The Study on Microbial Polymers: Pullulan and PHB

Alemzadeh, Iran*[+]

Department of Chemical and Petroleum Engineering, Sharif University of Technology,
P.O. Box 11155-9465 Tehran, I.R. IRAN

ABSTRACT: *Microbial cells are producers of natural polymers present in plant cells. Production of pullulan (an extracellular microbial polysaccharide) by Aureobasidium pullularia pullulans (P. pullulans) was studied under fermentation conditions, and kinetic parameters were determined. Pullulan formation obeyed a growth and non-growth associated term. PHB (polyhydroxybutyrates) an intracellular biopolymer production by Rastonia eutropha (Alcaligen eutrophus), R. eutropha was studied under different culture media, including synthetic and natural carbon sources. Molasses as a natural carbon source in the culture media presented high efficiency in cell and biopolymer accumulation.*

KEY WORDS: *Natural polymers, Pullulan, Polyhydroxybutyrate (PHB), Kinetic parameters.*

INTRODUCTION

Biopolymers produced by a wide variety of micro-organisms, are generally water soluble gums which have novel and unique physical properties. Because of their wide diversity in structure and physical characteristics these polysaccharides have found a wide range of applications in the food, pharmaceutical and other industries. Some of these applications include their use as emulsifiers, stabilizers, binders, gelling agents, coagulants, lubricants, film formers, thickening and suspending agents.

The biopolymers are rapidly emerging as a new and industrially important source of polymeric materials which are gradually becoming economically competitive with natural gums produced from marine algae and other plants. The potential use of genetically modified micro-organisms under controlled fermentation conditions may result in the production of new exopolysaccharides having novel superior properties which will open up new areas of industrial applications and thus increase their demand.

Biopolymers, which serve different functions in a microbial cell, may be distinguished into three main types: (a) extracellular polysaccharides referred as exopolysaccharides such as pullulans, (b) intracellular polymers which may provide mechanisms for storing carbon or energy for the cell; such as PHB, and (c) structural components of cell structures such as lipopol-ysaccharides and teichoic acids present as integral components of cell walls. Depending on the microbial system, some exopolysaccharides form capsules around the cell thus becoming part of the cell wall, while others form slimes which accumulate outside the cell wall and have the ability to diffuse away into the liquid phase during the course of fermentation. As a result of exopolyaccharide production, the viscosity and rheology of the fermentation broth may undergo profound changes, starting out as a low-viscosity Newtonian fluid and

* *To whom correspondence should be addressed.*
+ *E-mail: alemzadeh@sharif.edu*
1021-9986/09/1/13 *9/$/2.90*

Table 1: Production of polysaccharides by various microorganisms [1].

Product	Substrate	Microorganism
Alginate	Sucrose	Azotobacter vinelandii NCB 9068
Polymer of: D-glucose, D-mannose, D-ribose, 6-deoxy-L-mannose	2 % glucose	Xanthomonas fuscans
Curdlan succinoglucan	Glucose	Alcaligenes faecalis Var. myxogenes 10C3 LFO 13-140
Erwina gun (Zanflo)	Lactose, Hydrolyzed Starch	Erwina tahitica
Polymer of:D-glucose 81.9 %, L-rhamnose 14 %, L-glucose 0.7 % D-mannose 1.9 % D-galactose 1.5 %	1 % w/v Methanol	Methylocytis parvus OBBP
Polysaccharide	4.55 % methanol	Methylomonas mucose NRRL B-5696
Polyhydroxy Butyrate (PHB)	16 % molasses	Alealigen eutrophus
PS-60 gum: glucose 41 %. Rhamnose 30 % uronic		Pseudomonas sp.
Levan	2 % sucrose	Zymomonas mobilis NCIB 8938
Scleroglucan	3 % glucose	Sclerothium rolfsii ATCC 15206
Scleroglucan	5 % w/v starch	Sclerotum delphinii, S.glucanicum
Pullulan	5 % sucrose	Aureobasidium pullulans S-1

ending up as a highly viscous non-Newtonian fluid. Those microorganisms that produce large amounts of polysaccharide slimes have the greatest potential for commercialization since these exopolymers may be recovered easily from the fermentation broth, table 1 [1].

