Turkish Journal of Biology

Volume 27 | Number 1 Article 5

1-1-2003

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Turk J Biol
27 (2003) 37-42

Determination of Poly-β-hydroxybutyrate (PHB) Production by Some Mesophilic and Thermophilic Lactic Acid Bacteria

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Received: 05.03.2002

Abstract: Accumulated poly-β-hydroxybutyrate (PHB) was determined in lactic acid bacteria belonging to the genera *Lactobacillus*, *Lactococcus* and *Streptococcus*. Lactobacilli were grown in MRS broth and the others were grown in Elliker broth medium. Cell biomass was obtained by centrifugation. The cell walls were lysed with sodium hypochlorite. Poly-β-hydroxybutyrate was extracted using chloroform in a Soxhlet system. Then it was converted to crotonic acid using sulfuric acid and the amount of crotonic acid was measured spectrophotometrically. The yield of poly-β-hydroxybutyrate % of cell dry weight of *Lactobacillus* species was 0.52-25.55%. The values for *Lactococcus* and *Streptococcus* species were 0.61-14.81 and 1.20-13.69%, respectively. It was observed that one of the *Streptococci* and six of *Lactococcus* species did not produce poly-β-hydroxybutyrate. Generally, *Lactobacillus* species produced more poly-β-hydroxybutyrate than the other tested bacteria did and no significant correlation was observed between poly-β-hydroxybutyrate production and cell density of the cultures. Additionally, no significant difference was observed between mesophilic and thermophilic lactic acid bacteria according to PHB yield.

Key Words: *Lactobacillus*, *Lactococcus*, *Streptococcus*, poly-β-hydroxybutyrate production

Bazı Mesofilik ve Termofilik Laktik Asit Bakterilerinin Poli-β-Hidroksibütirat (PHB) Üretim Yeteneklerinin İncelenmesi

Özet: Bu çalışmada, *Lactobacillus*, *Lactococcus* ve *Streptococcus* cinslerine dahil olan bazı laktik asit bakterilerinin PHB üretimleri tespit edilmiştir. Laktobasiller MRS besiyerinde ve diğerleri Elliker Broth besiyerinde geliştirilmiştir. Hücre süspansiyonu santrifüj ile elde edilmiş ve hücre huvarları sodyum hipoklorit ile parçalanmıştır. Poli-β-Hidroksibütirat sokslet sisteminde kloroform kullanılarak ekstrakte edilmiştir. PHB, sülfürik asitle krotonik asite dönüştürülerek krotonik asit miktarı spektrofotometrik olarak (235 nm) ölçülmüştür. Bakteri türlerinin PHB verimi (hücre kuru ağırlığına göre) *Lactobacillus* türlerinde % 0.52-25.55, *Lactococcus* ve *Streptococcus* türlerinde % 0.61-14.81 ve % 1.20-13.69 olarak saptanmıştır. *Streptococcus* 'un bir, *Lactococcus*'un altı suşunun PHB üretmediği gözlenmiştir. Genellikle, *Lactobacillus* türlerinin diğer bakteri türlerine kıyasla daha fazla PHB ürettikleri gözlenmiştir. PHB üretimi ve kültürlerin hücre yoğunluğu arasında önemli bir ilişki gözlenmemiştir. Ayrıca, PHB üretimine göre mesofilik ve termofilik laktik asit bakterileri arasında önemli bir fark belirlenmemiştir.

Anahtar Sözcükler: *Lactobacillus*, *Lactococcus*, *Streptococcus*, PHB

Introduction

Poly-β-hydroxybutyrate (PHB) is a carbon-energy storage material that is accumulated intracellularly in a variety of microorganisms under controlled concentrations of nutrients such as nitrogen, oxygen and/or mineral ions (1). It was first isolated by Lemoigne about 50 years ago (2). It is known that PHB is synthesized from acetyl-CoA by three sequential reactions catalyzed by β-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase, and PHB synthase (2-4).

