HNUE JOURNAL OF SCIENCE DOI: 10.18173/2354-1059.2020-0050
Natural Sciences 2020, Volume 65, Issue 10, pp. 73-81
This paper is available online at http://stdb.hnue.edu.vn

PRODUCTION OF POLY(3-HYDROXYBUTYRATE) FROM RAW CASSAVA STARCH BY *Bacillus megaterium* D8

Doan Van Thuoc[1], Tran Thi Loan[2] and Pham Thi Hong Hoa[1]
[1]*Faculty of Biology, Hanoi National University of Education*
[2]*Student of the Faculty of Biology, Hanoi National University of Education*

Abstract. Biosynthesis of polyhydroxyalkanoate (PHA) from raw cassava starch as the carbon source by bacteria was investigated in this study. About 300 bacterial colonies were isolated from soil samples. Among them, sixteen bacterial strains were found to produce PHA from cassava starch. Strain D8 produced the highest poly(3-hydroxybutyrate) (PHB) content of 69.8 wt% was selected for further studies. Strain D8 was classified under the *Bacillus megaterium* group based on 16S rRNA gene sequences. High cell dry weight (CDW) of 6.5 g/L and poly(3-hydroxybutyrate) (PHB) content of 66.2 wt% were obtained by strain D8 after 21 h of cultivation in a bioreactor using batch culture mode. In this study, *Bacillus megaterium* D8 exhibited high promise for reducing the production cost of PHB.

Keywords: *Bacillus megaterium*, cassava starch, poly(3-hydroxybutyrate), carbon source.

1. Introduction

In nature, many microorganisms accumulate polyhydroxyalkanoate (PHA) as reserves of carbon and energy, usually when grown in the presence of excess carbon and limitation of nutrients such as nitrogen, oxygen, phosphorus, and sulfur [1]. To date, there are over 150 PHA monomer subunits that have been found [2]. The properties of PHA are similar to those of common petrochemical-based synthetic thermoplastics and can hence potentially replace them in many application areas such as packaging and coating, as well as biodegradable carriers for a long-term dosage of drugs, medicines, hormones, insecticides, and herbicides. After use, they become completely degraded to carbon dioxide and water under aerobic conditions and methane and carbon dioxide under anaerobic conditions by various microorganisms in the environment [3, 4].

However, the production cost of PHA is currently too high as compared to that of the non-biodegradable plastics of fossil origin. Up to 50% of the total production cost is attributed to the carbon source [5]. Therefore, many studies have been focused on the use of inexpensive carbon substrates such as waste lipids, cheese whey, starch, starchy residues, lignocellulosic residues, and crude glycerol [2, 6, 7].

Received October 12, 2020. Revised October 22, 2020. Accepted October 29, 2020.
Contact Doan Van Thuoc, e-mail address: thuocdv@hnue.edu.vn

Cassava is the world's fourth most important staple crop after rice, wheat, and maize, and plays an essential role in food security. Cassava is adapted to growing on poor degraded soils and can tolerate low pH, high levels of exchangeable aluminum, and low concentrations of phosphorus. Cassava starch is an important source of biomaterial for different food and non-food industrial applications. With more than 10 million metric tons in 2017, Vietnam is the seventh cassava producing country [8]. Recently, cassava starch was considered as a cheap carbon source for PHA production. The mixture of cassava starch and valerate have been used as carbon sources for copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) production by *Caldimonas taiwanensis* [9].

The study aims to isolate bacteria that can use cassava starch as a sole carbon substrate for PHA production. The selected strain will be identified using 16S rRNA gene. The ability to use cassava starch as the source of carbon for PHA production by selected strain will also be evaluated.

2. Content

2.1. Materials and methods

2.1.1. Isolation of bacteria

Soil samples collected from Hanoi were serially diluted with 0.9% NaCl solution, and then 100 µL of the dilution was spread on solid MPA (Meat-Peptone-Agar) medium containing (g/L): NaCl, 5g; meat extract, 5g; peptone, 5g; and granulated agar, 20. The plates were incubated at 35 ^{o}C for 48 h. More than 200 colonies were isolated by plating them again on a fresh agar medium.

