Turk J Med Sci
30 (2000) 535-541
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Biosynthesis of Polyhydroxybutyrate and its Copolymers and Their Use in Controlled Drug Release

Received: May 5, 2000

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Abstract: Our aim was to prepare antibiotic loaded rods with biotechnologically produced biodegradable polymers and use them in the treatment of osteomyelitis by providing a high local dose of antibiotic at the infected site. For that purpose, first the production of PHB and its copolymers (P(3HB-*co*-3HV) and P(3HB-*co*-4HB)) by *Alcaligenes latus* and *Alcaligenes eutrophus* in the shake-flask cultures and in a fed-batch fermenter and their purification and characterization were performed. The polymers were then used in the preparation of the sulbactam-cefoperazone loaded rods. To predict the *in vivo* behavior of the controlled release system, the *in vitro* release kinetics of the rods were studied in PBS at 37°C. Release from 50 % w/w loaded P(3HB-*co*-3HV) and P(3HB-*co*-4HB) rods showed that the drug was completely released in less than 3 days. To retard the rate, dip coatings of these rods using the same polymer solution were done and the release profiles were obtained. After coating, cumulative release was about 70 % of its initial content at the end of 12 days. It was concluded that PHB and its copolymers may be a promising alternative to the materials of petrochemical origin in the treatment of osteomyelitis, due to being biodegradable, and eliminating the need for a second operation.

Key Words: polyhydroxyalkanoates, *Alcaligenes latus, Alcaligenes eutrophus,* osteomyelitis, controlled drug delivery

Introduction

Bone infections can be defined as the inflammation of bone and surrounding cortical tissue. Management of bone infections is difficult, often requiring multiple surgical operations and prolonged and sometimes repeated courses of antibiotic administration [1-6]. Conventional treatment using systemic antibiotics is expensive, toxic and prone to complications, and the eradication of microorganisms is very difficult with this approach.

Another method that is currently in use is the controlled antibiotic release to the infected site by antibiotic-impregnated polymethylmethacrylate (PMMA) beads. This method provides high local antibiotic concentration at the desired area but requires an additional surgical procedure since the non-biodegradable PMMA beads must be removed after they release their antibiotic content. This procedure, in addition to being uncomfortable for the patient, increases the chance of reinfection [1]. The disadvantages of additional surgery would be eliminated if the local delivery system were constructed from a biodegradable material.

Polyhydroxyalkanoates (PHAs) are naturally occurring polyesters produced as energy storage materials by many bacteria. Their general structural formula is shown in Figure 1. The most common representative is polyhydroxybutyrate (PHB) together with the copolymer containing hydroxyvalerate (PHBV). These microbial polyesters have unique physicochemical properties such as thermoplasticity, biodegradability, biocompatibility and piezoelectricity. Recently, it has become of industrial interest to evaluate these polyesters for a wide range of medical applications [7].

In this study, 2 microorganisms, namely *Alcaligenes eutrophus* and *Alcaligenes latus*, were used as producer organisms. Both organisms can use inexpensive carbon sources, which is important in industrial-scale production [8]. The organisms show differences in their growth and polymer production conditions but they were chosen because of their high polymer production capacity. The other criterium for their selection is the ease of the separation of the polymer from cells.

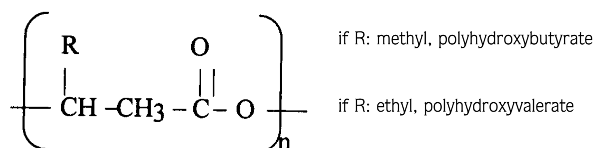


Figure 1. The General Structure of PHAs.

Materials and Methods

Materials

P(3HB-*co*-3HV) copolymers containing up to 11.3 % (mol) HV and P(3HB-*co*-4HB) copolymers containing up to 9.5 % (mol) 4-HB were obtained by cultivation of *A. eutrophus* and *A. latus* under controlled conditions. Sulperazone™ (consists of sulbactam and cefoperazone in 1:1 ratio), the selected wide spectrum antibiotic, was obtained from Pfizer (Turkey) and was used as received.

