

Biodegradable bacterial polyester, poly (hydroxybutyrate-co-hydroxyvalerate) copolymer, produced by moderately halophile bacterium *Halomonas* sp. PR-1 isolated from marine environment

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Abstract: PHA (polyhydroxyalkanoate) production by halophiles has attracted much attention in recent years. It was certified that the halophile bacteria *Halomonas* sp. PR-1 isolated from saltern soil synthesised poly (hydroxybutyrate-co-hydroxyvalerate) (PHBV) intracellularly from simple carbon substrate by means of FT-IR spectra analysis. The carbon and nitrogen source suitable for PHA production were selected as glucose and NH₄Cl, respectively. The optimal ratio of glucose to NH₄Cl was 20, when PHA content was 2.85 g/L and PHA yield was 50.85%. The optimal NaCl concentration for PHA biosynthesis was 30 g/L, when PHA yield was 63.3%. The halophile bacterium *Halomonas* sp. PR-1 was considered as a promising candidate for PHA production.

Keywords: bacterial polyester; poly (hydroxybutyrate-co-hydroxyvalerate); halophile; C/N ratio

1. Introduction

Polyhydroxyalkanoate (PHA) is a group of bacterial polyesters and synthesised by numerous bacteria as an intracellular energy storage material under nutrient-limiting conditions with excess carbon. The bacterial polyesters may be considered as the alternatives to plastic materials as their structural properties are similar to polyethylene and polypropylene [1–3]. Their good biodegradability and biocompatibility make them profitable biomaterial for application in packaging, agriculture and medical field [4–6]. Recently, COVID-19 pandemic increased the demand for plastics and consequently highlighted the associated environmental challenge [7,8]. In relation to this situation bioplastics such as PHA have increasingly become a more sustainable solution [9].

The most common polyhydroxyalkanoate is polyhydroxybutyrate (PHB), a homopolymer formed by polymerization of 3-hydroxybutyrate. PHB is easy to break down during melting stage because its melting temperature is nearly few degrees low than its degradation temperature. In addition, its structural nature is brittleness, hard and crystalline, and therefore high crystallinity results in slow biodegradation of PHB [10,11]. A way to overcome the drawbacks posed by homopolymer for its applications is biosynthesis of copolymers consisting of 3-hydroxybutyrate (3HB) and the other hydroxyalkanoate. It was reported that copolymers had faster hydrolytic and enzymatic degradation rate than homopolymers due to their low crystallinity [12].

Many studies on biosynthesis and characterization of various copolymers including copolymers of 3HB with 3-hydroxyvalerate (3HV), 3-hydroxyhexanoate (3HH) and 4-hydroxybutyrate (4HB) have been reported. For biosynthesis of copolymers such as poly (hydroxybutyrate-co-hydroxyvalerate) (PHBV) the precursors such as propionic acid and valeric acid are accustomed to be added [13,14].

PHA production by halophiles has attracted much attention in recent years [15–18]. PHA biosynthesis in halophilic microbe is a certain countermeasure for adaptation to hypertonic conditions and osmotic fluctuations. PHA granules intracellularly accumulated help bacterial cells to preserve cell integrity when exposed to sudden osmotic imbalances [19]. PHA production by halophiles provides additional advantages: PHA biosynthesis can be performed in open unsterile environment and PHA granules can be easily obtained from bacterial cells by osmotic lysis [20,21].

Especially halophiles synthesize PHA with relatively high molecular weight including PHBV without addition of any precursors. Extremely halophilic archaeobacterium, *Haloferax mediterranei* accumulates PHBV on from enzymatic hydrolyzed starch [22]. Moderately halophilic and alkalitolerant *Halomonas campisalis* MCM B-1027 produces copolymer PHBV with molecular weight of 1.3×10^6 when provided of simple carbon source like maltose [23].

This study describes the biosynthesis of PHBV by a moderately halophilic strain, *Halomonas* sp. PR-1 isolated from saltern soil in the western of DPR Korea.