2.1.2. Screening of PHA producing bacteria

PHA producing bacteria were then detected by Nile blue A staining method [10]. For that, bacterial isolates were grown on modified MPA medium containing (g/L): NaCl, 5; meat extract, 1; peptone, 1; glucose, 20; granulated agar, 20; and Nile blue A (Sigma) (dissolved in dimethylsulfoxide) with a final concentration of 0.5 µg dye per mL of the medium. The agar plates were incubated at 35 ^{o}C for 48 h and then exposed to ultraviolet light (312 nm). The colonies with fluorescent bright orange were chosen for further studies.

The selected bacterial strains were grown in 20 mL of liquid MPA medium in 100 mL Erlenmeyer flasks with rotary shaking at 180 rpm for 13 h. Subsequently, 1 mL of each culture was inoculated in 50 mL of modified MPA medium in 250 mL Erlenmeyer flasks. The medium contains (g/L) NaCl, 5; meat extract, 1; peptone, 1; cassava starch, 20, the pH of this medium was initially adjusted to 7.0. The cultures were incubated at 35 ^{o}C with rotary shaking at 180 rpm. Samples were withdrawn at 48 h of cultivation for cell dry weight (CDW) determination and PHA content analysis.

2.1.3. Phylogenetic characterization of the selected PHA producing bacterium

The genomic DNA of the selected strain was extracted by Thermo Scientific GeneJET Genomic DNA Purification Kit according to the manufacturer's recommendations. The 16S rRNA gene was amplified using the universal primers, 341F (5'-CCTACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTCCTTTGAGTTT-3').

Sequencing of the amplified DNA fragment was performed at 1st Base (Singapore), and the GenBank database was used to search for 16S rRNA genes similarities. Phylogenic analysis based on 16S rRNA gene was performed with the aid of MEGA6 software [11] using the neighbor-joining distance correlation method [12].

2.1.4. Effect of different nitrogen sources on growth rate and PHA accumulation of the selected strain

The selected strain was grown in a modified liquid minimum medium containing (g/L): NaCl, 5; $MgSO_4.7H_2O$, 0.4; $FeSO_4.7H_2O$, 0.02; 0.05 M phosphate buffer (pH 7); starch, 20; and 2 g of different nitrogen sources (ammonium chloride - AC, ammonium sulfate - AS, ammonium nitrate - AN, Urea, sodium nitrate - SN, potassium nitrate - PN, Glutamate - Glu, meat extract and peptone - ME+P). After 30 h of cultivation at 35 °C and 180 rpm, the bacterial cells were collected by centrifugation for CDW and PHA analysis.

2.1.5. PHA production in batch fermentation

The selected bacterial strain was initially grown in 6 different 250 mL flasks containing 50 mL MPA medium at 35 ^oC with rotary shaking at 180 rpm for 15 h. The culture was then used to inoculate 2.7 liters of modified MPA medium containing 20 g/L cassava starch as a carbon source in a 10-liter bioreactor. The cultivations were performed in batch mode during which temperature was kept constant at 35 ^oC and pH was maintained at 7.0 by adding 5 M HCl/NaOH. Stirring velocity and aeration, initially set at 250 rpm and 1 L/min, were increased during the fermentation and reached 500 rpm and 3 L/min, respectively. Samples were taken at different time intervals for CDW, PHA, and reducing sugar analysis.

2.1.6. Analytical methods

CDW was determined by centrifuging 3 ml of the culture samples at 10 000 rpm for 10 min in a pre-weighed centrifuge tube, the pellet was washed once with 3 mL distilled water, centrifuged and dried at 105 ^oC until a constant weight was obtained. The centrifuge tube was weighed again to calculate the CDW.

The amylase activity was determined based on the release of reducing sugar from starch (Merck) using the dinitrosalicylic acid (DNS) method [13]. A standard curve was made using glucose (Merck) as the standard. One unit of amylase activity was defined as the amount of enzyme releasing 1 μmol reducing sugar equivalent to glucose/min under the standard assay conditions.

