pH: 5.0	2.7306	1648	1.656
pH: 6.0	2.3431	1733	1.352
pH: 7.0	10.4981	2489	4.217
pH: 8.0	2.9704	1374	2.161
pH: 9.0	4.2250	1329	3.179

DISCUSSION

PHB synthesis in *Bacillus subtilis* ATCC 6633 strain was constantly reaching its peak level (10.4981 µg/ml) at 24th h *(Table 1)*. The decrease in PHB level after a definite time despite the increase in cell number may be due to insufficient carbon and nitrogen levels in the environment owing to the uptake of PHB by the bacteria [7]. 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 [7-10].

Table 3. *Effects of different carbon and nitrogen sources on PHB production, and productivity in Bacillus subtilis ATCC 6633*

Tablo 3. *Bacillus subtilis ATCC 6633'de farklı karbon ve azot kaynaklarının kullanımına bağlı olarak üreme, phb üretimi ve verimlilik*

Carbon and Nitrogen Sources	PHB (µg/ml)	Cell Count ($\times 10^6$)	Yield of PHB (µg PHB / Total Cell $\times 10^{-9}$)
Sucrose*	8.7638	3124	2.805
Glucose*	5.2952	2644	2.002
D-Mannitole*	23.6623	2544	9.301
Arabinose*	7.3708	71	0.0001
Protease Peptone**	3.0904	2128	1.452
Glycine**	14.6217	1903	7.683
Amonium Sulfate**	0.1014	2196	0.046
Potassium Nitrate**	11.4022	2155	5.291
Cysteine**	0	114	0

* Carbon Sources (2 g/ml), ** Nitrogen Sources (0.2 g/ml)

Table 4. *Effects of different C/N ratios on PHB production, and productivity in Bacillus subtilis ATCC 6633*

Tablo 4. *Bacillus subtilis ATCC 6633'de farklı karbon/azot oranına bağlı olarak üreme, phb üretimi ve verimlilik*

Carbon/Nitrogen Ratios	PHB (µg/ml)	Cell Count ($\times 10^6$)	Yield of PHB (µg PHB / Total Cell $\times 10^{-9}$)
0.5	1.0027	2260	0.4367
1.0	0.8773	2227	0.3939
1.5	2.3238	2173	1.0693
2.0	1.2915	2188	0.5902
2.5	3.2481	2132	1.5230

Required results could not be obtained at the highest and the lowest incubation temperatures *(Table 2)*, probably due to the low enzyme activity at these temperatures. Optimum temperature was found to be 30°C, where both PHB synthesis (10.4981 µg/ml) and amount of the cells were determined at their highest values. At higher and lower temperatures, PHB synthesis and amount of the cells were decreased. These results show similarity with other studies about incubation temperatures [11,12].

In the present study, optimum pH for PHB synthesis was determined as 7.0 (10.4981 µg/ml) *(Table 2)*. Different incubation times, incubation temperatures, carbon and nitrogen sources, and C/N ratios were investigated at pH 7.0. However, in a previous study, starting pH value for PHB synthesis was found as 6.5 [11].

Maximum PHB synthesis (23.6623 µg/ml) was found in *B. subtilis* ATCC 6633 when mannitol was used as the carbon source *(Table 3)*. The PHB content was lower when glucose, sucrose and arabinose were used as the carbon sources. Yet, mannitol is generally not preferred in industrial applications as a carbon sources due to its higher cost. Therefore, PHB synthesis was assessed in a cheaper and easily found carbon source that contained glucose culture medium instead of mannitol as a carbon source. Yüksekdağ et al.[7] similarly reported that the highest PHB synthesis was found in *B. subtilis* 25 strain and *B. megaterium* 12 strain when glucose was used as the carbon source. The production of PHB in *B. megaterium* was studied by Hori et al.[13] and found the highest value of PHB contents when glucose was used.

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 (14.6217 µg/ml) in *Bacillus subtilis* ATCC 6633 was obtained when glycine was used as the nitrogen source *(Table 3)*. Mercan et al.[14] also reported that PHB accumulation was high in two strains of *Rhizobium* sp. when L- cysteine and glycine were used as the nitrogen source.

Different carbon/nitrogen ratio (0.5, 1.0, 1.5, 2.0 and 2.5) on PHB synthesis was all assessed in this study. The highest PHB synthesis in *B. subtilis* ATCC 6633 strain (3.2481 µg/ml) was observed when the ratio of C/N was 2.5 *(Table 4)*. The necessity and the importance of suitable carbon/nitrogen ratio were determined by various researchers to raise the productivity of polymer products [11]. The increasing amount of carbon was found to be related to diminished cell number, while the rate of PHB synthesis was elevated. Thus, it may be suggested that when glucose was used as the carbon source, the nitrogen source, glycine, may act as the growth-limiting factor.

In conclusion, an important raw material, for various industries the bacteria-derived PHB, was investigated in this study. For this purpose, *Bacillus subtilis* ATCC 6633 strain was used and PHB synthesis on cultural parameters and bacteria production were investigated. On the basis of data obtained from this study, *B. subtilis* ATCC 6633 strain may be used for industrial production after the optimization of the PHB synthesis conditions.

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