Table 2 is based on published literature data and shows the estimated US consumption and approximate price for different types of commercial polysaccharides used in food and other industrial applications. These data show that the majority of industrial polysaccharides used in the USA are starch based, comprising about 41 % of the total market. Plant-derived polysaccharides, such as gum arabic, guar gum, constitute about 6.6 % of the total market, while algae-derived alginates account for about 6.7 % of the total market value. Xanthan gum is a microbial exopolysaccharide derived from Zanthomonas canperstris fermentation and accounts for 4 % of the total market value.

Although the xanthan gum production has increased over the last few years, the relative market value contribution considering all other industrial polysaccharides did not vary significantly from the estimated data shown in table 2 [1].

However, the competitive advantage of microbial exopolysaccharides will improve with the development of more efficient fermentation processes coupled with the discovery of new microorganisms which produce exopolysaccharides in high yields and having unique physical and chemical properties. Other interesting developments are the production of exopolysaccharides, pullulans by Aureobasidium pullulans.

One of the most important factors for biopolymers is their rheological characteristics, which is influenced by their structure and composition. The structure and composition of microbial biopolymers depends on many different factors, such as microbial species, nature of substrate and other fermentation conditions. This section describes the structure of a few selected biopolymers as examples to illustrate the nature and diversity of these polymers.

Pullulan

The black yeast-like fungus Aureobasidium pullularia pullulans (*A. pullulans*) shows activities for enzymes such as invertase, amylases, glucose oxidase, ß-glucosidase, fructosyltransferase, and small quantities of proteolytic enzyme, pullulans synthetase for elaboration of many

Table 2: Estimated Consumption and Price of Industrial Polymers in the US [1].

Industrial polysaccharide	Food usage (tones)	Industrial Usage (tones)	Total usage (tones)	Price ($ kg^{-1})	Approx. total value (million $)	% of total value
Corn starch	203 000	1 013 000	1 216000	0.20	243.2	40.77
Carboxymethyl cellulose	6100	40000	46100	2.00	92.2	15.46
Methylcellulose	820	21500	22320	3.30	73.7	12.36
Alginate	3700	3600	7300	5.50	40.2	6.74
Pectin	4900	0	4900	4.85	23.8	3.99
Pullulans	—	—	—	—	—	—
PHB	—	—	—	—	—	—
Xanthan	950	2500	3450	6.90	23.8	3.99
Gum Arabic	9340	2850	12190	1.65	19.4	3.20
Guar gum	6070	14180	20250	0.96	19.4	3.20
Carrageenan	3700	90	3790	4.40	16.7	2.80
Tragacanth	526	81	607	26.50	16.1	2.77
Locust bean gum	3650	1620	5270	2.00	10.5	1.76
Karaya	410	2900	3310	2.10	6.9	1.15
Ghatti	4050	410	4460	1.15	5.1	0.85
Agar	125	165	290	16.6	4.8	0.80

many by products of industrial importance like pullulans, an extracellular water soluble polysaccharide [2-11]. The biopolymer pullulans with molecular weight of 10000 to 400000 is produced in the medium containing starch or many other sugar sources. Pullulans is a natural glucan consisting of α-maltotriose units polymerized in linear fashion through 1-6 linkage on the terminal glucose residue of trisaccharide. Maltotetrose units are also present in some polymers (Fig. 1). Microbial polysaccharides have been proposed for a wide variety of industrial applications like, high viscosity syrups substitutes in low calorie foods and drinks, adhesives, dentifrice and other hygienic preparations and soil stabilizers [10].

Pullulans biopolymer resembles styrene in gloss, hardness and transparency and much more elastic. Oxygen permeability of 0.01 mm thick pullulans film varies from 0.6, to 2.compared with cellophanes 4.7 and polypropylene's 1.1. Application of pullulans biopolymer are based on biodegradability of the polymer so it is suggested in food packaging films, coating of food containers for perishable fruit and vegetables [2].