2. Materials and methods

2.1. Microorganism and PHA production medium

A gram-negative halophilic bacterium was isolated from saltern soil in the western of DPR Korea and was identified as a *Halomonas* sp. PR-1 by 16S rRNA phylogenetic analysis. For PHA production, *Halomonas* sp. PR-1 was cultivated in mineral medium consisting of (g/L): KH_2PO_4 (1.5); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5); $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05) nitrogen source (2.0); carbon source (20); NaCl (10–150). The pH of the medium was adjusted to 7.5 with 1 N NaOH/5 N HCl.

2.2. PHA production studies

PHA level was measured by crotonic acid assay method. Biomass was determined turbidimetrically: turbidity of culture broth was measured at 660 nm (OD_{660}) and then was converted to cell dry weight via a standard curve. All experiments were carried out in shakable Erlenmeyer flasks and values were measured 3 times and estimated statistically.

2.2.1. Effect of carbon source and nitrogen source

Halomonas sp. PR-1 was grown in 500 mL Erlenmeyer flask containing 50 mL mineral medium with individual addition of glucose, fructose, sucrose, lactose and glycerol as a carbon source. The carbon source type which recorded the highest PHA level was used in subsequent experiments. *Halomonas* sp. PR-1 was cultivated in medium with individual addition of $(\text{NH}_4)_2\text{SO}_4$, NH_4Cl , NH_4NO_3 , $(\text{NH}_4)_3\text{PO}_4$ and

NaNO₃ as a nitrogen source. The nitrogen source which provides the maximum PHA production was utilized in the subsequent experiments. The effect of carbon to nitrogen (C/N) ratio on the PHA production by *Halomonas* sp. PR-1 was investigated in mineral medium formulated by varying carbon source level at a fixed concentration of nitrogen source. Experiments were performed by using a fixed NaCl concentration of 50 g/L.

2.2.2. Effect of sodium chloride

For investigating the effects of NaCl on PHA production, *Halomonas* sp. PR-1 was grown in minimal medium inclusive of varying concentrations of NaCl (10, 30, 50, 70, 100, 120 and 150 g/L).

2.3. FT-IR spectroscopy

The absorption spectrum curves were obtained using a Fourier transform infrared (FT-IR) spectrometer WQB-520A by scanning. The extracted and purified PHA samples were mixed with pulverized potassium bromide (KBr) and then compacted in tab form. The scanning was performed at wavelength range between 4000 and 500 cm⁻¹.

3. Results and discussion

3.1. FT-IR spectroscopy

Purified PHA sample was characterised with respect to FT-IR spectra analysis (**Figure 1**). FT-IR spectra displayed the absorption band at 1724 cm⁻¹ corresponding to carbonyl group in ester bond. The FT-IR result of PHA formed from *Halomonas* sp. PR-1 in complete agreement with standard FT-IR spectra of copolymer PHBV. PHA sample analysed was made from glucose, the simplest carbon source.