PHB can be isolated from microorganisms quite readily. Perhaps because of the increased awareness of depleting fossil fuels as precursors for the synthesis of high polymers and the general upsurge in biotechnology, it was realized that this thermoplastic polyester could be put to commercial use (5).

PHB is considered an ideal storage material because it is highly reduced and water insoluble; therefore, no osmotic pressure effects are induced inside the cell. PHB is also a family of biodegradable and biocompatible polymers having many interesting properties, such as piezoelectricity and nonlinear optical activity, which may be useful in some high-value applications (6).

The purpose of this study was to determine PHB production by different genera of lactic acid bacteria.

Materials and Methods

Bacterial strains and culture media: The species of *Lactobacillus*, *Lactococcus* and *Streptococcus* used in this study were isolated from different kefirs and cheese products were obtained from the culture collection of the Biotechnology Laboratory of Gazi University, Department of Biology Faculty of Arts and Science, in Turkey. The strains used, their sources and optimal growth temperature (7) are listed in Tables 1 and 2.

Mesophilic and thermophilic *Lactobacillus* strains were grown twice in MRS broth, *Lactococcus* and *Streptococcus* strains were grown twice in Elliker broth medium at the appropriate temperature (Tables 1 and 2) for 24 h (8,9).

Table 1. Bacterial strains and their sources.

Strain	Identification no	Source	Optimal growth temperature (ºC)*
Lactobacillus acidophilus	Z1L	Biotechnol. Lab. [a]	45 ± 1
Lactobacillus helveticus	Z2L	Biotechnol. Lab. [a]	45 ± 1
Lactobacillus helveticus	Z5L	Biotechnol. Lab. [a]	45 ± 1
Lactobacillus bulgaricus	Z8L	Biotechnol. Lab. [a]	40 ± 1
Lactobacillus bulgaricus	Z14L	Biotechnol. Lab. [a]	40 ± 1
Lactobacillus bulgaricus	Z18L	Biotechnol. Lab. [a]	40 ± 1
Lactobacillus casei	Z3L	Biotechnol. Lab. [a]	37 ± 1
Lactobacillus casei	Z4L	Biotechnol. Lab. [a]	37 ± 1
Lactobacillus casei	Z6L	Biotechnol. Lab. [a]	37 ± 1
Lactobacillus casei	Z7L	Biotechnol. Lab. [a]	37 ± 1
Lactobacillus casei	Lc1	Dept. Microbiol. [b]	37 ± 1
Lactobacillus casei	Lc11	Dept. Microbiol. [b]	37 ± 1
Lactobacillus lactis	Z9L	Biotechnol. Lab. [a]	30 ± 1
Lactobacillus lactis	Z10L	Biotechnol. Lab. [a]	30 ± 1
Lactobacillus lactis	Z16L	Biotechnol. Lab. [a]	30 ± 1
Lactobacillus lactis	Z17L	Biotechnol. Lab. [a]	30 ± 1
Lactobacillus lactis	Z19L	Biotechnol. Lab. [a]	30 ± 1
Lactobacillus lactis	Z21L	Biotechnol. Lab. [a]	30 ± 1
Lactobacillus plantarum	Z11L	Biotechnol. Lab. [a]	30 ± 1
Lactobacillus plantarum	Z12L	Biotechnol. Lab. [a]	30 ± 1
Lactobacillus plantarum	Z15L	Biotechnol. Lab. [a]	30 ± 1
Lactobacillus plantarum	Lp6	Dept. Microbiol. [b]	30 ± 1
Lactobacillus plantarum	Lp9	Dept. Microbiol. [b]	30 ± 1
Lactobacillus plantarum	Lp10	Dept. Microbiol. [b]	30 ± 1
Lactobacillus plantarum	Lp11	Dept. Microbiol. [b]	30 ± 1
Lactobacillus plantarum	Lp12	Dept. Microbiol. [b]	30 ± 1
Lactobacillus plantarum	Lp13	Dept. Microbiol. [b]	30 ± 1
Lactobacillus plantarum	Lp15	Dept. Microbiol. [b]	30 ± 1
Lactobacillus plantarum	Lp16	Dept. Microbiol. [b]	30 ± 1
Lactobacillus plantarum	Lp17	Dept. Microbiol. [b]	30 ± 1
Lactobacillus plantarum	Lp18	Dept. Microbiol. [b]	30 ± 1
Lactobacillus plantarum	Lp19	Dept. Microbiol. [b]	30 ± 1
Lactobacillus plantarum	Lp22	Dept. Microbiol. [b]	30 ± 1
Lactobacillus brevis	Z13L	Biotechnol. Lab. [a]	30 ± 1
Lactobacillus brevis	Z20L	Biotechnol. Lab. [a]	30 ± 1