Methods

The Biosynthesis of the Polymers

The polymer production conditions differ for the 2 organisms. When *A. eutrophus* cells were used, the biosynthesis was realized in 2 steps. Firstly, the growth of the cells was promoted since a high number of cells was needed for high polymer yield. In this step, the cells were grown until the late logarithmic phase. The second step was the production step in which the polymer production and deposition was encouraged by nutrient deficiencies (i.e., nitrogen, phosphate, etc.). The grown cells were collected and transferred to the production medium. As the carbon source, fructose was used. Valerolactone and 4-hydroxybutyrate were added to the production medium as co-substrates for the production of copolymers P(3HB-*co*-3HV) and P(3HB-*co*-4HB), respectively.

Polymer production by *A. latus* cells was performed in a single step in contrast to *A. eutrophus* cells because *A. latus* is a growth-associated producer. Because of this characteristic, *A. latus* cells were selected for the biosynthesis of the polymers at the fed-batch reactor. The production was realized by the addition of the extra carbon source, sucrose, at the late logarithmic phase [9].

Shake-flask Studies

A. latus and *A. eutrophus* cells were cultivated in 250, 500 and 1000 mL media. The cultivation conditions, temperature 30°C and shaking speed 150-200 rpm, were kept constant. The cells were harvested after 48 and 72 h with *A. latus* and *A. eutrophus*, respectively.

Fed-batch Fermenter Studies

Fermentative production of polyhydroxybutyrate (PHB) and its copolymers was carried out in a 2-liter LH fermentation system using *A. latus* as the producer organism. Control of temperature, oxygen concentration, pH and foam were automatic. In the reactor studies, sucrose was added twice to the growth medium as the sole carbon source at the beginning of the fermentation and after nitrogen depletion. The operation conditions of different runs were tabulated (Table 1). The medium was inoculated with *A. latus* (40 mL per liter of fermentation medium) and 12 h later, determination of sugar, nitrogen and dry cell weight (DCW) were initiated (with 2 h intervals). Temperature (30°C) and agitation rate (500 rpm) were kept constant throughout the experiment.

Table 1. Operation Conditions of the Fermenter.

	RUN 1	RUN 2	RUN 3
Sucrose (g/L)	15 + 15	20 + 15	20 + 15
Nitrogen (g/L)	0.460	0.264	0.410
Oxygen (%)	20	20	30

The polymer synthesized during fermentation was monitored with the use of crotonic acid assay in 10 mL samples. An equal amount of sample was filtered (Whatman cellulose acetate filters, pore size: 0.45 µm), dried in an oven (50°C, 2 h) and weighed for DCW determination. Ammonium nitrogen and sucrose analyses were carried out with the filtrate. Ammonium nitrogen concentration was measured using a Spectroquant 14752 Ammonium kit (Merck AG, Germany). For sucrose determination, the sample was treated with glucose oxidase and peroxidase after acid hydrolysis and using o-dianisidine dye. H_2O_2 production was monitored at 530 nm by UV-Vis spectrophotometry.

Extraction and Purification of the Polymers

After the cells were collected, they were lyophilized. The dried cells were extracted for 6 h in chloroform to achieve the destruction of the cells and solubilization of polymer. Purification of the polymers was done by precipitation of the polymers in alcohol. The precipitated polymers were collected by filtration and dried at room temperature.

Preparation of Sulperazone Loaded Rods

Rod preparation was carried out as described elsewhere [10]. In brief, a fraction of the weighed polymer powder was dissolved in chloroform and poured into a mortar, and the rest of the polymer was mixed with antibiotic to yield a 1:1 w/w drug to polymer ratio (i.e., 50 % (w/w) loading). The paste formed was ground until it was homogeneous. This was then put into a glass mold to produce the final rod in the shape of a rectangular prism (20 x 0.3 x 0.3 cm^3). Following complete evaporation and solidification, antibiotic rods of the required dimension and weight (ca. 1.0 x 0.3 x 0.3 cm^3 and 90±10 mg) were cut and stored in sealed bags until use in further studies. In order to modify the release behavior, a set of these rods was dip-coated twice using the corresponding polymer solution (10% w/w in chloroform).

In vitro Release Studies

Release studies were carried out in previously autoclaved (121°C, 14 min) flasks containing sterile PBS solution (0.1M, pH: 7.4, 100 mL). The release behavior of sulperazone loaded uncoated and coated rods were investigated in a shaking waterbath (rate: 70 rpm) maintained at 37°C. At certain time intervals, aliquots were taken from the release medium and their UV absorbances were spectrophotometrically measured at λmax=224 nm. Release profiles of triplicate samples were constructed.