The concentration of reducing sugar during the fermentation process was also analyzed by using the DNS method [13].

PHA content (wt%) in dried cell mass and its composition were determined by gas chromatography (GC) [14]. Approximately 10-15 mg lyophilized cells were mixed with 2 mL methanolysis solution (contains 15% H_2SO_4 and 85% methanol, v/v) and 2 mL analytical grade chloroform. The methanolysis process was carried out for 140 min at 100 ^oC by using thermoblock. After cooling down to room temperature, 1 mL MiliQ water was added to the mixture and vortexed for 30 seconds. The bottom layer containing methyl ester was transferred to sodium sulfate anhydrous to remove the remaining water and analyzed by using Trace 1310 GC system (Thermo Scientific, Italy)

equipped with capillary HP-5 column. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) containing 12 % valerate (Sigma) was used as a standard for calibration.

2.2. Results and discussion

2.2.1. Isolation and screening of PHA producing bacteria

Soil samples were serially diluted and spread on an MPA medium. After 48 h of cultivation, about 300 bacterial colonies were collected by plating on fresh MPA medium. They were then grown on agar plate medium containing Nile blue A for the screening of PHA producers**.** After 48 h of cultivation, the plates were exposed under UV light and the colonies showed fluorescent bright orange were labeled. The cells of labeled strains were then stained with 1% safranin and observed under a light microscope, 31 bacterial strains accumulated PHA granules were selected. The 31 bacterial strains were cultured in the modified MPA medium containing 20 g/L cassava starch. As shown in Table 1, sixteen bacterial strains could use cassava starch as a carbon source for PHA production with the PHA content ranged from 24.7 to 69.84 wt%. Besides, all 16 bacterial strains were found to synthesize extracellular amylase with activities ranged from 0.7 to 1.73 IU/mL. Among them, strain D8 exhibited the highest PHA content of 69.84 wt% and a PHA concentration of 3.36 g/L was chosen for further studies (Figure 1 and Table 1). The data of GC analysis showed that strain D8 accumulated homopolymer poly(3-hydroxyburtyrate) (PHB) (a typical polymer found in the PHA family) from cassava starch.

Table 1. PHA production by isolated strains using cassava starch as carbon substrate

Strain	CDW (g/L)	PHA content (wt%)	PHA conc. (g/L)	Amylase (IU/mL)
D3	4.82	58.8	2.83	1.73
D6	5.24	52.88	2.77	1.46
D7	5.02	59.94	3.01	0.7
D8	4.81	69.84	3.36	1.14
D11	5.82	54.48	3.17	0.82
D43	5.78	46.59	2.69	1.18
D44	4.83	66.71	3.22	1.26
D77	4.03	65.19	2.63	1.56
D92	4.56	64.98	2.96	0.95
D96	3.04	67.22	2.04	0.64
D117	4.33	67.73	2.93	0.8
D138	5.65	59.59	3.37	1.69
D164	4.48	48.16	2.16	0.7
D188	3.88	56.17	2.18	1.53
D189	3.67	57.23	2.1	1.12
D241	1.06	24.7	0.26	1.05

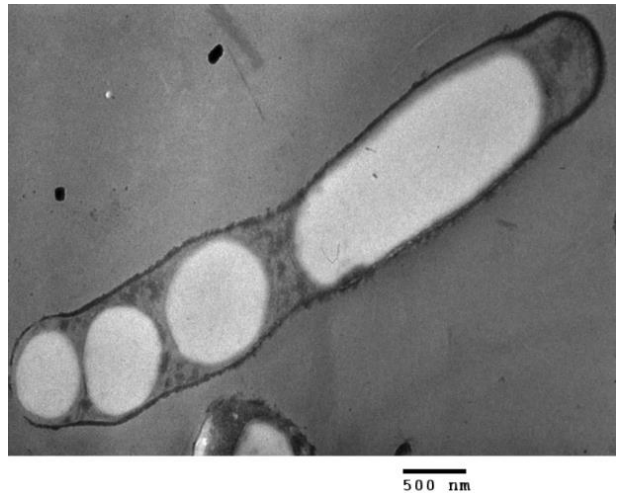

Figure 1. Transmission electron micrographs showing PHA granules in bacterial cell