SCP is an important by product during pullulans formation. Fungal cells contain high level of protein (approximately 40 %). The fungi contain less nucleic acid than yeast and bacterial cells. The filamentous nature of fungal mycel facilitates recovery of fungal SCP from fermentation broths. Food products from fungi are preferable in the world. This paper reports the results from investigation on the pullulans biopolymer production from different sugar sources: glucose, sucrose and an agricultural by product, molasses. Kinetics model and kinetics parameters were also determined.

Poly-β-hydroxybutyrate

Many bacterial species produce the polymeric compound, poly-β-hydroxybutyrate (PHB) as a reserve storage polymer, as an energy source [12-32]. It has recently received high attentions in utilizing PHB as polymeric materials in drug delivery systems (Fig. 2) [14].

Although PHB is not a fatly acid-containing lipid, it is nevertheless classified as a lipid in view of its solubility in chloroform and similar solvents. Like the lipids of eukaryotic microorganisms, PHB is produced in increased

quantities when nitrogen is exhausted from the medium. However, its synthesis responds to the concentration of O_2. The difficulty of the fermentation has been due to the recovery process involved in extracting the intracellular material [13-16].

Co-culture of *Lactobacillus delbrueckii* and *Ralstonia eutropha* with programmed control of carbon and nitrogen feed rate in simple batch fermentation by controlling the mixing intensity resulted a high yield [23].

PHB is currently being considered for commercial name of Biopol at Billingham, UK and is classed as a biodegradable thermoplastic [17]. Its applications vary from acting as a substitute for plastic in roles where biodegradability would be an important attribute [20].

The organism of choice for PHB production is Alcaligen euthrophus (Ralstonia eutropha), Reuthropha, which produces between 70 % and 80 % of its biomass as the polymer, the substrate currently used is glucose [2].

Various approaches including efficient PHB production, increasing the intracellular concentration of PHB, optimizing a fermentation process, and efficient PHB recovery / purification system have been studied [12-19]. Different bacteria in pure [19, 29], wild type [31], co-cultures [23] and recombinant strains [26] were studied in PHB production.

Several microbial processes are presently being optimized to produce PHB at a lower cost. The development and optimization of biotechnological processes requires rapid and accurate qualification of PHB in microbial cells. The methods used to serve this purposes including gravimetry or IR spectrometry [21], after solvent extraction, spectrophotometry after quantitative conversion to Crotonic acid [12], gas chromatography (GC, FID) [21], high pressure liquid chromatography after conversion to Crotonic acid [24].

In this investigation cell growth and PHB production were studied under synthetic and natural sugar sources as fructose in synthetic medium, molasses, whey and starch.

The aim of this study is an investigation on natural polymers such as pectin from two different sources, apple and sugar beet pulp, extraction and modification for improving gel quality are the point to be concluded, besides, microbial polymers such as pullulans and PHB production were optimized.

Table 3: Natural polymers, sources and methods of evaluation.

Polysaccharide	Source	Method
Pullulans	Aureobasidium pullulans ATCC9348	Fermentation
PHB	Ralstonia eutropha ACM 1296	Fermentation

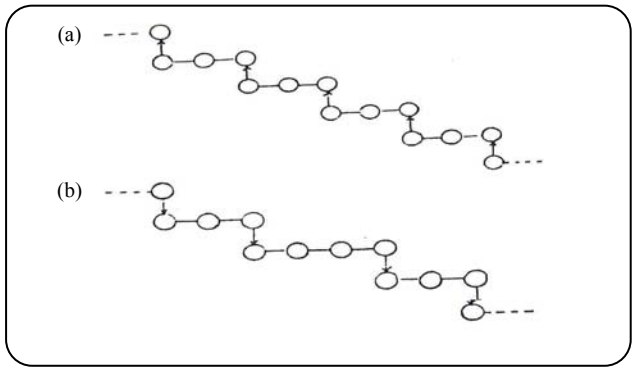

Fig. 1: (a) polymaltotriose structure of pullulans polymer (b) Maltotetrose unit in pullulans polymer. O Glucose residue, 0-0 α-1,4 glucosidic linkage [3].

