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Original paper

Studies on polyhydroxyalkanoates biosynthesis by some Pseudomonas spp. strains

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Abstract Medium-chain-length polyhydroxyalkanoates *(mcl*-PHAs) are naturally produced by bacteria and accumulates in cytoplasm in the form of granules, in particular culture broth conditions. PHAs are biodegradable, biocompatible and have useful mechanical properties that recommend them for divers applications in various fields. In order to obtain *mcl*-PHAs of microbial origin we used two *Pseudomonas* spp. strains, namely *Pseudomonas putida* ICCF 391 and *Pseudomonas fluorescens* ICCF 392. Researches have focused the ability of these two strains to use structurally related or not related substrates, to obtain biopolymers with controlled composition, and growth the amount of PHAs in reproducible conditions. Moreover, bioprocess conditions for *mcl*-PHAs biosynthesis, fermentation broth processing, and polymers composition – were reproducible. As the results achieved with the two strains were similar, researches continued with *Pseudomonas fluorescens* strain, which is less studied regarding the potential of PHA biosynthesis. Were carried out (co) polymer films containing more than 85% PHO, as determined by GC-FID.

Keywords Precursors, biopolymers, bioprocess, fermentation, fatty acids

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Introduction

Polyhydroxyalcanoates are polyesters of aliphatic hydroxy acids. They have properties similar to petroleum-derived polymers and form a class of thermoplastic materials whose mechanical properties vary between elasticity similar to rubber and hardness comparable to crystalline textolite. These characteristics recommend PHAs use in various forms and in different areas (R. RAI & al. [1]). They have gained much interest because of their biodegradability (R.W. LENZ & al. [2], S.P. LIM & al. [3]) and biocompatibility (G. CHEN & al. [4]). PHAs are naturally produced by bacteria and accumulates in the form of granules in the cytoplasm as energy reserve and carbon (C) atoms, in particular culture broth conditions.

Biosynthesis of these polymers depends on the genetic characteristics of microorganisms and environmental factors, including: the nature and concentration of the substrate used as C and energy source, presence and concentration of other nutrients in the culture medium, temperature, pH, dissolved oxygen concentration, cultivation system (batch, in steps, fed-batch or continuous). These factors influence the rate growth of the microorganism, cell density at the end of fermentation, the degree conversion of the substrate and intracellular PHAs content (A. MUANGWONG, & al. [5]).

Optimization studies for obtaining growing quantities of PHAs occupy the forefront of current researches.

A large number of bacteria are able to store the carbon in the form of PHAs. Currently only PHB which is a *scl*-PHA type, is produced at industrial scale. *Mcl*-PHAs production is still inferior to that of *scl*-PHAs and is probably due to the toxicity of the substrate (Z. Sun & al. [6]). Only a few bacterial species, including *Pseudomonas* (D.M. Cirstea & al. [7]), can synthesize *mcl*-PHAs that consist mainly of 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD). Many studies confirmed that they appear to be much more flexible and resistant (A.V. SAMROT & al. [8], S. KABILAN & al. [9], P. WECKER & al. [10]) than *scl*-PHAs. These extraordinary properties of *mcl*-PHAs make them good candidates for further utilization in many domains, notably, for the biomedical area or in manufacturing films and coatings.

Our experiments focused on the ability of the strains *Pseudomonas putida* ICCF 391 and *Pseudomonas fluorescens* ICCF 392 to use different fatty acids to produce PHAs. These fatty acids, namely, octanoic, nonanoic and decanoic acid, were carbon and energy sources, unique, mixed or combined with other carbon sources (structurally related or not).

The aim of the researches presented in this paper was to develop an experimental model for microbial biosynthesis of *mcl*-PHAs with controlled compositions.

Materials and Methods

For PHAs biosynthesis were used two *Pseudomonas spp.* strains namely *Pseudomonas putida* ICCF 391 and *Pseudomonas fluorescens* ICCF 392. The strains were grown at 29±1°C and periodically transferred on on M44 (M.G. Soare & al. [11]) solid medium, with the following composition (% g/vol): yeast extract 1.0%, peptone 1.0%, glycerol 5.0%, and agar 2.0%. All the components were dissolved in distilled water, pH adjusted to 6.5, then sterilized at 120ºC, 20 minutes. Stock cultures were stored at 4°C.

The inoculum broth containing (% g/v): glucose 1.00%, corn extract 1.50%, KH2PO4 1.00%, NaCl 1.00%, MgSO4 0.05%, was sterilized at 115°C, for 20 minutes. The inoculum was developed in 500 ml shake flasks (100 ml medium), at 29±1°C and 220 rpm for 24 h.