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* Determined according to Bergey's Manual of Determinative Bacteriology.

Table 2. Bacterial strains and their sources.

Strain	Identification no	Source	Optimal growth temperature (°C)*
Lactococcus lactis	Z1S	Biotechnol. Lab. [a]	30 ± 1
Lactococcus lactis	Z2S	Biotechnol. Lab. [a]	30 ± 1
Lactococcus lactis	Z3S	Biotechnol. Lab. [a]	30 ± 1
Lactococcus lactis	Z13S	Biotechnol. Lab. [a]	30 ± 1
Lactococcus lactis	SL1	Dept. Microbiol. [b]	30 ± 1
Lactococcus lactis	SL3	Dept. Microbiol. [b]	30 ± 1
Lactococcus lactis	SL7	Dept. Microbiol. [b]	30 ± 1
Lactococcus lactis	SL15	Dept. Microbiol. [b]	30 ± 1
Lactococcus lactis	SL32	Dept. Microbiol. [b]	30 ± 1
Lactococcus lactis	SL33	Dept. Microbiol. [b]	30 ± 1
Lactococcus lactis	SL34	Dept. Microbiol. [b]	30 ± 1
Lactococcus lactis	SL35	Dept. Microbiol. [b]	30 ± 1
Lactococcus lactis	SL37	Dept. Microbiol. [b]	30 ± 1
Lactococcus lactis	SL46	Dept. Microbiol. [b]	30 ± 1
Lactococcus lactis	SL47	Dept. Microbiol. [b]	30 ± 1
Lactococcus cremoris	Z6S	Biotechnol. Lab. [a]	30 ± 1
Lactococcus cremoris	Z9S	Biotechnol. Lab. [a]	30 ± 1
Lactococcus cremoris	Z10S	Biotechnol. Lab. [a]	30 ± 1
Lactococcus cremoris	Z11S	Biotechnol. Lab. [a]	30 ± 1
Lactococcus cremoris	Z14S	Biotechnol. Lab. [a]	30 ± 1
Lactococcus cremoris	Z16S	Biotechnol. Lab. [a]	30 ± 1
Lactococcus cremoris	Z17S	Biotechnol. Lab. [a]	30 ± 1
Lactococcus cremoris	Z18S	Biotechnol. Lab. [a]	30 ± 1
Lactococcus cremoris	Z19S	Biotechnol. Lab. [a]	30 ± 1
Lactococcus cremoris	Z20S	Biotechnol. Lab. [a]	30 ± 1
Lactococcus cremoris	Z21S	Biotechnol. Lab. [a]	30 ± 1
Streptococcus durans	Z7S	Biotechnol. Lab. [a]	30 ± 1
Streptococcus durans	Z8S	Biotechnol. Lab. [a]	30 ± 1
Streptococcus durans	Z15S	Biotechnol. Lab. [a]	30 ± 1
Streptococcus thermophilus	Z4S	Biotechnol. Lab. [a]	40 ± 1
Streptococcus thermophilus	Z5S	Biotechnol. Lab. [a]	40 ± 1
Streptococcus thermophilus	Z12S	Biotechnol. Lab. [a]	40 ± 1

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* Determined according to Bergey's Manual of Determinative Bacteriology.