2.2.1. Identification of selected PHB producer

Strain D8 was an aerobic, Gram-positive, rod-shaped, and spore-forming bacterium. The strain was mesophilic with optimum temperatures for growth of between 32 ^{o}C and 35 ^{o}C, and grows well at pH between 7 and 8. Strain D8 was able to produce extracellular enzymes such as amylase and protease. The phylogenetic characterization of the strain D8 was analyzed using its 16S rRNA gene partial sequences. The results showed that strain D8 belonged to genus *Bacillus*, and showed the closest similarity with *Bacillus megaterium* LY6 and *B. megaterium* S29 (100%), *B. aryabhattai* B8W22, and *B. aryabhattai* POD1 (99.8%) (Figure 2).

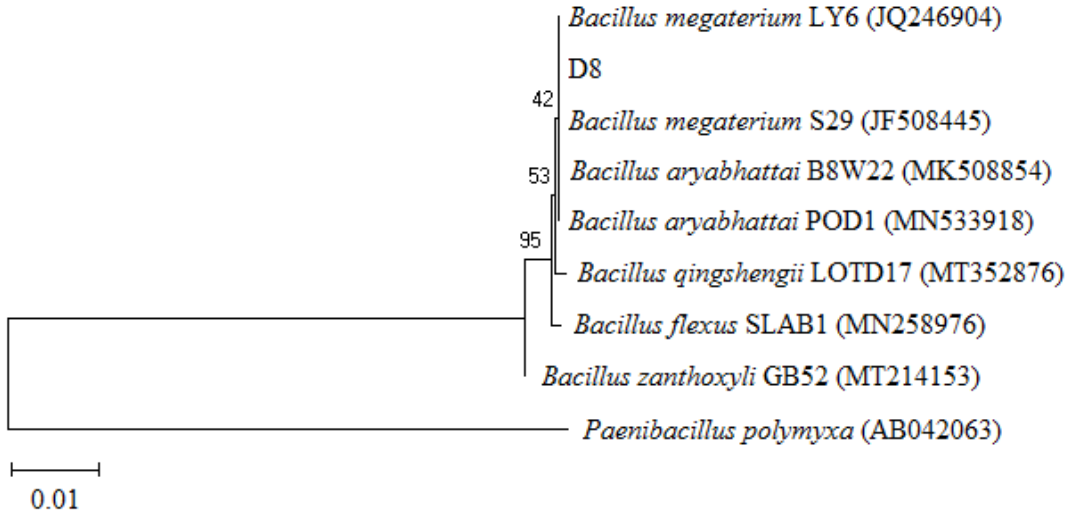

Figure 2. Neighbor-joining phylogenetic tree based on the comparison of 16S rDNA sequences, showing the relationships between the selected strain and other strains of the genus Bacillus. Bar, 0.01 subtitutions per nucleotide position

Bacillus species are extensively studied in the PHA world since the exploration of PHB in the cells of *Bacillus megaterium* by the French Lemoigne in 1926 [15]. Some *Bacillus* species have been reported to produce high PHA content up to 80 wt% of bacterial cells when growing under nutrients imbalance [16]. *Bacillus* species are also capable of producing PHA copolymers utilizing relatively simple, inexpensive, and structurally unrelated carbon sources such as sugars, starch, or glycerol [17].