A well-developed inoculum culture shows an optical density ($OD_{\lambda = 550\ nm}$) of 12.5 which provide a good start for bioprocess.

The culture broth used in experiments had the following composition: $NaNH_4HPO_4 \cdot 4H_2O$ 3.5 g/L, K_2HPO_4 7.5 g/L, KH_2PO_4 3.7 g/L, structural uncorrelated carbon source (glucose or trisodium citrate) 20.0g/L, structural correlated carbon source (sodium octanoate, nonanoate or decanoate) 0.835 – 2.5 g/L, trace element solution I 1.0 mL/L, trace element solution II 1.0 mL/L. Trace element solution I contains 120.0 g/L $MgSO \cdot 7H_2O$. Trace element solution II contains per liter: 2.78 g of $FeSO_4 \cdot 7H_2O$, 1.47 g of $CaCl_2 \cdot 2H_2O$, 1.98 g of $MnCl_2 \cdot 4H_2O$, 2.81 g of $CoSO_4 \cdot 7H_2O$, 0.17 g of $CuCl_2 \cdot 2H_2O$, and 0.29 g of $ZnSO_4 \cdot 7H_2O$ in 1M HCl. As carbon sources correlated with biopolymers were investigated C8, C9, and C10 fatty acids and as not related C sources, glucose or trisodium citrate. Before inoculation all culture media were sterilized at 120°C for 20 minutes.

Bioprocesses were carried out in 500 ml flasks containing 100 ml of culture broth on a rotary shaker at 220 rpm, for 48-72 hours, maintaining the temperature at 29±1°C. The cultivation went in two steps (fed batch) with periodically nutrient addition. Optical density (OD) of cell suspension was measured at λ = 550 nm, 1:25 dilution, with a UV-VIS spectrophotometer Jasco V-Able 630. The quantity of dry biomass was determined with a thermobalance A & D, MF-50.

Results and Discussions

Initial tests were aimed at determining: the bioproductivity of strains, the optimum period for addition of the precursor, the type of precursor with the highest conversion in *mcl*-PHA.

1. Biomass development with octanoic acid as precursor for PHA biosynthesis

Results obtained from a first set of tests are presented in the following table.

Table 1. The influence of C8 used as carbon and energy source, added at different intervals in association with glucose on strains biomass development

No	Total C8 added (g/100mL)	$OD_{(\lambda = 550nm,\ 1:25\ dilution)}$ / C8 Added (g/100mL)					Total C8 consumed (%)	Dry biomass (g/L)
		24h	30h	48h	54h	72h		
Pseudomonas putida								
1	0.7515*	10 / 0.25	6.75 / 0.083	5.75 / 0.167	5.25/ 0.25	6 / 0	78%	1.775
2	0.7515**	12.5 / 0.25	8.5/ 0.25	7.25 / 0	-	-	93%	2.58
Pseudomonas fluorescens								
3	0.7515*	9 / 0.25	7 / 0.083	6.5 / 0.167	6.5 / 0.25	6.25 / 0	80%	1.903
4	0.7515**	10.5 / 0.25	7.25 / 0.25	6.5 / 0	-	-	85%	2.57

Observations: - $NaNH_4HPO_4 \cdot 4H_2O$ was 6g/L instead of 3.5 g/l in all tests above mentioned
- C8 - represent octanoic acid;
* glucose 2% added without C8 at 0 hours
** glucose 2%, C8 (0.25% g/v final concentration in fermentation broth) added starting with 0 hour

Examining the data contained in table 1 we consider that:

- using a 2-stage bioprocess was adequate – the first stage of 24 hours, is aimed at developing cell mass and the next at PHA biosynthesis and accumulation induced by C8 addition at well-defined intervals;

- cells of both strains grow better in the first 24 hours using octanoic acid as the only C source (according to OD values and quantities of dry biomass) compared to baseline strategy that starts with glucose fermentation, followed by octanoic acid added starting at 24 hours;

- in terms of developing cellular biomass on C8, the results were similar for the two strains studied so the research continued with *P. fluorescens* strain.