Lactic acid cultures were maintained in glycerol-skim milk at –10 °C. Prior to use they were subcultured twice in MRS or Elliker broth medium.

Analytical procedures: Lactic cultures grown were inoculated at 2% (v/v) in sterile MRS or Elliker broth medium. Cultures were incubated for 48 h at the optimal growth temperature (Tables 1 and 2). Bacterial cells were centrifuged at 6000 rpm for 15 min and were dried at 40 ± 1 °C for 24 h. The dry weight of the pellets was determined. Bacterial cell walls were lysed by adding sodium hypochloride, mixing and incubating at 60 °C for 1 h. Supernatant was obtained by centrifugation and transferred to a Soxhlet system. Cell lipids and other molecules were treated with 5 ml 96% ethanol and acetoin. PHB was extracted by hot chloroform (adding 10 ml chloroform in a water bath). Then chloroform was evaporated to obtain PHB crystals. By adding 10 ml of 98% sulfuric acid at 60 °C for 1 h, PHB crystals were converted into crotonic acid. The absorbance of the

solution was measured at 235 nm in a UV spectrophotometer against a sulfuric acid blank. The amount of PHB per gram dry weight of bacterial cells was determined using a standard curve of PHB (10,11). All experiments were repeated three or sometimes four times; the average values of this procedure are given in the related tables (3-4).

Statistical analysis: The correlation between bacterial cell dry weight (g l^{-1}) and PHB production (g l^{-1}) of the bacteria was determined according to Spearman's ρ correlation coefficient test.

The ρ value was estimated with the formula

$$\rho = 1 - \frac{6\sum(x_i - y_i)^2}{n(n^2 - 1)}$$

and explained using Conver's table (12).

Results and Discussion

The cell dry weight and yield of PHB produced by *Lactobacillus* species are shown in Table 3. *Lactobacillus* species were grown in MRS broth medium for 48 h. The

Table 3. PHB production by some *Lactobacillus* species.

Bacteria	Cell dry weight (g l^{-1})	PHB[a] (g l^{-1})	%PHB[b] (%)
Lactobacillus acidophilus Z1L	1.34 ± 0.04	0.01 ± 0.001	0.52
Lactobacillus helveticus Z2L	1.39 ± 0.13	0.06 ± 0.01	4.32
Lactobacillus helveticus Z5L	0.61 ± 0.07	0.01 ± 0.001	2.13
Lactobacillus bulgaricus Z8L	1.26 ± 0.04	0.02 ± 0.01	1.59
Lactobacillus bulgaricus Z14L	0.94 ± 0.02	0.02 ± 0.004	1.70
Lactobacillus bulgaricus Z18L	1.30 ± 0.08	0.13 ± 0.01	10.00
Lactobacillus casei Z3L	1.61 ± 0.01	0.07 ± 0.02	4.34
Lactobacillus casei Z4L	1.95 ± 0.01	0.11 ± 0.02	5.64
Lactobacillus casei Z6L	1.59 ± 0.03	0.20 ± 0.01	12.57
Lactobacillus casei Z7L	1.16 ± 0.12	0.07 ± 0.01	6.03
Lactobacillus casei Lc1	3.74 ± 0.76	0.05 ± 0.01	1.34
Lactobacillus casei Lc11	7.71 ± 1.11	0.16 ± 0.01	2.15
Lactobacillus lactis Z9L	1.27 ± 0.23	0.05 ± 0.01	3.93
Lactobacillus lactis Z10L	1.13 ± 0.09	0.02 ± 0.003	1.42
Lactobacillus lactis Z16L	0.90 ± 0.04	0.23 ± 0.05	25.55
Lactobacillus lactis Z17L	1.01 ± 0.07	0.14 ± 0.00	13.86
Lactobacillus lactis Z19L	0.96 ± 0.10	0.08 ± 0.01	8.33
Lactobacillus lactis Z21L	1.37 ± 0.03	0.13 ± 0.01	9.49
Lactobacillus plantarum Z11L	1.09 ± 0.05	0.14 ± 0.00	12.84
Lactobacillus plantarum Z12L	1.16 ± 0.06	0.01 ± 0.004	1.03
Lactobacillus plantarum Z15L	1.07 ± 0.03	0.11 ± 0.01	10.28
Lactobacillus plantarum Lp6	4.26 ± 0.48	0.07 ± 0.002	1.71
Lactobacillus plantarum Lp9	6.62 ± 0.48	0.07 ± 0.002	1.09
Lactobacillus plantarum Lp10	6.96 ± 0.54	0.08 ± 0.03	1.19
Lactobacillus plantarum Lp11	6.21 ± 0.84	0.09 ± 0.03	1.51
Lactobacillus plantarum Lp12	7.11 ± 0.58	0.10 ± 0.06	1.74
Lactobacillus plantarum Lp13	8.98 ± 1.39	0.06 ± 0.01	0.76
Lactobacillus plantarum Lp15	0.07 ± 0.56	0.06 ± 0.01	0.76
Lactobacillus plantarum Lp16	6.31 ± 0.83	0.05 ± 0.005	0.92
Lactobacillus plantarum Lp17	5.59 ± 1.22	0.05 ± 0.002	0.97
*Lactobacillus plantarum*Lp18	3.45 ± 0.41	0.05 ± 0.01	1.59
*Lactobacillus plantarum*Lp19	6.34 ± 0.12	0.08 ± 0.04	1.36
Lactobacillus plantarum Lp22	8.73 ± 0.19	0.10 ± 0.002	1.20
Lactobacillus brevis Z13L	1.36 ± 0.10	0.05 ± 0.01	3.67
Lactobacillus brevis Z20L	0.85 ± 0.03	0.04 ± 0.01	4.71