2.2.3. Effect of different nitrogen sources

The effect of different nitrogen sources on cell growth rate and PHB accumulation by strain *Bacillus megaterium* D8 was investigated in flask experiments. Figure 3 showed that *B. megaterium* D8 was able to grow and accumulate PHA from all tested nitrogen sources. The highest CDW of 5.01 g/L was reached when a mixture of meat extract and peptone (ME+P) was used, whereas high PHB content of 52.3 wt% was obtained when and ammonium sulfate (AS) was used as a nitrogen source. Among 8 different tested nitrogen sources, ammonium chloride, ammonium nitrate, glutamate, and a mixture of meat extract and peptone were found to be favorable nitrogen sources for both cell growth rate and PHB accumulation by *B. megaterium* D8 (Figure 3). The highest PHB concentration of 2.35 g/L was obtained on the medium using a mixture of meat extract and peptone as nitrogen sources.

2.2.4. Production of PHB in a bioreactor

The production of PHA by *B. megaterium* D8 was carried out in a 10-L bioreactor using batch cultivation mode. Bacterial cell mass and PHA accumulation were increased during fermentation, maximum CDW of 6.5 g/L was achieved after 21 h, and PHB content of 69.5 wt% was reached after 24 h of cultivation. Reducing sugar was increased and reached a maximum value of 4.8 g/L after the first 10 h of fermentation; it was then consumed by strain *B. megaterium* D8 and finished after 27 h of cultivation. A maximum PHB concentration of 4.3 g/L was obtained after 21 h of cultivation (Figure 4). The PHB yield from cassava starch under batch cultivation mode was 0.215 g/g (product/substrate, P/S).

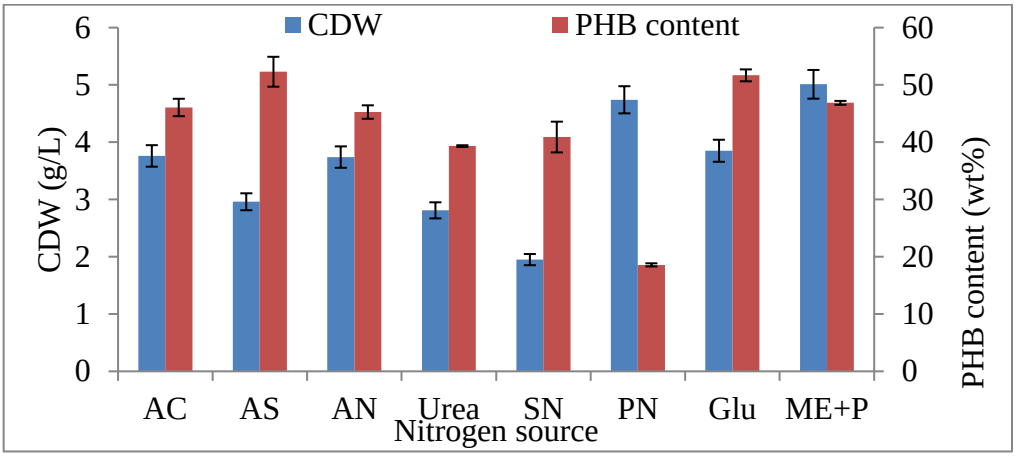

Figure 3. Effect of different nitrogen sources on the cell growth rate and PHB accumulation by strain B. megaterium D8