2. Consumption of different precursors (C8, C9, C10, C8+C10) for PHAs biosynthesis

In terms of comparative experiences on the use of fatty acids C8 and C10 (Table 2) by *Ps. fluorescens* strain, as additional C and energy sources (the initial source was trisodium citrate) were noted:

- confirmation of previous results regarding octanoic acid tolerance of *Ps. fluorescens*, which is consumed in 99% during the 48 hours, in fed-batch culture system. Cell density is increasing, which indicates the possibility of extending the bioprocess duration to get a higher biomass amount;

- decanoic acid is metabolized much harder than octanoic acid in the presence of trisodium citrate. The small amount of biomass and low optical densities of cell suspensions at different periods of fermentation suggest that decanoic acid was consumed only for breathing processes necessary for culture survival, without biomass increasing and development;

- co-feeding with octanoic and decanoic acids at a mass ratio of 1:1, promoted decanoic acid consumption but reduced the use of octanoic acid. Nonanoic acid was also tested as a precursor for PHAs biosynthesis (Table 3).

Table 2. The influence of different precursors used as C and energy source on *Ps. fluorescens* biomass development

No	Precursor (g at 0h)	Total Precursor added (g)	$OD_{(\lambda = 550nm,\ 1:25\ dilution)}$ / Precursor added (g)						Total Precursor consumed (%)	Dry Biomass (g/L)
			6 h	12 h	24 h	30 h	36 h	48 h		
1	C8 0.167	0.918	0.18/ 0.083	0.19/ 0.167	0.32/ 0.167	0.46/ 0.167	0.42/ 0.167	0.47/ 0	99%	3.1
2	C10 0.167	0.626	0.04/ 0.083	0.03/ 0.167	0.07/ 0.167	0.06/ 0.042	0.05/ 0	0.05/ 0	22%	nd*
3	C10 0.0835	0.5845	0.03/ 0.083	0.05/ 0.126	0.09/ 0.083	0.07/ 0.083	0.05/ 0.125	0.05/ 0	15.9%	nd*
4	C10 0.0835	0.501	0.08/ 0.083	0.06/ 0.083	0.089/ 0.083	0.073/ 0.083	0.063/ 0.083	0.053/ 0	15.7%	0.77
5	C8 0.0835 + C10 0.0835	0.3131 + 0.3131	0.02 / 0.04 + 0.04	0.024/ 0.08 + 0.08	0.01/ 0.08 + 0.08	0.08/ 0.021 + 0.021	0.08/ 0 + 0	0.07/ 0 + 0	C8 38% C10 35.4%	nd*

Observations: - in all experiments was used as the initial carbon source trisodium citrate in a concentration of 2% g/v.
- C10 - represent decanoic acid
- nd*- not determined

Table 3. *Ps. fluorescens* biomass development with nonanoic acid (C9) as precursor

No	Total Precursor (g/100mL)	$OD_{(\lambda = 550nm,\ 1:25\ dilution)}$ / C9 added (g/100mL)						Total Precursor consumed (%)	Dry Biomass (g/L)
		6 h	12 h	24 h	30 h	36 h	48 h		
1	0.585	3 / 0.126	4.25/ 0	4.75 / 0.167	5.75/ 0.126	6.75/ 0	6.75/ 0	69%	1.62
2	0.668	3.25 / 0	4.75/ 0.167	5.5 / 0.167	7.5/ 0	7/ 0.167	8/ 0	96%	1.97
3	0.835	2.75/ 0.083	4.5/ 0.167	6.75 / 0.167	7.25/ 0.0835	6.75/ 0.167	8/ 0	67.9%	2.74

Observations: - 0.167 g C9 were added at 0 hours of bioprocess;
- in all batches trisodium citrate (2% g/v) was also added.

The microorganism used nonanoic acid up to 96% in fed-batch cultivation system.

The results of tests with nonanoic acid lead to the following conclusions:

- nonanoic acid is tolerated equally as the octanoic acid;
- the highest quantity of biomass (test no. 3 in the table) was obtained when the precursor (C9) was in excess in media;
- if the period of addition and the amount of precursor are similar to those from experiments with octanoic acid then the amount of biomass produced is similar;
- because similar results were obtained in tests in which have used C8 and C9 as precursors, the experimental model developed for PHA biosynthesis with C8 is validated also in fermentations with C9.

3. Establishing the optimal periodicity for adding the precursors in order to elaborate the experimental model for PHA biosynthesis.

The next set of experiments was performed to develop the experimental model for PHAs biosynthesis.

