Available online at https://jcst.rsu.ac.th
Formerly Rangsit Journal of Arts and Sciences (RJAS)

Journal of Current Science and Technology, July-December 2020 JCST Vol.10 No.2, pp. 195-201

ISSN 2630-0656 (Online)

Selection and cultivation conditions of *Bacillus thuringiensis* L25.1 for poly (3-hydroxybutyrate-co-3-hydroxyvalerate) production using native rice bran waste as the main nutrient

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Received 18 February 2020; Revised 7 July 2020; Accepted 17 July 2020
Published online 20 July 2020

Abstract

Among the bioplastics produced by microorganisms, polyhydroxyalkanoate (PHA) copolymers are particularly biocompatible. These compounds do not cause inflammation or allergies to human tissue, and they are more elastic than homopolymers; hence, they are suitable for medical applications. However, PHA production costs are still relatively high, and the development of a low-cost PHA is, therefore, necessary. This research aimed to study the cultivation conditions for the production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV)) by *Bacillus thuringiensis* L25.1 using low-cost native rice bran waste from rice bran oil production via a cold press method as the main carbon source. The cultivation of *Bacillus thuringiensis* L25.1 on minimal salt agar (MSA) at various medium pHs, cultivation temperatures, percentages of native rice bran waste, incubation times, percentages of levulinic acid (LA), and enrichment of the bacterium in LA were studied. Production of P(3HB) and P(3HB-co-3HV) was determined via gas chromatography (GC). Using the 5th subculture of *Bacillus thuringiensis* L25.1 cultured on MSA with additional 24% native rice bran waste with citric acid, a medium pH of 7.0, and incubation at 30 ^{o}C for 48 hours, the % and weight per plate of poly (3-hydroxybutyrate) P(3HB):P(3HB-co-3HV) was 2.07%, 3.47 mg and 12.90%, 21.59 mg, respectively. This finding suggested that P(3HB-co-3HV) in the presence of low P(3HB) content could be produced by using low-cost waste as a carbon source and LA as a co-substrate in a semi-solid medium.

Keywords: *Bacillus thuringiensis L25.1, cultivation condition, native rice bran waste, polyhydroxybutyrate P(3HB), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV))*

1. Introduction

The usage of petroleum-derived synthetic plastics in our daily lives has grown rapidly in recent years. Packaging and service ware represent a significant use of single-use plastic. It has been forecast that nearly 12,000 Mt of plastic waste will be accumulated in the environment by 2050 (Geyer, Jambeck, & Law, 2017); plastic objects and particles that are categorized into micro-, meso-, or macro particles act as pollutants and adversely affect the environment and humans. Moreover, the degradation rates of petroleum-derived plastics in landfills are extremely slow. Incineration of the plastics can release toxic by-products into the environment (Castilho, Mitchell, & Freire, 2009), and the recycling of plastics is limited by a time-consuming sorting of discarded plastics (Khanna & Srivastava, 2005). One effective solution to reduce impacts of plastics pollution is replacing petroleum-derived plastics with decomposable ones.

Polyhydroxyalkanoates (PHAs) are a family of microbiologically produced biodegradable polymers. Their properties are rather similar to petroleum-derived synthetic plastics (Heinrich et al., 2016). PHAs are biocompatible, with no toxic compounds formed during their biodegradation. Therefore, PHAs are promising plastics for use in medical applications, including medical implants, medical scaffolds, and drug delivery carriers (Zheng, Chen, Ma, & Chen, 2020). PHAs consist of homopolymers such as polyhydroxybutyrate or poly (3-hydroxybutyrate) (P(3HB)) and copolymers such as poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV)) (Singh, Patel, & Kalia, 2009). P(3HB-co-3HV) is a copolymer of P(3HB) that is arranged randomly between 3-hydroxybutyrate (HB) groups and 3-hydroxyvalarate (HV) groups and is more elastic and more suitable for medical applications than P(3HB). Recently, a number of low-cost renewable raw materials, and both agricultural and industrial wastes have been studied to reduce PHA

production costs (Reddy, Thirumala, & Mahmood, 2009; Hassan, Bakhiet, Hussein, & Ali, 2018; Dalsasso, Pavan, Bordignon, Aragão, & Poletto, 2019). The productivity of PHA content produced via the inexpensive substrates is relatively low (Choi & Lee, 1999); therefore, a pre-treatment process, which is typically high cost and time consuming, is required. In a previous study, we isolated a P(3HB)-producing bacterium that utilized native rice bran waste taken from a rice bran oil cold press-production as the main carbon source without any pre-treatment process (Chobchuenchom, 2016). In this study, attempts were made for the utilization of cheap, native rice bran waste as the main carbon source and levulinic acid (LA) as a co-substrate for the production of P(3HB-co-3HV).