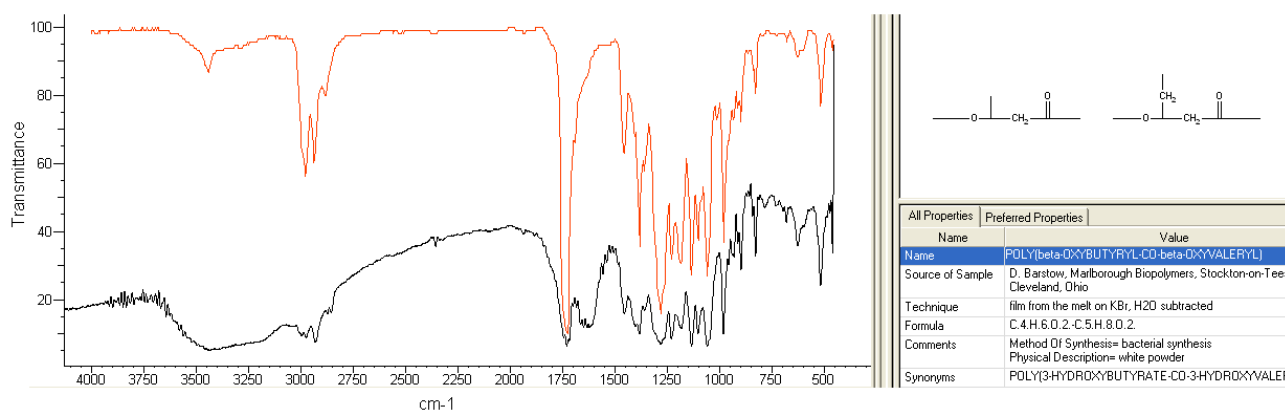


Figure 1. FTIR spectrum of PHA extracted from *Halomonas* sp. PR-1.

3.2. Effect of carbon source type on PHA biosynthesis by *Halomonas* sp. PR-1

The type of carbon substrate significantly affects bacterial growth and PHA production. In our study, simple carbon sources such as glucose, sucrose, fructose, lactose and glycerol were compared in terms of biomass and PHA production. As shown in **Figure 2** glucose supported the highest production level of biomass of 5.52

g/L and PHA of 1.58 g/L among the carbon sources tested. Glucose is the simplest carbon substrate that can be easily metabolized by microbe. *Halophile* bacterium *Halomonas* sp. PR-1 formed intracellularly PHBV from glucose (**Figure 1**). To our knowledge, so far there is no report on copolymer biosynthesis from glucose by halophile bacterium without addition of any precursor. Moderately halophilic and alkalitolerant *Halomonas campisalis* MCM B-1027 produces copolymer PHBV using maltose [23].

The production level of biomass and PHA formed from sucrose and fructose was quantitatively like. Sucrose has been commonly used in PHA production by the other species such as *Halomonas boliviensis* and *Alcaligenes latus* [24]. The amount of PHA produced from lactose was trivial in comparison with those from the others, indicating that lactose is not a proper carbon source for PHA production.

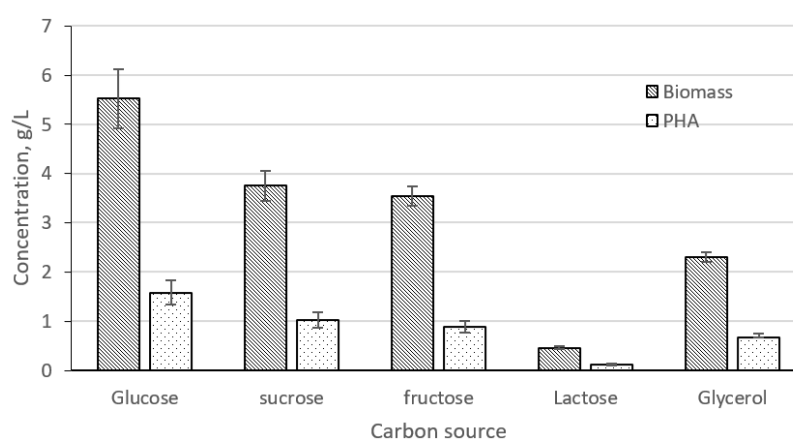


Figure 2. Effect of carbon source type on biomass and PHA biosynthesis in a batch culture of *Halomonas* sp. PR-1 grown in a mineral medium at a temperature of 30 °C and an agitation rate of 200 rpm for 70 h.