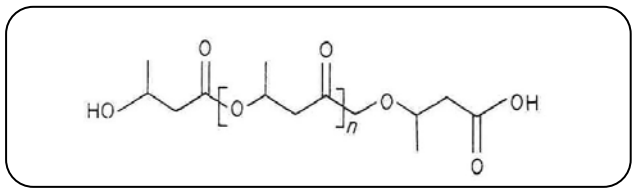

Fig. 2: Structure of PHB [14].

MATERIALS AND METHODS

This investigation was studied under laboratory scale and all the data presented in the results section was the mean of three replicates

Table 3 Presents the biopolymers studies in this investigation. Biopolymers which were studied were pullulans and PHB.

Pre-culture for pullulans production

Preculture contained, 25 g/L sucrose; 4 g/L yeast extract; 3 g/L K_2HPO_4; 1 g/L NaCl; 0.6 g/L $(NH_4)_2SO_4$, 0.1 g/L $FeSO_4$, 7 H_2O and tap water up to 1 liter, pH 6.5. Preculture in 500 mL Erlenmeyer is inoculated by 1 % of the fungi from 48 h slant of PDA culture. Fermentation time is 48 h at 30 ºC and agitation speed of 250 rpm in the Labcon model shaking incubator.

The fermentation medium compositions are identical to pre-culture unless in sugar source. In the first

fermentation medium 50 g/L glucose, in the second medium 50 g/L sucrose and in the third case 100 g/L beet molasses was utilized. Batch cultivation was carried out in a standard agitated fermentor of 5 L capacity with temperature control. The fermentor was loaded with 11 of the fermentation culture. 2 % inoculation from 48 h preculture medium was aseptically transferred into the fermentation medium. Temperature was maintained at 30 °C and agitation speed of 650 rpm.

Analytical procedure

Ten milliliter samples were removed from the fermentor for measuring pH and fungal cell biomass and sugar analysis at different time intervals were effected. pH was measured by a pH meter and fungal cell biomass by filtration followed by drying and weighing. Sugars were analyzed by HPLC with a "sphere-5-Amino 5-micron" column and a RI detector system operated at 26 °C using a mixture of acetonitrile and water as the mobile phase at a ratio of 80 to 20.

Pullulan extraction

After fungal cell separation by centrifugation at 3500 rpm for 15 minutes by Heriss 11KS model centrifuge, supernatant decanted and saved for polysaccharide extraction. Crude polysaccharide contained within the supernatant culture broth was precipitated with cold ethanol-acetone. A volume of ethanol-acetone equal to that of the cell free supernatant was added with vigorous stirring and then allowed to stand for 4-6 hours in an ice bath at 0 °C. The precipitated polysaccharide was recovered and centrifuged at 3000 rpm for 10 minutes. The precipitated crude polysaccharide was dissolved in hot water and then precipitated with an equal volume of ethanol-acetone. The crude polysaccharide was dried under vacuum in a Helious model incubator at 40 °C to a constant weight.

PHB production

Microorganism: Rastonia eutropha (Alcaligen eutrophus) ACM 1296. Bacterial cells were stored on TSA (tryptic soy agar) on slants.

In this study 4 cultures media, synthetic, molasses, whey and starch according to the compositions were utilized for cell growth and PHB production.

Synthetic medium

Na_2HIPO_4, 3.57 g, KH_2PO_4, 1.5 g; $(NH_4)_2SO_4$, 1.35 g; D(-) Fructose, 9.5 g; Trace element solution, 0.5 mL; Distilled water, 1 L: pH=7.

Molasses medium

Molasses, 250 g; $(NH_4)_2SO_4$, 1.35 g; KH_2PO_4, 1.5 g; Distilled water, 1 L; pH=7.