Results

Polymer Production

Shake-Flask Studies

It was observed that the carbon:co-substrate ratio played an important role in the polymer productivity. The compositions that yielded maximum productivity are given in Table 2. The highest result was obtained with *A. eutrophus* cells as 71.5 % polymer recovery based on dry cell weight. In that case, the primary carbon source was fructose (16 g/L) and the co-substrate was butyricacid lactone (4 g/L).

Fermenter Studies

The results of the fed-batch reactor are given in Table 3. The fermentation profile of Run 1 is given in Figure 2. The results show that nitrogen was depleted after 15 h and polymer production started at the 12th hour. Between 12 h and 21 h, the polymer production rate was maximum (0.0547 g PHB produced/ g DCW/ h), and after that point it started to slow down and a second stage, where the production rate was about 10 % of the first stage (0.0055 g PHB produced/ g DCW/ h), was reached.

Characterization of the Polymers

The composition and the molecular weight of the produced polymers were investigated and the results are summarized in Table 4.

Table 2. Polymer Production with Different Co-substrates.

Substrate (g/L)	Y I E L D			
	WCW (g/L)	DCW (g/L)	Polymer Yield (g/L)	Polymer Yield (% w/w)
F+4HBA (18+2) n:4	23.9 ± 1.7	8.3 ± 0.9	3.5 ± 0.7	42.6 ± 5.8
F+VL (18+2) n: 8	20.6 ± 4.5	7.2 ± 2.2	3.1 ± 1.6	41.2 ±12.1
S+VL (24+1) n: 3	17.2 ± 0.9	5.3 ± 0.4	1.8 ± 0.7	33.2 ±12.6
S+4HBA (24+1) n: 7	21.1 ± 4.2	7.2 ± 1.9	2.9 ± 1.3	39.1 ± 13.6

F: fructose, 4HBA:4-hydroxybutyrate, S: sucrose, VL: valerolactone
WCW: wet cell weight, DCW: dry cell weight

Table 3. The Results of Fed-Batch Reactor.

Run No.	YIELD		
	Dry Cell Weight (g/L)	Polymer Yield (g/L)	Polymer : DCW (%)
RUN 1	13.70	4.61	33.60
RUN 2	7.91	2.83	35.80
RUN 3	13.60	6.43	47.30

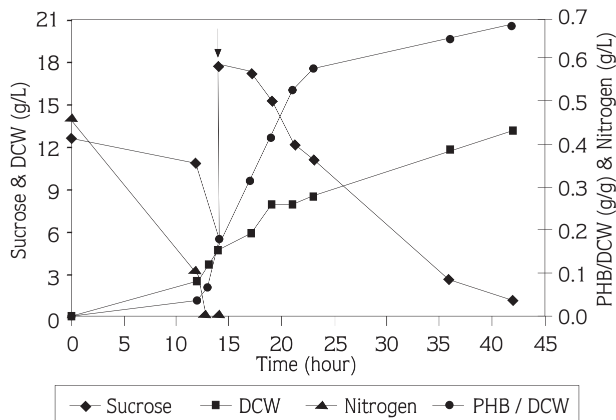


Figure 2. The Fermentation Profile of Run 1; Production of PHB by *A. latus* in Fed-Batch Fermenter.

Table 4. Polymer Properties.

Producer Organism	Carbon Source and Amount (g/L)		PHA Type and Composition (% mol)	Molecular Weight (daltons)
A. eutrophus	F	(10)	P(3-HB) 100.0	3.53×10^5
A. eutrophus	F + VL	(18+2)	P(3HV-*co*-3HB) 11.3 : 88.7	3.31×10^5
A. eutrophus	F + 4-HB	(18+2)	P(3HB-co-4HB) 90.5 : 9.5	2.91×10^5
A. latus	S	(15)	P(3-HB) 100.0	2.90×10^5
A. latus	S + VL	(24+1)	P(3-HB) 100.0	3.84×10^5
A. latus	S + 4-HB	(24+1)	P(3HB-*co*-3HV) 92.5 : 7.7	2.90×10^5

F: Fructose, S: Sucrose, 4HBA:4-hydroxybutyrate, VL: Valerolactone

In vitro Drug Release

The uncoated rods released their contents quite rapidly. Release of the drug is apparently controlled only by the solubility of the drug and the tortuosity of the paths within the rods. Uncoated *A. eutrophus* (3HB-*co*-3HV) rods (1:1 drug:polymer ratio) released all of their drug contents in 24 h (Figure 3). Similar results were obtained with the *A. latus* (3HB-*co*-4HB) rods. They released their complete drug contents in 3 days (Figure 4). When uncoated rods were examined under SEM before and after release (Figures 5a, b), it was observed that before release, the antibiotic crystals were close to the surface of the rods.