3.3. Effect of nitrogen source type on PHA biosynthesis by *Halomonas* sp. PR-1

The effect of nitrogen source type on PHA biosynthesis by *Halomonas* sp. PR-1 is examined (**Figure 3**). Among the various nitrogen sources screened, ammonium chloride supported the growth and PHA production of *Halomonas* sp. PR-1. Ammonium chloride provides the highest biomass of 5.8 g/L with PHA of 1.66 g/L. This agrees with results of other study in which *H. boliviensis* produced PHA using ammonium chloride as the nitrogen source. However, Gao et al. reported that the most suitable nitrogen source for PHA production by *H. venusta* was $(\text{NH}_4)_2\text{SO}_4$ [25]. Kawata et al. [24] reported NaNO_3 as the nitrogen source supported the greatest PHA biosynthesis by *Halomonas* sp. KM-1. However, in this study the biomass and PHA production showed the lowest level when using NaNO_3 as nitrogen source. This may be explained by diversity of strain preference and variation. Like carbon source, the nitrogen source is important compound for microbial metabolism. The deprivation or reduction of the nitrogen supply to microbial cells results in the limitation of bacterial cell multiplication thereby decreasing the volumetric productivity of PHA. However, bacteria have been found to accumulate PHA when there is a limitation of nutrients, specifically nitrogen sources. Therefore, the ratio of

carbon to nitrogen in growth medium should be essentially examined for PHA production studies.

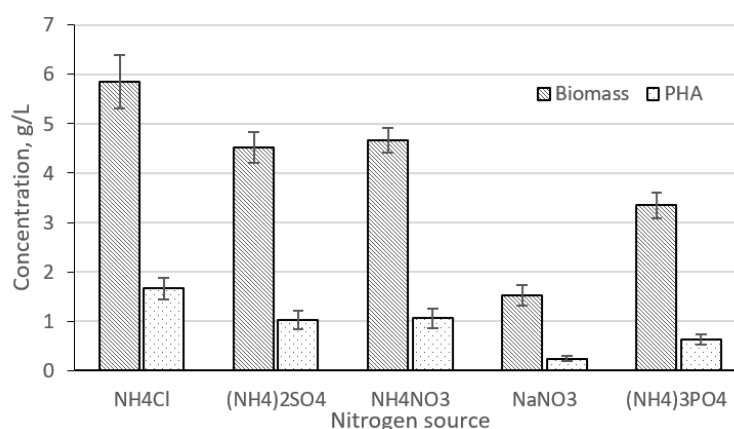


Figure 3. Effect of nitrogen source type on biomass and PHA biosynthesis in a batch culture of *Halomonas* sp. PR-1 grown in a mineral medium at a temperature of 30 °C and an agitation rate of 200 rpm for 70 h.

3.4. Effect of C/N ratio on PHA biosynthesis by *Halomonas* sp. PR-1

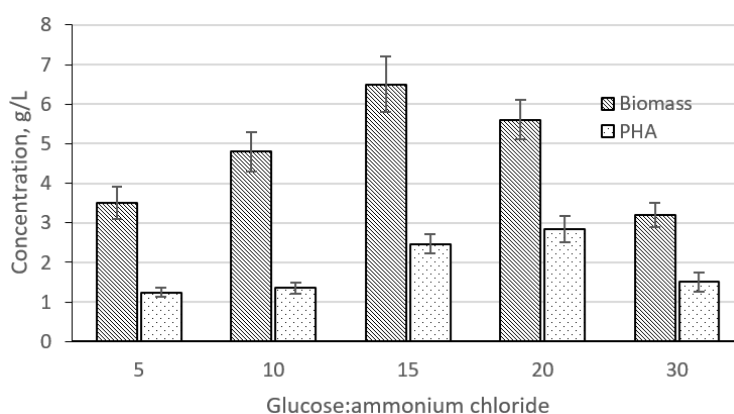


Figure 4. Effect of C/N ratio on biomass and PHA biosynthesis in a batch culture of *Halomonas* sp. PR-1 grown in a mineral medium at a temperature of 30 °C and an agitation rate of 200 rpm for 70 h.