Whey medium

Whey powder, 30 g; $(NH_4)_2SO_4$, 4 g; KH_2PO_4, 13.3 g; $MgSO_4.7H_2O$, 1.2 g; Citric acid, 1.7 g; Trace element solution, 1 mL; Distilled water, 1 L; pH=7.

Starch medium

Na_2HPO_4, 3.57 g, KH_2PO_4, 1.5 g; $(NH_4)2SO_4$, 1.35 g, Soluble Starch, 10 g, Trace element solution, 0.5 mL; Distilled water, 1 L; pH=7.

Cell growth was studied under batch fermentation at 30 ºC with 180 rpm shaking and 10 % inoculation from nutrient broth cell suspension.

Growth was measured by OD at 550 nm, centrifugation of the cell suspension after growth at 4000 rpm for 45 min and separation of the cell body in 5 mL quantity water and extraction of PHB according to the methods [13] and measuring the quantity of PHB at 235 nm by a UV-visible Spectronic 21D model system.

RESULTS AND DISCUSSION

Pullulans production and kinetics

Growth and pullulans production from P. pullulans was investigated under different sugar sources, glucose sucrose and molasses and kinetics constants were determined.

Kinetic Model

Under optimal growth conditions and when any inhibitory effect of substrate and product does not exist, the rate equation for biomass (X) obeys the population theory [1, 2].

$$dX/dt = \mu X\{1-(X/X_m)^n\} \quad (1)$$

where μ is the specific growth rate, X_m is the maximum attainable biomass concentration. From equation (1) specific growth rate decreases with an increase in the cell concentration. When X approaches X_m, the specific

growth rate approaches zero. Equation (1) is applicable in polysaccharide fermentation systems [1, 2].

The product formation rate in this investigation obeys from Luedeking and Piret model [1, 2].

$$dP/dt = \alpha\, dX/dt + \beta\, X \quad (2)$$

X is a non growth term and dX/dt is a growth associated term α is stoichiometric constant and β is product formation activity/mass of cell.

The substrate consumption equation in polysaccharide fermentation is according to the equation (3):

$$-dS/dt = 1/y_x.\, dX/dt + 1/y_p.\alpha.\, dP/dt + m_cX \quad (3)$$

Substrate consumption depends on biomass growth (first term), product formation (second term), and a biomass maintenance function (m_cX). Maintenance is the amount of substrate used to support cell viability even in the absence of growth. Replacing dP/dt from equation (2), the equation (3) becomes:

$$-dS/dt = \gamma\, dX/dt + \delta\, X \quad (4)$$

$$\gamma = 1/y_x + \alpha / y_p \quad (5)$$

$$\delta = \beta / y_p + m_c \quad (6)$$

The value of kinetic parameters, α, β, γ, and δ are determined from drawing 1/X.dP/dt, and 1/X.dS/dt against μ and calculating slope and intercept in each case (table 4).

The results in table 4 presents the kinetics parameters of Luedeking and Piret in this study; it presents that polymer formation is a growth and non growth associated model as the stoichiometric constant and product forming activity are greater than zero.

PHB production

Poly hdroxybulyrate production by *R. eutropha* was studied under different culture media. The results of growth and PHB production are presented in table 5.

The result of table 5 presents that medium containing molasses resulted a high amount of cell growth and PHB production. Effect of molasses concentration on cell growth and PHB production is shown in table 6. According to table 5, more than 70 % of the cell biomass is PHB.

Optimal molasses concentration for cell growth and PHB production was observed at 160 g/L. Our results are comparable with some of the investigators.

Table 4: The value of kinetic parameters for P. pullulans in various medium.

Carbon source	β	α	γ	δ
Glucose	0.040	1.925	1.40	0.08
Sucrose	0.040	0.383	9.40	0.20
Molasses	0.040	0.265	12.88	0.27

Table 5: Effect of culture media composition on growth and PHB production by R. eutropha.

Culture	Growth (g/L)	PHB(g/L)
Synthetic	3	2
Molasses	22	17
Whey	3	1
Starch	8	5

Table 6: Effect of molasses concentration on growth and PHB production.