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

biomasses of the cultures were different (Table 3). *L. lactis* Z16L produced more PHB than the other *Lactobacillus* species did, while *L. acidophilus* Z1L produced less PHB than the other *Lactobacillus* species did. In one of the studies conducted by Aslım et al. (13), it was reported that PHB yields (%) accumulated in cells according to dry weight were also different: 13.8% for *L. plantarum* A, 7.2% for *L. plantarum* B, 29.4% for *L. brevis*, 17.1% for *L. acidophilus*, 29.0% for *L. casei*, 35.8% for *L. bulgaricus*, 19.1% for *L. bifidus* and 6.6% for *L. fermentum*.

Specifically, there was no relation between high cell density and the PHB content of *Lactobacillus* cultures. *L. casei* Z6L, *L. bulgaricus* Z18L, *L. lactis* Z16L, *L. lactis* Z17L, and *L. plantarum* Z15L produced more PHB than the other *Lactobacillus* strains did. The ρ value was calculated to be ρ = 0.172. This value was compared with the 0.05 level of the critical table value ρ = 0.150 < 0.305 (12). The results showed that there was no significant correlation between cell dry weight (g l^{-1}) and PHB amount (g l^{-1}).

The amounts of PHB produced by *Lactococcus* and *Streptococcus* are reported in Table 4. The highest yield of PHB accumulation in comparison to dry weight was obtained in *Lactococcus lactis* SL 33 (14.81%).

Table 4. PHB production by some *Lactococcus* and *Streptococcus* species.