2. Objectives

This study aimed to determine the most appropriate cultivation conditions for the accumulation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by the selected bacterial isolate—*Bacillus thuringiensis* L25.1—using native rice bran waste as the main nutrient and LA as a co-substrate.

3. Materials and methods

3.1 Isolation, selection, and identification of *Bacillus thuringiensis* L25.1

The soil samples collected from a refuse site in Pathum Thani, Thailand were diluted using sterilized distilled water. By using a standard spread plate technique, each soil suspension was spread on minimal salt agar (MSA) with additional 3% native rice bran waste obtained from rice bran oil production using the cold press method. MSA with the addition of native rice bran waste was prepared as previously described (Chobchuenchom, 2016); briefly, 2.0 g potassium dihydrogen phosphate, 0.6 g disodium hydrogen phosphate, 0.2 g magnesium sulfate heptahydrate, 1.0 g ammonium sulfate, 0.75 g citric acid, 2.0 g sodium acetate, 0.05 g yeast extract, 15.0 g agar, and native rice bran waste were mixed in 1 L distilled water to obtain a final concentration (w/v) of 3%. The prepared agar medium was sterilized (121 ^{o}C, 15 mins). The bacteria were cultured on the MSA at 30 ^{o}C for 48 hours, and the subculture was performed for the pure isolates. By using a modified method previously described by Okwuobi and Ogunjobi (2012), isolate L25.1 was smeared on a glass slide, air-dried, heat-fixed, and subsequently stained with Sudan Black solution. The capability of production of P(3HB) by isolate L25.1 was confirmed by using gas chromatography-mass spectrometry (GC-MS) at the Scientific Equipment Center, Prince of Songkla University. Isolate L25.1 was identified as *Bacillus thuringiensis* via Gram staining, spore staining, and 16S rRNA gene sequencing and was designated as *Bacillus thuringiensis* L25.1. The 16S rRNA gene sequencing was performed at Mahidol University-Osaka University Collaborative Research Center for Bioscience and Biotechnology, Mahidol University, Bangkok, Thailand.

3.2 Culture conditions

For cultivation, 100 □L of starter in nutrient broth was spread onto MSA, which was prepared as described above. The native rice bran waste was added into the MSA to obtain final concentrations (w/v) of 6, 9, 12, 15, 18, 21, and 24%. The pH of the MSA was adjusted to 6.0, 7.0, and 8.0 at 3% concentration of the native rice bran waste. Plates were incubated at various temperatures, including room temperature, 30 ^{o}C, or 37 ^{o}C for 24, 48, and 72 hours.

3.3 Extraction and determination of P(3HB) and P(3HB-co-3HV)

After incubation, *Bacillus thuringiensis* L25.1 cell on MSA with additional native rice bran waste was harvested, washed with minimal salt broth two times by centrifugation, and adjusted to a volume of 2 mL. After that, 300 μL of the cell suspension was dried at 85 ^{o}C for 36 hours and weighed. The extraction of P(3HB) in the dried cell was performed by mixing 2 mL of chloroform and 2 mL of 3% H_2SO_4 (v/v) in methanol, with 10 mg/mL of benzoic acid as the internal standard and approximately 50 mg of the dried cell and heated at 100 ^{o} C for 4 hours, with inverse mixing at the first 30 minutes. After heating, the mixture was kept at room temperature overnight to cool. Then, 2 mL of distilled water was added and mixed vigorously by vortexing for 4 minutes to disrupt the bacterial cell membrane and allow the PHAs to be extracted from the chloroform. The chloroform layer was filtered and used for determination of P(3HB) and P(3HB-co-3HV) in milligrams by reading from P(3HB) and P(3HB-co-3HV) standard curves. Gas chromatography was carried

out on a gas chromatography instrument (Agilent technologies 7890A). The percentages of P(3HB) or P(3HB-co-3HV) per cell dry weight (% w/w) and weight of P(3HB) or P(3HB-co-3HV) in mg per plate were further calculated.

3.4 Calculations

The percentage of P(3HB) or P(3HB-co-3HV) per cell dry weight (% w/w) was calculated by comparing the amount of P(3HB) or P(3HB-co-3HV) in mg per 100 mg of cell dry weight. Further, the weight of P(3HB) or P(3HB-co-3HV) was calculated in mg per plate.