These went through all the specific stages for a bioprocess, as presented below:

I. the maintenance of strains, to preserve viability and bio productivity, by regular passages on a solid nutrient medium that contains: glycerol, yeast extract and peptone;

II. obtaining inoculum culture for seeding fermentation broth. Inoculum culture was developed in a liquid broth with above presented composition;

III. the fermentation broth used to establish experimental model, had an identical composition to that used in the tests above and was seeded with an inoculum cell suspension of 10 ml/100 ml medium. Precursor concentration was maintained around 0.25g% in fed-batch fermentation. Bioprocess parameters were: stirring 220 rpm, initial pH 7-7.2, and temperature 30°C, duration 48-72 hours. During fermentation usual measurements were made: optical density, pH, precursor content. Dry biomass and PHA concentration were determined at the end of the bioprocess. The differences between batches consisted in the amount of supplied and consumed precursor and in appropriate periodicity of supplementation as we concluded after latest experiments.

The adding of precursors and their consumption are shown in the tables 4 and 5.

Correlating the results from previous tables regarding octanoic acid using as PHAs precursor, the following aspects can be noticed:

- the precursor was completely consumed in 48 hours in batches 2, 3, 5, 6 and 7; the best results were obtained with batches 5 and 7;
- we extended the time of fermentation to 72 hours at batch 7 in the assumption that additional intake of C8 will favor biosynthesis and accumulation of polymer, which was confirmed. If one compares the batches 3 (of 48 hours) and 7 (of 72 hours) finds that the extension of fermentation resulted in a higher coefficient of total carbon conversion, a larger amount of cell mass and PHA/liter of fermentation and a higher content of dry biomass PHAs reported (Table 5). In both cases, precursors additions were done in the same quantity at the same intervals up to 48 hours;
- best results were obtained in batch 5, where precursor supply was more balanced, avoiding octanoate sudden drop recorded at batches 2, 6 and 7 as shown in table 4;
- in batches where decanoic acid was used, we noticed that supplying with low and constant quantities of precursor at specified intervals, ensure full and constant consumption after 48 hours of cultivation, as happened in batch 8;
- although in batches 1 and 8 were used the same quantities of precursors C8 respectively C10, the amount of PHAs obtained from fermentation with C10 was much lower. This means that C10 was used for cell growth rather than PHAs biosynthesis;
- considering the results obtained during batches, the experimental model proposed for the biosynthesis of PHAs, containing mostly PHO (over 85%) is represented by batch 5.

Table 4. Evolution of precursors consumption during fermentations

	Time (h)	0	6	12	24	30	36	48	54	72	Total
C8											
1	Add. (g/2L)	5.01			2.506	2.506					10.02
	Cons. (g/2L)				0.2	1.25		3.99			0.55%
2	Add. (g/2L)	3.34	2.506		3.304	2.06					11.69
	Cons.(g/2L)		1.25		4.548	2.902		2.99			100%
3	Add. (g/2L)	3.34		3.34	3.34		3.34				13.36
	Cons. (g/L)			3.32	3.32		3.32	3.34			99.7%
4	Add. (g/2L)	5.01			5.01	5.01					15.03
	Cons. (g/2L)				2.226	3.47		5.63			75%
5	Add.(g/2L)	3.34	1.67	3.34	3.34	1.67	3.34				16.7
	Cons. (g/2L)		1.39	2.96	3.4	3.1	3.1	2.75			100%
6	Add. (g/2L)	3.34	3.34	3.34	3.34	1.67	3.34				18.37
	Cons (g/2L)		1.608	3.534	5.02	3.34	1.788	3.08			100%
7	Add. (g/2L)	3.34		3.34	3.34		3.34	3.34	3.34		20.04
	Cons. (g/2L)			3.12	5.72		2.17	3.20	3.20	2.63	100%
C10											
8	Add. (g/2L)	1.67	1.67	1.67	1.67	1.67	1.67				10.02
	Cons. (g/2L)		1.67	1.67	1.67	1.67	1.67	1.59			99.2%
9	Add.(g/2L)	1.67	1.67	2.505	1.67	1.67	2.505				11.69
	Cons. (g/2L)		1.67	1.67	2.10	2.00	0	0			63.4%

Observation: in all batches trisodium citrate (2% g/v) was added; Add. = Added; Cons. = Consumed

Table 5. Experimental values obtained in fed-batch fermentation for PHAs biosynthesis