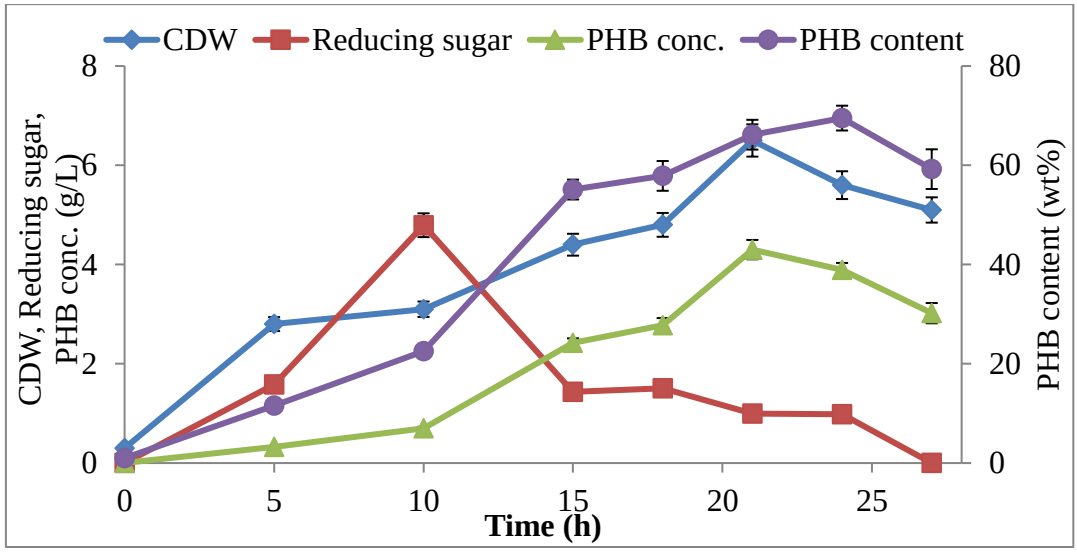

Figure 4. Profile of the growth rate and PHB accumulation by strain D8 in a bioreactor using batch culture mode

There are only a few bacteria can use directly raw starch as a carbon source for PHA production. For example, *Caldimonas taiwanensis* produced copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) when cultivated on starch and valerate [9], *Bacillus cereus* [18], *Saccharophagus degradans* [19], *Bacillus aryabhattai* [20] synthesized PHB when used starch as carbon substrate, and wild type strain *Bacillus megaterium* could also synthesize PHBV when starch was used as a carbon substrate [21]. Table 2 showed that PHA productivity and yield obtained by *B. megaterium* D8 in this study were much higher than those obtained by other bacteria, suggested that *B. megaterium* D8 could be a promising candidate for PHA production from cassava starch.

Table 2. PHA production by bacterial strains using starch as a carbon source

Strain	PHAs	Carbon source	CDW (g/L)	PHA content (wt%)	PHA productivity (g/L/h)	Yield (g/g) P/S)	References
B. cereus	PHB	S	1.0	48	0.007	0.024	[18]
S. degradans	PHB	S	7.44	7.12	0.022	0.028	[19]
B. aryabhattai	PHB	S	4.4	46	0.067	0.067	[20]
C. taiwanensis	PHBV	CaS+V	2.8	67	0.058	0.121	[9]
		CoS+V	3.3	65	0.067	0.138	
		WS+V	4.1	42	0.054	0.111	
B. megaterium	PHBV	S	1.72	24	0.023	0.083	[21]
B. megaterium	PHB	CaS	6.5	66.2	0.205	0.215	This work

Note: *S – soluble starch; CaS – cassava starch; CoS – corn starch; WS – wheat starch; V - valerate*

3. Conclusions

Sixteen bacterial strains were found to produce PHA when cultivated on starch medium. Among them, strain *B. megaterium* D8 can produce high PHB content of 69.8 wt% from cassava starch and a mixture of meat extract and peptone. This is an interesting feature considering that cassava starch could be renewable, cheap, and widely available carbon source. This work will contribute to finding the most effective way to reduce PHA production costs.

Acknowledgment. The authors acknowledge the Hanoi National University of Education, Vietnam for providing infrastructure facilities.

REFERENCES

[1] Sudesh, K., Abe, H., Doi, Y., 2000. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Prog. Polym. Sci.*, 25, pp. 1503-1555.

[2] Surendran, A., Lakshmanan, M., Chee, J.Y., Sulaiman, A.M., Thuoc, D.V., Sudesh, K., 2020. Can polyhydroxyalkanoates be produced efficiently from waste plant and animal oils? *Front. Bioeng Biotechno*l., 8, 169.

[3] Bugnicourt, E., Cinelli, P., Lazzeri, A., Alvarez, V., 2014. Polyhydroxyalkanoate (PHA): Review of synthesis, characteristics, processing and potential applications in packaging. Express. *Polym. Lett*., 8, pp. 791-808.

[4] Ali, I., Jamil, N., 2016. Polyhydroxyalkanoates: Current applications in the medical field. *Front. Biol*., 11, pp. 19-27.