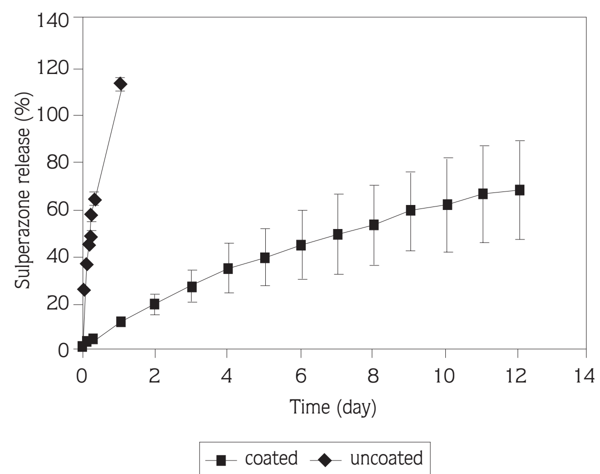


Figure 3. Drug Release From Uncoated and Coated *A. eutrophus* (3HB-*co*-3HV) rods.

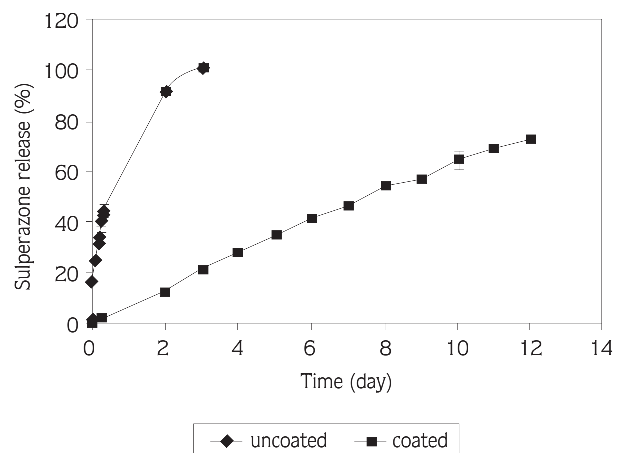


Figure 4. Drug Release From Uncoated and Coated *A. latus* (3HB-*co*-4HB) rods.

The dip-coating procedure yielded a coating of 10.6-10.9 mg/cm^2. Coating of the rods significantly reduced the burst effect and resulted in a much slower release of the antibiotic (Table 5).

Coated AE(3HB-*co*-3HV) samples released 70% of their initial drug content in about 2 weeks (Figure 3). The cumulative release versus time plot of the coated rods is quite linear and the release rate calculated directly from the slope was found to be 6.00 mg Sulperazone/day. The *A. latus* (3HB-*co*-4HB) counterpart released about 72% of the initial drug within 12 days (Figure 4) at a rate of 5.4 mg Sulperazone/day.

SEM micrographs of coated rods differed significantly from the uncoated ones. A uniform surface porosity can be seen on the coated rod surfaces (Figure 6a). After the release, the honeycomb structure loosened and the continuity was lost at several points (Figure 6b).

Discussion

In this study, biodegradable polyhydroxyalkanoate polyesters were produced using *A. eutrophus* and *A. latus*, which were extracted, and after purification, they were characterized. Sulperazone loaded rods were then prepared and their *in vitro* release behavior was investigated to predict their behavior in vivo.