4. Results

4.1 Identification of P(3HB) by *Bacillus thuringiensis* L25.1

Isolate L25.1 was a Gram-positive, spore-forming bacillus. By using 16S rRNA gene sequencing, this isolate was found to be *Bacillus thuringiensis* and was designated *Bacillus thuringiensis* L25.1. After cultivation of *Bacillus thuringiensis* L25.1 on MSA with additional 3% native rice bran waste at 30 ^{o}C for 48 hours, the cells were smeared and stained with Sudan Black B for PHA production screening. The bacterium demonstrated positive cells for PHA granules. By using the chloroform extraction method, P(3HB) produced by *Bacillus thuringiensis* L25.1 was separated and further identified via GC-MS—the mass spectrum of the polymer corresponded to standard P(3HB) (data not shown).

4.2 Investigation of cultivation conditions for P(3HB) production by *Bacillus thuringiensis* L25.1 cultured on MSA with native rice bran waste

Bacillus thuringiensis L25.1 was grown on MSA (pH 7), which consisted of 3% native rice bran waste and supplemented with yeast extract and/or citric acid and incubated at 30 ^{o} C for 48 hours. It was found that the highest wt.% P(3HB) per cell dry weight and P(3HB) weight per plate were 16.53% and 13.12 mg, respectively, when *Bacillus thuringiensis* L25.1 was cultured on MSA with 3% native rice bran waste with citric acid (Table 1). The pH of the MSA varied from 6, 7, and 8. It was found that the highest wt.% P(3HB) per cell dry weight and weight of P(3HB) per plate were 20.13% and 1.74 mg, respectively, when *Bacillus thuringiensis* L25.1 was cultured on MSA with 3% native rice bran waste and citric acid adjusted to pH 7 (Table 2). For the optimized incubation period, the bacterium was cultured on MSA with 3% native rice bran waste and citric acid adjusted to pH 7 and incubated for various times. As shown in Table 3, the optimized incubation time was 48 hours. In Table 4, MSA supplemented with 24% native rice bran waste demonstrated the highest % P(3HB) per cell dry weight and weight of P(3HB) per plate. It is worth noting that the addition of more than 24% of native rice bran waste resulted in an MSA formation that was excessively loose. Figure 1 provides an example of the GC chromatogram of P(3HB) extracted from *Bacillus thuringiensis* L25.1.

Table 1 P(3HB) production by *Bacillus thuringiensis* L25.1 cultured on MSA (pH7) with additional 3% native rice bran waste with and without yeast extract and/or citric acid supplementation when incubated at 30 ^{o}C for 48 hours.

Yeast extract	Citric acid	P(3HB) per cell dry weight [%]	Weight of P(3HB) per plate [mg]
No	No	10.52	9.26
Yes	No	11.16	11.24
No	Yes	16.53	13.12
Yes	Yes	11.24	9.37

Table 2 P(3HB) production by *Bacillus thuringiensis* L25.1 cultured on MSA with additional 3% native rice bran waste with citric acid supplementation at pH 6, 7, and 8 when incubated at 30 ^{o}C for 48 hours.

pH	P(3HB) per cell dry weight [%]	Weight of P(3HB) per plate [mg]
6	8.82	1.59
7	20.13	1.74
8	10.43	0.63

Table 3 P(3HB) production by *Bacillus thuringiensis* L25.1 cultured on MSA (pH7) with additional 3% native rice bran waste with citric acid supplementation incubated at 30 ^{o}C for 24, 48, and 72 hours.

Incubation period	P(3HB) per cell dry weight [%]	Weight of P(3HB) per plate [mg]
24	2.78	0.82
48	22.56	7.22
72	16.53	13.12

Table 4 P(3HB) production by *Bacillus thuringiensis* L25.1 cultured on MSA (pH7) with additional 6, 9, 12, 15, 18, 21, and 24% (w/v) native rice bran waste with citric acid supplementation incubated at 30 °C for 48 hours.