For microbial growth the carbon and nitrogen substrates are essential. In general, Carbon requirement of microorganism is larger than nitrogen requirement and affects the organisms' ability to utilize the nutrition material. Especially, the ratio of carbon to nitrogen concentration is very important for PHA production because PHA accumulation in bacteria cell is usually triggered under abnormal growth environment. As shown in **Figure 4**, the effect of the ratio of glucose to NH₄Cl on PHA production was studied at various levels. The highest PHA level of 2.85 g/L was achieved when glucose and ammonium chloride ratio was 20, with biomass production of 5.6 g/L corresponding to PHA yield of 50.89%. *Halomonas* sp. PR-1 biosynthesized maximum biomass of 6.5 g/L at the ratio of glucose to ammonium chloride of 15, but at which its PHA productivity was worse than that at 20 of glucose and ammonium chloride ratio. When glucose and ammonium chloride

ratio was 30 in growth medium PHB yield was relatively high, but volumetric productivity of PHA decreased. These results indicate that C/N ratio is very important factor affects PHA production by microorganism. Therefore, the optimal ratio of glucose to ammonium chloride was set for 20. The optimal C/N ratio for PHA production varies with the bacterial species. In *Haloferax mediterranei*, an extreme halophilic archaeobacterium, the maximum PHA yield of 47.22% was achieved at glucose and NH_4Cl medium with C/N ratio of 35 [22]. When the C/N ratio of 8 was selected for *Cupriavidus taiwanensis* 184, PHA productivity was the highest [26].

3.5. Effect of NaCl on PHA biosynthesis by *Halomonas* sp. PR-1

The growth of marine bacteria and its PHA accumulation are subjected to influence of the salinity. To examine the effect of NaCl concentration on PHA biosynthesis by *Halomonas* sp. PR-1, the NaCl concentrations in medium were conditioned to 10, 30, 50, 70, 100, 120 and 150 g L^{-1} . The highest amount of PHA (3.25 g/L) was obtained at 30 g/L of NaCl concentration with 56.03% of PHA yield (Figure 5). The largest cell density (6.8 g/L) was obtained at 70 g/L of NaCl concentration with low PHA yield of 37.5%. As the NaCl concentration increased up to 70 g/L of NaCl concentration the amount of biomass and PHA decreased indicating that high NaCl concentration inhibited cell growth and PHA accumulation. This finding reflects the necessity of controlling the salinity in medium to overcome osmotic stress and its effect on PHA production [19]. Therefore, the optimal NaCl concentration for PHA production by *Halomonas* sp. PR-1 was 30 g/L being different from those reported the by previous studies of the other halophile bacteria. For example, the proper NaCl concentration for PHA production by the moderately halophile *H. boliviensis* was 45 g/L , and marine bacteria *Vibrio* sp. BM-1 produced the maximum PHA at 18 g/L of NaCl [27,28].

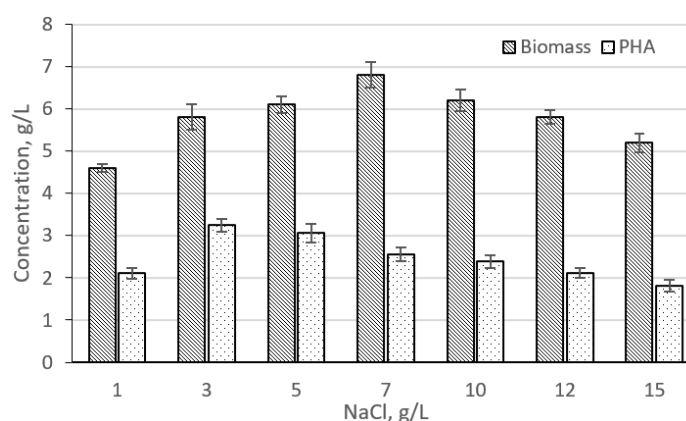


Figure 5. Effect of NaCl concentration on biomass and PHA biosynthesis in a batch culture of *Halomonas* sp. PR-1 grown in a mineral medium at a temperature of 30 °C and an agitation rate of 200 rpm for 70 h.

4. Conclusion

The halophile bacterium *Halomonas* sp. PR-1 isolated from saltern soil synthesised PHBV intracellularly synthesise from glucose. The proper C/N ratio was

20, in which PHA of 2.85 g/L was obtained with 50.85% of PHA yield. The optimal NaCl concentration for PHA production was 30 g/L with 63.3% of PHA yield. The halophile bacterium *Halomonas* sp. PR-1 was considered as promising candidate for PHA production.