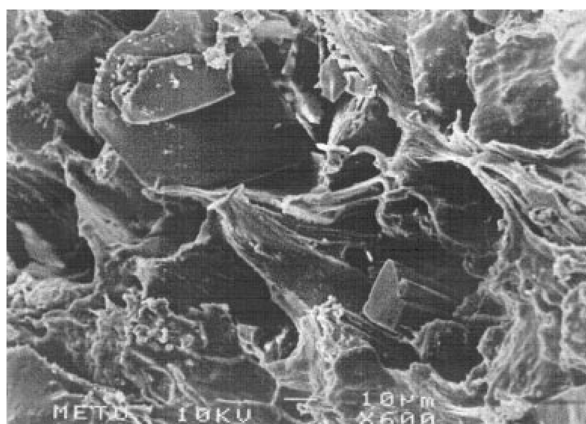
Polymer Production

In these preliminary studies, our aim was to determine the optimum growth and production

Table 5. Properties of the Antibiotic Loaded Rods.

Sample (m.o. / polymer) Coating	Weight of Rod (mg)	Sulperazone Content (mg)	Coat Weight (mg)	Percent Release (%) in Duration (day)
AE/(3HB-3HV) UC	85.5 ± 2.5	42.7 ± 1.3	-	113.5 ± 7.0 (1)
AE/(3HB-3HV) C	100.5 ± 25.2	50.2 ± 12.6	115.2 ± 29.8	69.8 ± 19.9 (12)
AL/(3HB-4HB) UC	92.0 ± 8.1	46.0 ± 4.0	-	100.8 ± 2.1 (3)
AL/(3HB-4HB) C	85.6 ± 0.1	42.8 ± 0.0	100.7 ± 1.1	72.9 ± 1.4 (12)

AE: *A. eutrophus*, AL: *A. latus*, C: coated, UC: uncoated



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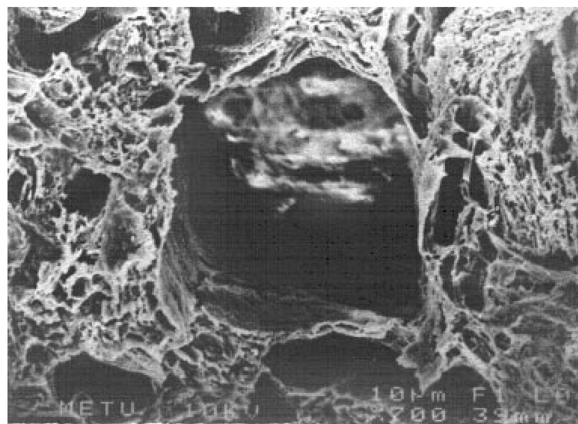

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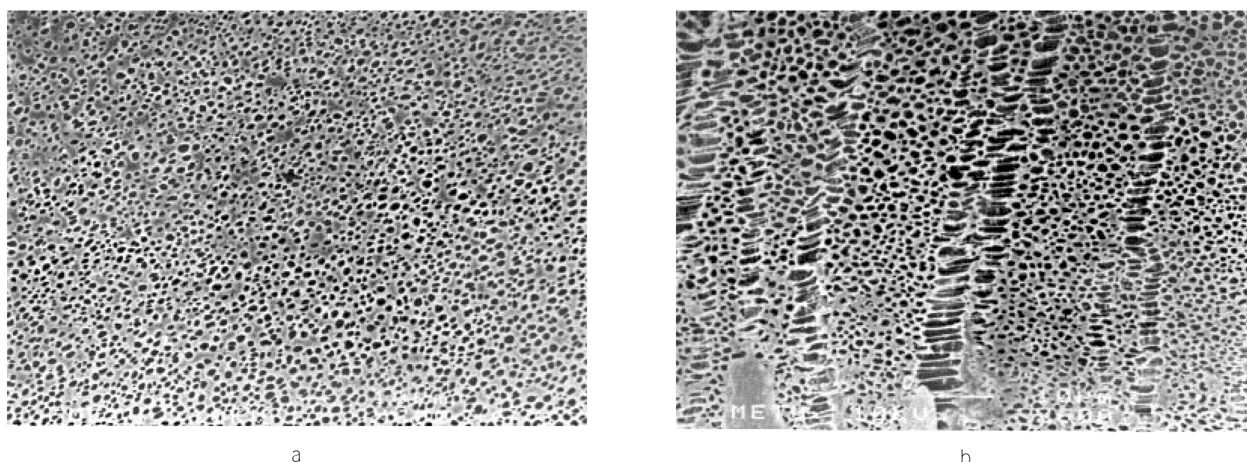
Figure 5. SEM Micrographs of Uncoated Rods Before (a) and After (b) Release.



a b

Figure 6. SEM Micrographs of Coated Rods Before (a) and After (b) Release.

conditions. It was observed that by increasing oxygenation, the time necessary for the whole process decreased, an important achievement for industrial production. Sucrose (30 g/L) was depleted in 42 h at 20 $\%O_2$ (Run 1), whereas when it was increased to 35 g/L and O_2 was adjusted to 30%, the complete utilization period could be reduced to 36 h (Run 3).