[5] Choi, L., Lee, S.Y., 1999. Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. *Appl. Microbiol. Biotechnolo*., 51, pp. 13-21

[6] Jiang, G., Hill, D.J., Kowalczuk, M., Johnston, B., Adamus, G., Irorere, V., Radecka, I., 2016. Carbon sources for polyhydroxyalkanoates and an intergrated biorefinery. Int. *J. Mol. Sci.*, 17, 1157.

[7] Favaro, L., Basaglia, M., Casella, S., 2019. Improving polyhydroxyalkanoate production from inexpensive carbon sources by genetic approaches: a review. *Biofuels Bioprod. Bioref*., 13, pp. 208-227.

[8] Sivamani, S., Chandrasekaran, A.P., Balajii, M., Shanmugaprakash, M., Hosseini-Bandegharaei, A., Baskar, R., 2018. Evaluation of the potential of cassava-based residues for biofuels production. *Rev. Environ. Sci. Biotechnol.*, 17, pp. 553-570.

[9] Sheu, D.S., Chen, W.M., Yang, J.Y., Chang, R.C., 2008. Thermophilic bacterium *Caldimonas taiwanensis* produces poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from starch and valerate as carbon sources. *Enzyme Microb. Technol.*, 44, pp. 289-294.

[10] Spiekermann, P., Rehm, B.H., Kalscheuer, R., Baumeister, D., Steinbüchel, A., 1999. A sensitive, viablecolony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. *Arch. Microbiol*., 171, pp. 73-80.

[11] Tamura, K., Stecher, G., Peterson, D., Filipski, A., 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Bio. Evol*., 30, pp. 2725-2729.

[12] Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*., 4, pp. 406-425.

[13] Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.*, 31, pp. 426-428.

[14] Huijberts, G.N.M., van der Wal, H., Wilkinson, C., Eggink. G., 1994. Gas-chromatographic analysis of poly(3-hydroxyalkanoates) in bacteria. *Biotechnol. Tech.*, 8, pp. 187-192.

[15] Lemoigne, M., 1926. Products of dehydration and of polymerization of β-hydroxybutyric acid. *Bull. Soc. Chim. Biol*., 8, pp. 770-782.

[16] Mohapatra, S., Samantaray, D.P., Samantaray, S.M., 2014. Phylogenetic heterogeneity of the rhizospheric soil bacterial isolates producing PHAs revealed by comparative analysis of 16s-rRNA, Int. *J. Curr. Microbiol. Appl. Sci.*, 3, pp. 680-690.

[17] Mohapatra, S., Maity, S., Dash, H.R., Das, S., Pattnaik, S., Rath, C.C., Samantaray, D., 2017. *Bacillus* and biopolymer: prospects and challenges. *Biochem. Biophys. Rep.*, 12, pp. 206-213.

[18] Halami, P.M., 2008. Production of polyhydroxyalkanoate from starch by the native isolate *Bacillus cereus* CFR06. *World J. Microbiol. Biotechnol*., 24, pp. 805-812.

[19] González-García, Y., Rosales, M.A., González-Reynoso, O., Sanjuán-Duenas, R., Córdova, J., 2011. Polyhydroxybutyrate production by *Saccharophagus degradans* using raw starch as carbon source. *Eng. Life Sci*., 11, pp. 59-64.

[20] Bomrungnok, W., Arai, T., Yoshihashi, T., Sudesh, K., Hatta, T., Kosugi, A., 2019. Direct production of polyhydroxybutyrate from waste starch by newly-isolated *Bacillus aryabhattai* T34-N4. Environ. Technol. https://doi.org/10.1080/09593330.2019.1608314.

[21] Porras, M.A., Ramos, F.D., Diaz, M.S., Cubitto, M.A., Villar, M.A., 2019. Modeling the bioconversion of starch to P(HB-co-HV) optimized by experimental design using *Bacillus megaterium* $BBST_4$ strain. *Environ. Technol*., 40, pp. 1185-1202.