Batch*	Precursor (g/L)		Conversion rate of total C (%)	**DB (g/L)	PHAs (g/L)	PHAs (%)
	Added	Consumed				
1	10.02	5.436	25.35	1.975	0.343	22.56
2	11.69	11.69	32,76	2.71	1.055	44.47
3	13.36	13.32	29.30	2.566	0.977	45.14
4	15.03	11.33	25.33	2.34	0.12	17.1
5	16.70	16,70	34.00	3.305	1.49	53.56
6	18.37	18.37	18.77	1.915	0.288	20.66
7	20.04	20.04	31.08	3.321	1.327	50.4
8	15.03	9.938	30.90	2.47	0.3	14.84
9	16.70	5.845	17.44	1.394	0.123	15.37

* in batches 1-7 octanoic acid was added and in batches 8-9 decanoic acid; **DB = dry biomass

4. Extraction of the intracellular polymer

Polymer extraction was achieved by processing bacterial biomass at the end of fermentation, with organic solvents. For extraction was first used acetone and then Soxhlet proceeding was applied. Isolation-purification yields ranged between 65-98% depending on the polymer content of biomass.

All polymers obtained through fermentation batches made at this stage were processed through this method, yielding polymeric films, which were characterized by FTIR spectra and GC-FID analysis at INCDCF-ICCF, Bucharest. The composition and the purity degree of polymers biosynthesized after fermentation (conducted in fed-batch cultivation system and isolated by Soxhlet (X. JIANG & al. [12]) proceeding of extraction with acetone, were determined by GC-FID and expressed in g/100 g of analyzed product or in moles %. For all biopolymers samples FTIR spectral analysis was performed and compared to standards of *mcl*-PHAs used to identify the characteristic absorption bands.

In table 6 are presented the results of GC-FID analysis performed for polymers produced in fermentation batches carried out during this stage of research.

The analytical results certify that under these conditions established as experimental model for biosynthesis, isolation and purification of PHAs, using C8 as precursor (supplied in fed-batch system) were obtained polymers with a C8 content ranging between 85.25-89.4%, C6 between 9-11% and C10 between 0.75-3%. Analytical

Table 6. The analytical characteristics of synthesized polymers

No	DB (g/L)	PHAs (g/L)	PHAs (%)	Hydroxyacids					
				C6 (%)	C8 (%)	C10 (%)	C11 (%)	C14 (%)	Molar Ratio C6:C8:C10:C11:C14
C8 precursor									
1	1.975	0.343	22.56	11,01	85,93	2,25	-	-	8:52:1,0
2	2.71	1.055	44.47	10,98	85,25	2,70	-	-	8:51:1,3
3	2.566	0.977	45.14	9,50	86,42	3,01	0,30	-	7:52:1,5
4	2.34	0.12	17.1	9,00	79,90	2,75	0,17	1,81	10:71:2:1,0
5	3.305	1.49	53.56	11,47	85,75	1,85	-	-	9:54:1,0
6	1.915	0.288	20.66	9,36	89,40	0,75	-	-	7:56:0,5
7	3.321	1.327	50.4	10,05	86,75	2,08	0,3	-	7:50:0,4:0,1
C10 precursor									
8	2.47	0.3	14.84	7,18	53,56	37,32	-	-	5,5:34:20
9	1.394	0.123	15.37	6,59	56,92	35,74	-	-	5:35,5:19

characterization of PHAs obtained from batches with C10 as precursor, revealed that PHO prevails in proportion of 53.5 to 57%, followed by PHD between 35.5-37.5% and PHHx between 6.5-7.5 %.

Conclusions

Microbial production of polyhydroxyalkanoates is influenced by genetic characteristics of microorganisms and medium factors which includes the nature and concentration of the substrate (used as energy and C source), the presence and concentration of other nutrients in the growth medium, temperature, pH, dissolved oxygen concentration, and cultivation system (batch, fed-batch or continuous). These factors ultimately influence the growth rate of the microorganism, cell density in the end of the fermentation, the degree of substrate conversion and intracellular PHAs content.

A first set of experiments aimed to study two strains (*Ps. fluorescens* and *Ps. putida* selected as producing PHAs) on their ability to use different fatty acids as C and energy sources single, mixed or combined with other carbon sources, structurally related or not related, for cellular growth. *Ps. fluorescens* strain led to results similar to *Ps. putida* in producing PHAs. Because *Ps. fluorescens* strain is less studied in terms of PHAs biosynthesis potential, we considered appropriate to continue research with this strain.

For this purpose, were conducted several sets of experiments aiming the effect each of fatty acids C8 – C10 and combinations of them or their combinations with citric acid (carbon source uncorrelated). The results showed the optimal conditions and maximum limit of the 3 fatty acid metabolism and the ability of the microorganism to metabolize easier and more productive octanoic acid than the other two fatty acids, these results are verified later in the experimental biosynthesis and post-processing model for obtaining PHAs.