It can be seen that the initial nitrogen amount is also important. After nitrogen depletion, there was no significant cell growth, indicating that at low nitrogen concentrations the corresponding number of cells was also low, which in turn would reflect on the overall polymer yield. For example, when the initial nitrogen was 0.46 g/L, DCW was 13.7 g/L (Run 1) and when it was 0.26 g/L, the DCW was reduced to 7.91 g/L (Run 2). Therefore, even if the polymer to DCW ratio were high, there would not be much polymer to recover and this is not desirable.

An important finding, worth studying in detail, is the effect of O_2 concentration on polymer yield. The resultant DCWs in Runs 1 and 3 were quite close and furthermore, the C-source in Run 3 was slightly higher than in Run 1 (30 g/L vs 35 g/L). The polymer yield, however, was found to be ca. 1.5-fold higher in Run 3 than Run 1, which represents an increase higher than that expected from the difference in the amount of C-sources. This increase can be correlated with the increase in the O_2 supplied to the fermenter (from 20% to 30%, an increase of 50%). A possible explanation could be an increase in efficiency of the enzyme system that is involved in the synthesis of PHA from simple C-sources. The increase in O_2 supply creating an additional stress condition, and forcing the organism to deposit PHA at a much higher efficiency and rate could be offered as another explanation for this observation.

In vitro Release Studies

Two polymers were used in the *in vitro* release studies and it was seen that both the polymers had the same release profile. The release rate, however, was highly affected by the presence of the coat around the antibiotic loaded road.

High release rates exhibited by the uncoated PHA rods may be desired at the beginning of the implant-related osteomyelitis therapy because the efficiency of the antibiotic often depends on the maintenance of high drug concentrations at the site of infection. This is especially true for initial effect. The release behavior of the uncoated rods may provide the high drug concentrations required at the beginning of the therapy. The lower but more constant releases can be provided by the coated rods to maintain the asceptic conditions.

Acknowledgements

We would like to acknowledge the support of the NATO Science for Stability Programme through TU-POLYESTERS, and the Turkish State Planning Organization (Grant No: AFP.97.08.01.01). We also would like to acknowledge the contribution of Prof. E. Bayramlı (METU) who determined the compositions and the molecular weights.

References

1. Haas DW, McAndrew MP. Bacterial osteomyelitis in adults: Evolving considerations in diagnosis and treatment. The American Journal of Medicine Nov: 550-561, 1996.
2. Nakamura, S., Scandola, M. and Doi, Y. Microbial synthesis and characterization of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate). Macromolecules, 25: 4236-4240, 1992.
3. Embleton, J.K., Tighe, B.J. Polymers for biodegradable medical devices. X. Microencapsulation studies: control of poly-hydroxybutyrate-hydroxyvalerate microcapsules porosity via polycaprolactone blending. Journal of Microencapsulation, 10: 341-352, 1993.
4. Gebelein, C.G. (ed), Biotechnological Polymers: Medical, Pharmaceutical and Industrial Applications, Technomic Publications, USA, 1993.
5. Doi, Y., Microbial Polyesters, VCH Publishers Inc., USA, 1990
6. Chasin, M., Langer, R. (eds), Biodegradable Polymers as Drug Delivery Systems, Marcel Dekker Inc., USA, 1990
7. Lee SY. Bacterial polyhydroxyalkanoates. Biotechnology and Bioengineering, 49: 1-14, 1996.
8. Pouton CW, Akhtar S. Biosynthetic polyhydroxyalkanoates and their potential in drug delivery. Advanced Drug Delivery Reviews 18: 133-162, 1996.
9. Gürsel I, Alaeddinolu NG. Bacterial PHAs: Biosynthesis, screening and characterization. Biomedical Science and Technology (Eds.A. Hıncal and H.S. Kaş) Plenum Press, 1998, pp: 81-88.
10. Türesin F. *MSc Thesis*, Middle East Technical University, Ankara, 1998