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References

1. Kushwah BS, Kushwah AVS, Singh V. RETRACTED ARTICLE: Towards understanding polyhydroxyalkanoates and their use. *Journal of Polymer Research*. 2016; 23(8). doi: 10.1007/s10965-016-0988-3
2. Mozejko-Ciesielska J, Kiewisz R. Bacterial polyhydroxyalkanoates: Still fabulous? *Microbiological Research*. 2016; 192: 271-282. doi: 10.1016/j.micres.2016.07.010
3. Raza ZA, Khalil S, Abid S. Recent progress in development and chemical modification of poly(hydroxybutyrate)-based blends for potential medical applications. *International Journal of Biological Macromolecules*. 2020; 160: 77-100. doi: 10.1016/j.ijbiomac.2020.05.114
4. Ray S, Kalia VC. Biomedical Applications of Polyhydroxyalkanoates. *Indian Journal of Microbiology*. 2017; 57(3): 261-269. doi: 10.1007/s12088-017-0651-7
5. Gadgil BST, Killi N, Rathna GVN. Polyhydroxyalkanoates as biomaterials. *MedChemComm*. 2017; 8(9): 1774-1787. doi: 10.1039/c7md00252a
6. Diniz MSF, Mourão MM, Xavier LP, et al. Recent Biotechnological Applications of Polyhydroxyalkanoates (PHA) in the Biomedical Sector—A Review. *Polymers*. 2023; 15(22): 4405. doi: 10.3390/polym15224405
7. Vanapalli KR, Sharma HB, Ranjan VP, et al. Challenges and strategies for effective plastic waste management during and post COVID-19 pandemic. *Science of The Total Environment*. 2021; 750: 141514. doi: 10.1016/j.scitotenv.2020.141514
8. Haque MdS, Sharif S, Masnoon A, et al. SARS-CoV-2 pandemic-induced PPE and single-use plastic waste generation scenario. *Waste Management & Research: The Journal for a Sustainable Circular Economy*. 2021; 39(1_suppl): 3-17. doi: 10.1177/0734242x20980828
9. Vicente D, Proença DN, Morais PV. The Role of Bacterial Polyhydroalkanoate (PHA) in a Sustainable Future: A Review on the Biological Diversity. *International Journal of Environmental Research and Public Health*. 2023; 20(4): 2959. doi: 10.3390/ijerph20042959
10. Meereboer KW, Misra M, Mohanty AK. Review of recent advances in the biodegradability of polyhydroxyalkanoate (PHA) bioplastics and their composites. *Green Chemistry*. 2020; 22(17): 5519-5558. doi: 10.1039/d0gc01647k
11. Kunioka M, Doi Y. Thermal degradation of microbial copolyesters: poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and poly(3-hydroxybutyrate-co-4-hydroxybutyrate). *Macromolecules*. 1990; 23(7): 1933-1936. doi: 10.1021/ma00209a009
12. Renard E, Walls M, Guérin P, et al. Hydrolytic degradation of blends of polyhydroxyalkanoates and functionalized polyhydroxyalkanoates. *Polymer Degradation and Stability*. 2004; 85(2): 779-787. doi: 10.1016/j.polymdegradstab.2003.11.019
13. Anjum A, Zuber M, Zia KM, et al. Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: A review of recent advancements. *International Journal of Biological Macromolecules*. 2016; 89: 161-174. doi: 10.1016/j.ijbiomac.2016.04.069
14. Thiré RMDSM, Arruda LC, Barreto LS. Morphology and thermal properties of poly(3-hydroxybutyrate-co-3-hydroxyvalerate)/attapulgit nanocomposites. *Materials Research*. 2011; 14(3): 340-344. doi: 10.1590/s1516-14392011005000046