Molasses (g/L)	Cell growth (g/L)	PHB (g/L)
30	3	2
60	7	5
120	16	12
160	22	17
200	14	10
300	6	7

Many other investigators studied PHB production under different conditions with various source of carbon [32]. PHB production by A. eutrophus H16 in a 3 liter fermentor using lactic acid as the only carbon source, resulted 7.5 g/L PHB, which about 80 % of cell biomass [17]. Genetic modification of E.coli using PHB gene from A. eutrophus and fermentation under semi continuous system with glucose as sugar source in the presence of the modified E. coli, resulted 88.8 g/L PHB production which is so significant and relates utilization of recombinant E. coli in this studies [18].

PHB production of 12 g/L have been reported in laboratory experiment on the co-culture *Lactobacillus delbrueckii* and *Ralstonia eutropha* but theoretically under fed-batch fermentation with control of carbon and nitrogen feed rate could be increased up to 40 g/L [23].

Recombinant E.coli harboring Alcaligen eutrophus in flask cultivation containing 21 % glucose resulted the yield of 85.2 g/L PHB [19].

Recombinant E.coli harboring Alcaligen eutrophus in flask cultivation containing 21 % glucose resulted the yield of 85.2 g/L PHB [19].

CONCLUSIONS

In this investigation natural polymers from microbial sources were studied. The biopolymer produced in this investigation was pullulans an extracellular biopolymer consist of polysaccharides units from P. pullulans which was studied under similar medium with different sugar sources, glucose, sucrose and molasses. Biopolymer production and growth were determined. Biopolymer production in all three cases obeyed growth and non growth associated kinetic model.

PHB a type of microbial polymer was produced from *R. eutrorpha* producing intracellular biopolymer was investigated under different culture media (synthetic, molasses, whey and starch medium) containing different sources of carbon: fructose, molasses, lactose (in whey) and starch. The results presented that molasses showed optimal production for cell growth and PHB production. Molasses at 160 g/L showed high cell mass and PHB production. Higher molasses concentration due to unfavorable conditions for cell growth, resulted low biopolymer, (PHB) production in this investigation.

Nomenclatures

μ	Specific growth rate (h^{-1})
α	Stoichiometric constant
β	Product forming activity per mass of cell
y_x	Biomass yield based on substrate (g cell/ g substrate)
y_p	Product yield based on substrate (g cell/ g substrate)
γ	Constant defined in equation (5)
δ	Constant defined in equation (6)
m_c	Maintenance coefficient (g substrate / g cell)

Received: 25th September 2006 ; Accepted: 16th September 2008

REFERENCES

[1] Moo-Young, M., "Comprehensive Biotechnology", Microbial Polysaccharide, vol 3, Pergamon Press INC, London, UK, pp. 1005-1015 (1985).

[2] Alemzadeh, I., Vossoughi, M. and Naimpour, F., Biopolymer Production and Kinetics, International Seminar of Polymer Science and Technology, pp. 449- 455, Shiraz, I.R. IRAN (1994).

[3] Alemzadeh, I., Vossoughi M. and Fan, L.T., A Study on Fructose Production from Fermentation Media with High Sucrose Concentration by *P. pullulans,* Chisa Proceeding, Praha Chec, September (1994).

[4] Li, H., Chi, Z., Wang, X., Duan, X., Ma, L., Gao, L., Purification and Characterization of Extracellular Amylase from the Marine Yeast *Aureobasidium Pullulans* N13d and its Raw Potato Starch Digestion, *Enzyme and Microbial Technology*, **40**, p. 1006 (2007).

[5] Leite, R.S.R., Gomes, E., da Silva, R., Characterization and Comparison of Thermostability of Purified ß-Glucosidases from a Mesophilic *Aureobasidium Pullulans* and a Thermophilic *Thermoascus aurantiacus*, *Process Biochemstry*, **42**, p. 1101 (2007).