Native rice bran waste concentration [%]	P(3HB) per cell dry weight [%]	Weight of P(3HB) per plate [mg]
3	11.02	2.42
6	5.84	4.16
9	30.37	4.86
12	38.41	5.12
15	38.84	9.06
18	12.15	8.51
21	18.24	9.85
24	52.54	17.16

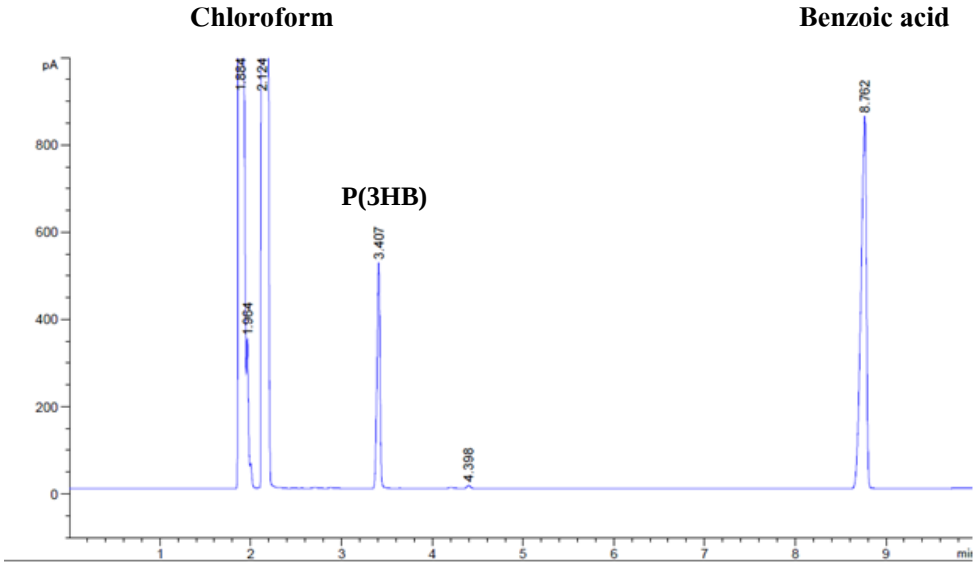

Figure 1 Example of GC chromatogram of the chloroform filtrate extracted from dried cells of *Bacillus thuringiensis* L25.1 cultured on MSA with additional native rice bran waste; peaks at 1.8 to 2.2, 3.4, and 8.7 are chloroform, P(3HB), and benzoic acid internal standard, respectively.

4.3 Cultivation conditions for P(3HB-co-3HV) production by *Bacillus thuringiensis* L25.1

Bacillus thuringiensis L25.1 was induced for P(3HB-co-3HV) production by an enrichment technique. After sub-culturing the bacterium 5 times on MSA (pH7) with additional 24% (w/v) native rice bran waste with citric acid and 0.3% LA supplementation incubated at 30 °C for 48 hours, it was found that the % and weight per plate of poly (3-hydroxybutyrate) P(3HB): P(3HB-co-3HV) was 10.37%, 5.81 mg and 15.55% 8.71 mg, respectively. Then, the concentration of LA was adjusted to 0.1%, and it was found that the % and weight per plate of poly (3-hydroxybutyrate) P(3HB): P(3HB-co-3HV) were 2.07%, 3.47 mg and 12.90%, 21.59 mg, respectively. An example of the P(3HB-co-3HV) GC chromatogram is provided in Figure 2.

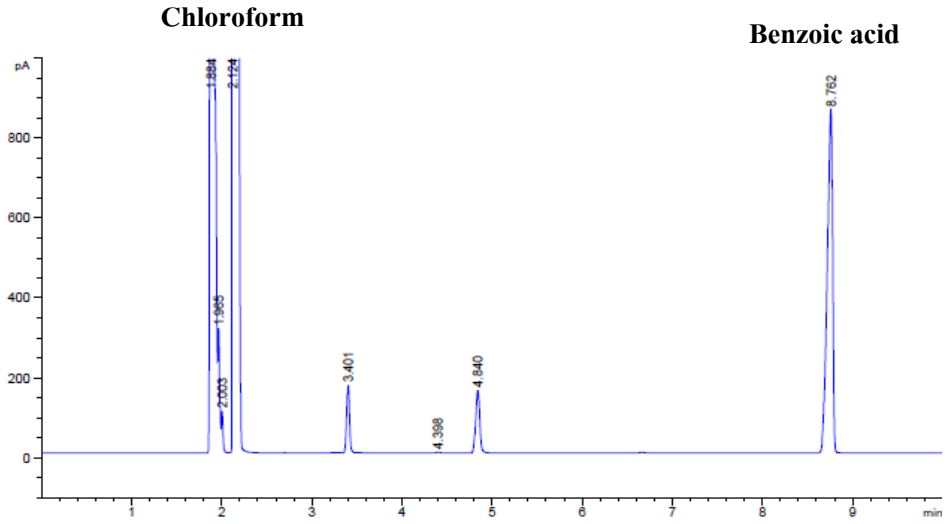

Figure 2 Example of GC chromatogram for the chloroform filtrate extracted from dried cells of *Bacillus thuringiensis* L25.1 cultured on MSA with the addition of native rice bran oil waste as the main carbon source and LA as co-substrate; peaks at 1.8 to 2.2, 3.4, 4.8, and 8.7 are chloroform, P(3HB), P(3HB-co-3HV), and benzoic acid internal standard, respectively.