15. Chen GQ, Jiang XR. Next generation industrial biotechnology based on extremophilic bacteria. *Current Opinion in Biotechnology*. 2018; 50: 94-100. doi: 10.1016/j.copbio.2017.11.016
16. Mitra R, Xu T, Xiang H, et al. Current developments on polyhydroxyalkanoates synthesis by using halophiles as a promising cell factory. *Microbial Cell Factories*. 2020; 19(1). doi: 10.1186/s12934-020-01342-z
17. Yin J, Chen JC, Wu Q, et al. Halophiles, coming stars for industrial biotechnology. *Biotechnology Advances*. 2015; 33(7): 1433-1442. doi: 10.1016/j.biotechadv.2014.10.008
18. Quillaguamán J, Guzmán H, Van-Thuoc D, et al. Synthesis and production of polyhydroxyalkanoates by halophiles: current potential and future prospects. *Applied Microbiology and Biotechnology*. 2009; 85(6): 1687-1696. doi: 10.1007/s00253-009-2397-6
19. Sedlacek P, Slaninova E, Koller M, et al. PHA granules help bacterial cells to preserve cell integrity when exposed to sudden osmotic imbalances. *New Biotechnology*. 2019; 49: 129-136. doi: 10.1016/j.nbt.2018.10.005
20. Chen X, Yin J, Ye J, et al. Engineering *Halomonas bluephagenesis* TD01 for non-sterile production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate). *Bioresource Technology*. 2017; 244: 534-541. doi: 10.1016/j.biortech.2017.07.149
21. Tan D, Xue YS, Aibaidula G, et al. Unsterile and continuous production of polyhydroxybutyrate by *Halomonas* TD01. *Bioresource Technology*. 2011; 102(17): 8130-8136. doi: 10.1016/j.biortech.2011.05.068
22. Chen CW, Don TM, Yen HF. Enzymatic extruded starch as a carbon source for the production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by *Haloferax mediterranei*. *Process Biochemistry*. 2006; 41(11): 2289-2296. doi: 10.1016/j.procbio.2006.05.026
23. Kulkarni SO, Kanekar PP, Nilegaonkar SS, et al. Production and characterization of a biodegradable poly (hydroxybutyrate-co-hydroxyvalerate) (PHB-co-PHV) copolymer by moderately haloalkalitolerant *Halomonas campisalis* MCM B-1027 isolated from Lonar Lake, India. *Bioresource Technology* 2010; 101: 9765-9771.
24. Kawata Y, Shi LH, Kawasaki K, et al. Taxonomic characterization and metabolic analysis of the *Halomonas* sp. KM-1, a highly bioplastic poly(3-hydroxybutyrate)-producing bacterium. *Journal of Bioscience and Bioengineering*. 2012; 113(4): 456-460. doi: 10.1016/j.jbiosc.2011.11.018
25. Gao S, Zhang LH. The Synthesis of Poly- β -Hydroxybutyrate by Moderately Halophilic Bacteria *Halomonas venusta* *Advanced Materials Research*. 2014; 1033-1034: 306-310. doi: 10.4028/www.scientific.net/amr.1033-1034.306
26. Wei YH, Chen WC, Huang CK, et al. Screening and Evaluation of Polyhydroxybutyrate-Producing Strains from Indigenous Isolate *Cupriavidus taiwanensis* Strains. *International Journal of Molecular Sciences*. 2011; 12(1): 252-265. doi: 10.3390/ijms12010252
27. García-Torreiro M, Lu-Chau TA, Lema JM. Effect of nitrogen and/or oxygen concentration on poly(3-hydroxybutyrate) accumulation by *Halomonas boliviensis*. *Bioprocess and Biosystems Engineering*. 2016; 39(9): 1365-1374. doi: 10.1007/s00449-016-1612-y
28. Wei YH, Chen WC, Wu HS, et al. Biodegradable and Biocompatible Biomaterial, Polyhydroxybutyrate, produced by an Indigenous *Vibrio* sp. BM-1 Isolated from Marine Environment. *Marine Drugs*. 2011; 9(4): 615-624. doi: 10.3390/md9040615