[6] De Wet, B.J.M., Van Zyl, W. H., Prior, B. A., Characterization of the *Aureobasidium Pullulans* α-Glucuronidase Expressed in *Saccharomyces Cerevisiae*, *Enzyme and Microbial Technology*, **38**, p. 649 (2006).

[7] Sangeetha, P.T., Ramesh, M.N., Prapulla, S.G., Production of Fructo-Oligosaccharide by Fructosyl Transferase from *Aspergillus Oryzae* CFR 202 and *Aureobasidium Pullulans* CFR 77, *Process Biochemistry*, **39**, p. 753 (2004).

[8] Duan, X., Chi, Z., Wang, L., Wang, X., Influence of Different Sugars on Pullulan Production and Activities of α-Phosphoglucose Mutase, UDPG-Pyrophosphorylase and Glucosyltransferase Involved in Pullulan Synthesis in *Aureobasidium Pullulans* Y68, *Carbohydrate Polymers,* **73**, p. 587 (2008).

[9] Zheng, W., Campbell, B. S., McDougall, B. M., Seviour, R.J., Effects of Melanin on the Accumulation of Exopolysaccharides by *Aureobasidium Pullulans* Grown on Nitrate, *Bioresource Technology*, **99**, p. 7480 (2008)

[10] Singh, R. S., Saini, G. K., Kennedy, J. F., Pullulan: Microbial Sources, Production and Applications, *Carbohydrate Polymers*, **73**, p. 515 (2008).

[11] Souguir, Z., Roudesli, S., Picton, E. L., Le Cerf, D., About-Jaudet, E., Novel Cationic and Amphiphilic Pullulan Derivatives I: Synthesis and Characterization, *European Polymer Journal*, **43**, p. 4940 (2007).

[12] Seidi, A. and Alemzadeh, I., Poly Hydroxyl Butyrate Production, 8th Chemical Engineering Congress, pp. 115-120, Mashad, I.R. IRAN (2003).

[13] Seung yoo, Woo-Sik kim, Cybemetic Model for Synthesis of Poly B-Hydroxy Butyric Acid in Alcaligenes Eutrophus, *Biotechnology and Bio-engineering*, **43**, p. 1043 (1994).

[14] Brandi, H., Gross, R.A., Lenz, R.W. and Clinton, R., Plastics from Bacteria: Polyhydroxyalkanoates as Natural, Biocompatible, and Biodegradable Polyesters, *Advances in Biochemical Engineering*, **41**, p. 77 (1990).

[15] Katircloglu, H., Aslim, B., Nuryuksek, D., Mercan, N. and Beyatli, Y., Production of Poly-B-Hydroxbutyrate (PHB) and Differentiation of Putative Bacillus Mutant Strains by SDS-PAGE of Total Cell Protein, *African Journal of Biotechnology*, **2**(6), p. 147 (2003).

[16] Aslim, B., Nuryuksek, D. and Beyatli, Y., Determination of PHB Growth Quantities of Certain Bacillus Species Isolated from Soil, *Turkish Electronic Journal of Biotechnology*, Special Issue, p. 24 (2002).

[17] Linko, S., Vaheri, H. and Seppala, J., Production of Poly-hydroxybutyrate on Lactic Acid by A. eutrophus H16 in a 3-l Bioreactor, *Enzyme and Microbial Technol*., **15**, p. 401 (1993).

[18] Kim, B.S., Lee, S.C., Chang, H.N. and Woo, Y.K., Production of Poly3- Hydroxy Butyrate Acid by Fed-Batch Culture of Recombinant E.coli, *Biotechnology. Letters*, **4**, p. 811 (1992).

[19] Jung, L., Phyo, K.H., Kim, K.C., Park, H.K., Kim, I.G., Spontaneous Liberation of Intracellular Polyhydroxy-Butyrate Granules in *Escherchia coli*, *Research in Microbilogy*, **156**, p. 865 (2005).

[20] Valappil, S. P., Misra, S. K., Boccaccini, A. R., Keshavarz, T., Bucke, C., Roy, I., Large-Scale Production and Efficient Recovery of PHB with Desirable Material Properties, from the Newly Characterized Bacillus Cereus SPV, *J. of Bio-technology*, **132**, p. 251 (2007).