For experimental model of PHAs biosynthesis were conducted several experiments that have gone through all the stages of a specific process of microbial biosynthesis. Fermentation batches made, using different feeding regimes with C8 or C10, as precursors, allowed establishing the conditions for biosynthesis of copolymers with controlled composition depending on the nature of precursors and growing conditions.

For post-processing of biomass, Soxhlet proceeding of extraction with acetone was investigated, for which were also established, optimum operating parameters.

The experimental model developed for biosynthesis, isolation and purification of PHAs led to obtaining copolymers (containing 9-11% C6, 85.25 – 89.4%, C8 and 0.75 – 3% C10), with yields between 65-98%, depending on the biopolymer content from the cell biomass.

Analyzing the results obtained during batches, the experimental model proposed for the biosynthesis of PHAs, containing mostly PHO (over 85%) is represented by batch 5.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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References

1. R. RAI, T. KESHAVARZ, J. ROETHER, A. BOCCACCINI, I. ROY, Medium chain length polyhydroxyalkanoates, promising new biomedical materials for the future. Mat. Sci. Eng. R., 72 (3), 29, 47 (2011).
2. R.W. LENZ, R.H. MARCHESSAULT, Bacterial Polyesters: Biosynthesis, Biodegradable Plastics and Biotechnology, Biomacrom., 6 (1), 1, 8 (2005).
3. S.P. LIM, S.N. GAN, I. TAN, Degradation of medium-chain-length polyhydroxyalkanoates in tropical forest and mangrove soils. Appl. Biochem. Biotech., 126, 23, 32 (2005)
4. G. CHEN, Y.C. WANG, Medical applications of biopolyesters polyhydroxyalkanoates, J. Polym. Sci., 31(5), 719, 736 (2013).
5. A. MUANGWONG, T. BOONTIP, J. PACHIM-SAWAT, S.C. NAPATHORN, Medium chain length polyhydroxyalkanoates consisting primarily of unsaturated 3-hydroxy-5-*cis*-dodecanoate synthesized by newly isolated bacteria using crude glycerol. Microb. Cell, 15 **(**55), DOI 10.1186/s12934-016-0454-2, (2016).
6. Z. SUN, J.A. RAMSAY, M. GUAY, B.A. RAMSAY, Fed-batch production of unsaturated medium-chain-length polyhydroxyalkanoates with controlled composition by *Pseudomonas putida* KT2440, Appl. Microbiol. Biotechnol., 82, 657, 662 (2009)
7. D.M. CIRSTEA, M. STEFANESCU, J.M. PAHONȚU, C. P. CORNEA, Use of some carbon sources by *Pseudomonas* strains for synthesizing polyhydroxyalkanoates and/or rhamnolipids, Romanian Biotechnological Letters, Vol. 19, No. 3, (2014).
8. A.V. SAMROT, R.B. AVINESH, S.D. SUKEETHA, P. SENTHILKUMAR, Accumulation of poly [(R)-3-hydroxyalkanoates] in *Enterobacter cloacae* SU-1 during growth with two different carbon sources in batch culture. Appl. Biochem. Biotechnol., 163, 195, 203 (2011).
9. S. KABILAN, M. AYYASAMY, S. JAYAVEL, G. PARAMASAMY, *Pseudomonas* sp. as a source of medium chain length polyhydroxyalkanoates for controlled drug delivery: perspective. Int. J. Microbiol., 2012, Article ID 317828, (2012).
10. P. WECKER, X. MOPPERT, C. SIMON-COLIN, B. COSTA, V. BERTEAUX-LECELLIER, Discovery of a mcl-PHA with unexpected biotechnical properties: the marine environment of French Polynesia as a source for PHA-producing bacteria. AMB Expr., 5 (74), (2015).
11. M.-G.SOARE (VLADU), C. TOMULESCU, M. PETRESCU, I. LUPESCU, M. MOSCOVICI, O. POPA, N. BABEANU, Antimicrobial activity of newly isolated *Bacillus* sp. and *Pseudomonas* sp. strains and their potential use as biocontrol agents, Scientific Bulletin. Series F. Biotechnologies, Vol. XXI, ISSN 2285-1364, 81-86, 2017.
12. X. JIANG, J.A. RAMSAY, B.A.RAMSAY, Acetone extraction of mcl-PHA from *Pseudomonas putida KT2440*, J. Microbiol. Methods, 67(2), 212, 219 (2006)