5. Discussion

The use of cheap, renewable carbon substrates, primarily agro-materials and industrial waste, for the cultivation of PHA-producing microbes appears to be a promising approach for the reduction of PHA production cost (Chaudhry, Jamil, Ali, Ayaz, & Hasnain, 2010). For increased biopolymer productivity, most of the raw materials require a pre-treatment process for high-content carbon-source formation, such as enzymes or acid-hydrolysis, and these approaches may be rather time consuming and costly (Saranaya, Vijayendra, & Shamala, 2012). Rice bran waste consists of various fatty acids, such as palmitic, linoleic, elaidic, adipic, mesitoic, myristic, and oleic acids, all of which could represent a desirable nutrient source for bacterial cultivation (Saranaya, Vijayendra, & Shamala, 2012; Chobchuenchom, 2016). In this study, the selected bacterial isolate—*Bacillus thuringiensis* L25.1—was found to utilize non-treated waste from rice bran oil production via the cold press method as its main carbon source for PHA production.

Although P(3HB) is the primary biopolymer produced by various microorganisms, it has a crystalline and brittle physical nature that makes it unsuitable for medical applications. The random incorporation of 3-hydroxybutyrate (HB) and 3-hydroxyvalarate (HV) groups into the P(3HB) biopolymer chain leads to the synthesis of copolymers P(3HB-co-3HV) that can decrease the crystallinity and melting point of P(3HB) and make them more suitable for medical applications. Recently, there have been reports that P(3HB)-produced bacteria can produce copolymers when organic (e.g., propionic and levulinic) acids are supplemented into bacterial media (Jain, 2001). For example, *Bacillus thuringiensis* EGU45 was found to produce P(3HB-*co*-3HV) with 13.4% 3HV content when cultured with high nitrogen-containing feed supplemented with propionic acid (Kumar, Ray, Patel, Lee, & Kalia, 2015), and *Cupriavidus necator* was able to synthesize P(3HB-*co*-3HV up to 18.1% (w/w) by using 6% sulfuric acid-digested rice straw hydrolysate as a carbon source (Ahn, Jho, Kim, & Nam, 2016). Thus, the utilization of cheap carbon sources without any supplementations or pre-treatment processes for P(3HB) and PHAs copolymer production is still required. In this study, *Bacillus thuringiensis* L25.1 used native rice bran waste as the main carbon source for P(3HB) production, and levulinic acid acted as a co-substrate that led to the formation of P(3HB-co-3HV). However, the appropriate scale-up method for the production of P(3HB-co-3HV) should be further studied.

6. Conclusions

A new bacterial strain—*Bacillus thuringiensis* L25.1—was isolated and found to be capable of utilizing native rice bran waste as the main carbon source and levulinic acid as a co-substrate for P(3HB-co-3HV) production. The bacterium was isolated from a soil sample obtained from the refuse site in Pathum Thani province, Thailand. The percentage and weight per plate of P(3HB):P(3HB-co-3HV) were 2.07%, 3.47 mg and 12.90%, 21.59 mg, respectively, when the bacterium was grown on MSA (pH7) with additional 24% (w/v) native rice bran waste supplemented with citric acid and 0.3% LA and incubated at 30 ^{o}C for 48 hours.

7. Acknowledgements

This study was financially supported by the research institute of Rangsit University, research fund 2014 (grant no. 25/2556).