[21] Monteil-Rivera, F., Betancourt, A., Tra, H.V., Yezza, A., Hawari, J., Use of Headspace Solid-Phase Micro Extraction for the Quantification of Poly(3-Hydroxybutyrate) in Microbial Cells, *J. of Chromatography* A, **1154**, p. 34 (2007).

[22] Theodorou, M.C., Panagiotidis, C.A., Panagiotidis, C.H., Pantazaki, A.A., Kyriakidis, D.A., Involvement of the AtoS-AtoC Signal Transduction System in Poly-(R)-3-Hydroxybutyrate Biosynthesis in *Escherchia Coli*, *Biochimica et Biophysica Acta*, **1760**, p. 896 (2006).

[23] Ganduri V.S.R.K., Ghosh, S., Patnaik, P.R., Mixing Control as a Device to Increase PHB Production in Batch Fermentations with Co-Cultures of *Lactobacillus Delbrueckii* and *Ralstonia Eutropha*, *Process Biochemistry*, **40**, p. 257 (2005).

[24] Taguchi, S., Maehara, A., Takase, K., Nakahara, M., Nakamura, H., Doi, Y., Analysis of Mutational Effects of a Polyhydroxybutyrate (PHB) Polymerase on Bacterial PHB Accumulation Using an in Vivo Assay System, *FEMS Microbiology Letters*, **198**, p. 65 (2001).

[25] Saad, G.R., Seliger, H., Biodegradable Copolymers Based on Bacterial Poly((R)-3-Hydroxybutyrate): Thermal and Mechanical Properties and Bio-degradation Behavior, Polymer Degradation and Stability, **83**, p. 101. (2004).

[26] Song, B.G., Kim, T.K., Jung, Y.M., Lee, Y.H., Modulation of *talA* Gene in Pantose Phosphate Pathway for Overproduction of Poly-ß-Hydroxy-butyrate in Transformant *Escherchia Coli* Harboring *phbCAB* Operon, *J. of Bioscience and Bio-engineering*, **102**, p. 237 (2006).

[27] Wu, G., Moir, A.J.G., Sawers, G., Hill, S., Poole, R.K., Biosynthesis of Poly-ß-hydroxybutyrate (PHB) is Controlled by CydR (Fnr) in the Obligate Aerobe *Azotobacter Vinelandi*, *FEMS Microbiology Letters*, **194**, p. 215 (2001).

[28] Takanashi, M., Saito, T., Characterization of Two 3-Hydroxybutyrate Dehydrogenases in Poly(3-Hydroxybutyrate)-Degradable Bacterium *Ralstonia Pickettii* T1, *J. of Bioscience and Bioengineering*, **101**, p. 501 (2006).

[29] Shih, W.J., Chen, Y.H., Shih, C.J., Hon, M.H., Wang, M.C., Structural and Morphological Studies on Poly(3-Hydroxybutyrate Acid) (PHB)/Chitosan Drug Releasing Microspheres Prepared by Both Single and Double Emulsion Processes, *J. of Alloys and Compouds*, **434-435**, p. 826 (2007).

[30] Khosravi Darani K., Vasheghani Farahani, E., Microorganisms and Systems for Production of

Poly(Hydroxybutyrate) az an Biodegradable Polymer, *Iran J. of Chem. Chem. Eng.*, **24**, 1 (2005).

[31] Khosravi Darani K., Vasheghani Farahani, E., Shojaosadati, S.A., Application of the Plackett-Burmann Design for the Optimization of Poly (ß-Hydroxybutyrate) Production by *Ralstonia Eutropha, Iran. J. Biotenchnology*, **1**, 155 (2003).

[32] Sangkharak, K., Prasertsan, P., Optimization of Polyhydroxybutyrate Production from a Wild Type and Two Mutant Strains of Rhodobacter Sphaeroides Using Statistical Method, *J. of Biotechnology*, **132**, p. 331 (2007).