Bacteria	Cell dry weight (g l^{-1})	PHB[a] (g l^{-1})	%PHB[b] (%)
Lactococcus lactis Z1S	0.99 ± 0.03	0.05 ± 0.01	4.54
Lactococcus lactis Z2S	1.10 ± 0.02	0.05 ± 0.01	4.09
Lactococcus lactis Z3S	1.12 ± 0.04	0.02 ± 0.01	1.79
Lactococcus lactis Z13S	0.50 ± 0.01	[c]	[c]
Lactococcus lactis SL1	0.59 ± 0.02	0.05 ± 0.01	8.81
Lactococcus lactis SL3	0.61 ± 0.10	0.07 ± 0.002	12.29
Lactococcus lactis SL7	0.53 ± 0.04	0.08 ± 0.005	15.14
Lactococcus lactis SL15	0.73 ± 0.01	0.07 ± 0.01	10.00
Lactococcus lactis SL32	0.73 ± 0.00	0.06 ± 0.003	8.36
Lactococcus lactis SL33	0.54 ± 0.08	0.08 ± 0.01	14.81
Lactococcus lactis SL34	0.72 ± 0.01	0.06 ± 0.01	8.33
Lactococcus lactis SL35	0.50 ± 0.03	0.06 ± 0.00	13.66
Lactococcus lactis SL37	0.62 ± 0.04	0.05 ± 0.01	9.35
Lactococcus lactis SL46	0.77 ± 0.02	0.05 ± 0.001	7.14
Lactococcus lactis SL47	0.55 ± 0.03	0.05 ± 0.004	10.18
Lactococcus cremoris Z6S	0.24 ± 0.00	[c]	[c]
Lactococcus cremoris Z9S	1.00 ± 0.04	0.05 ± 0.01	4.50
Lactococcus cremoris Z10S	0.63 ± 0.02	[c]	[c]
Lactococcus cremoris Z11S	0.51 ± 0.02	[c]	[c]
Lactococcus cremoris Z14S	1.26 ± 0.12	0.09 ± 0.01	6.75
Lactococcus cremoris Z16S	0.48 ± 0.08	0.01 ± 0.001	1.25
Lactococcus cremoris Z17S	0.66 ± 0.02	0.02 ± 0.01	2.88
Lactococcus cremoris Z18S	0.55 ± 0.01	[c]	[c]
Lactococcus cremoris Z19S	0.69 ± 0.03	[c]	[c]
Lactococcus cremoris Z20S	0.82 ± 0.00	0.01 ± 0.003	0.61
Lactococcus cremoris Z21S	0.86 ± 0.02	0.05 ± 0.01	5.23
Streptococcus durans Z7S	0.84 ± 0.00	0.12 ± 0.02	13.69
Streptococcus durans Z8S	0.74 ± 0.06	0.02 ± 0.01	3.24
Streptococcus durans Z15S	1.00 ± 0.02	0.01 ± 0.01	1.20
Streptococcus thermophilus Z4S	0.82 ± 0.02	0.04 ± 0.00	4.88
Streptococcus thermophilus Z5S	0.92 ± 0.00	0.06 ± 0.01	6.52
Streptococcus thermophilus Z12S	0.98 ± 0.01	[c]	[c]

[a] Determined at cell dry weight.
[b] According to cell dry weight.
[c] No PHB production.

Although *L. lactis* from thermophilic lactic acid bacteria had the highest PHB yield, no significant difference was observed between mesophilic and thermophilic lactic acid bacteria according to PHB yield.

Statistical analysis showed that there was no correlation between cell dry weight (g l^{-1}) and the PHB (g l^{-1}) content of the cultures.

In general, the amount of PHB produced by some *Lactococcus* species was higher than that produced by *Lactobacillus* and *Streptococcus* strains (Tables 3 and 4). In contrast, Aslim et al. (13) mentioned that the amount of PHB produced by some *Lactobacillus* species was higher than that produced by *Lactococcus*, *Pediococcus* and *Streptococcus* strains.

The ρ value between dry cell weight and PHB content of all bacteria listed in Tables 3 and 4 was calculated as ρ = 0.134. When this value was compared with the 0.05 level of the critical value, 0.134 < 0.336 (12), the results showed that at the 0.05 level there was no statistically significant correlation between cell dry weight and the PHB contents of the bacteria.

When compared to the values reported in the literature, the amount of PHB accumulated in lactic acid bacteria was generally lower than that accumulated by the soil bacteria *Ralstonia eutropha* (14), *Rhizobium* species (15) and *Bacillus cereus* UW85 (16).

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