8. References

Ahn, J., Jho, E.H., Kim, M., & Nam, K. (2016). Increased 3HV concentration in the bacterial production of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) copolymer with acid-digested rice straw waste. *Journal of Polymer and the Environment*, *24*, 98–103. DOI: 10.1007/s10924-015-0749-0

Chobchuenchom, W. (2016). Isolation of *Bacillus cereus* C042 capable of producing polyhydroxybutyrate (PHB) by using unhydrolysed waste from rice bran oil production as carbon source. *Rangsit Journal of Arts and Sciences*, *6*(1), 67-75. DOI: 10.14456/rjas.2016.6

Choi, J., & Lee, S.Y. (1999). Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. *Applied Microbiology and Biotechnology*, *51*, 13-21. DOI: https://doi.org/10.1007/s002530051357

Dalsasso, R. R., Pavan, F. A., Bordignon, S. E., Aragão, G. M. F. d., & Poletto, P. (2019). Polyhydroxybutyrate (PHB) production by *Cupriavidus necator* from sugarcane vinasse and molasses as mixed substrate. *Process Biochemistry*, *85*, 12-18. DOI: https://doi.org/10.1016/j.procbio.2019.07.007

Geyer, R., Jambeck, J. R., & Law, K. L. (2017). Production, use, and fate of all plastics ever made. *Science Advances*, *3*,(7), e1700782. DOI: https://doi.org/10.1126/sciadv.1700782

Hassan, M.A., Bakhiet, E.K., Hussein, H.R., & Ali, S.G. (2018). Statistical optimization studies for polyhydroxybutyrate (PHB) production by novel *Bacillus subtilis* using agricultural and industrial wastes. *International Journal of Environmental Science and Technology*, *16*(7), 3497–3512. DOI: https://doi.org/10.1007/s13762-018-1900-y

Heinrich, D., Raberg, M., Fricke, P., Kenny, S.T., Morales-Gamez, L., Babu, R.P., O'Connor, K.E., & Steinbüchel, A. (2016). Syngas-derived medium-chain-length PHA synthesis in engineered *Rhodospirillum rubrum*. *Applied Environmental Microbiology*, *82*(20), 6132–6140. DOI: 10.1128/AEM.01744-16

Kumar, P., Ray, S., Patel, S.K.S., Lee, J.K., & Kalia, V.C. (2015). Bioconversion of crude glycerol to polyhydroxyalkanoate by *Bacillus thuringiensis* under non-limiting nitrogen conditions. *International Journal of Biological Macromolecules*, *78*(July), 9-16. DOI: https://doi.org/10.1016/j.ijbiomac.2015.03.046

Castilho, L. R., Mitchell, D. A., & Freire, D. M. G. (2009). Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation. *Bioresource Technology*, *100*(23), 5996-6009. DOI: 10.1016/j.biortech.2009.03.088

Singh, M., Patel, S.K.,& Kalia, V.C. (2009). *Bacillus subtilis* as potential producer for polyhydroxyalkanoates. *Microbial Cell Factories*, 8, 38-49. DOI:10.1186/1475-2859-8-38

Okwuobi, P.N., & Ogunjobi, A. (2012). Characterization of polyhydroxyalkanoate (PHA) produced by *Bacillus species* isolated from garden soil. *New York Science Journal*, *5*(912), 159-163.

Reddy, S.V., Thirumala, M., & Mahmood, S.K. (2009). A novel *Bacillus* sp. accumulating

poly (3-hydroxybutyrate-co-3-hydroxyvalerate) from a single carbon substrate. *Journal of Industrial Microbiology & Biotechnology*, *36*, 837–843. DOI: https://doi.org/10.1007/s10295-009-0561

Saranaya D.E., Vijayendra S.V.N., & Shamala T.R. (2012). Exploration of rice bran, an agro-industry residue, for the production of intra and extra cellular polymers by *Sinorhizobium meliloti* MTCC 100. *Biocatalysis and Agricultural Biotechnology*, *1*(1), 80-84. DOI: https://doi.org/10.1016/j.bcab.2011.08.014

Khanna, S., & Srivastava, A. K. (2005). Recent advances in microbial polyhydroxyalkanoates. *Process Biochemistry*, *40*(2), 607-619. DOI: https://doi.org/10.1016/j.procbio.2004.01.053

Chaudhry, W., Jamil, N., Ali, I., Ayaz, M., & Hasnain, S. (2010). Screening for polyhydroxyalkanoate (PHA)-producing bacterial strains and comparison of PHA production from various inexpensive carbon sources. *Annals of Microbiology*, *61*(3), 623-629. DOI: 10.1007/s13213-010-0181-6

Zheng, Y., Chen, J-C., Ma, Y-M., & Chen, G-Q. (2020). Engineering biosynthesis of polyhydroxyalkanoates (PHA) for diversity and cost reduction. *Metabolic Engineering*, *58*, 82-93. DOI: https://doi.org/10.1016/j.ymben.2019.07.004