Classical and Molecular Detection of Polyhydroxybutyrate-Producing Bacteria obtained from Different Habitats

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Abstract: Polyhydroxybutyrate (PHB) was gained more interest in the recent years as a bio-based polymer that has many useful features. Polyhydroxybutyrate is a biodegradable plastic material, which used in different beneficial applications because the properties of bio-based polymer become more desirable than petroleum-based plastic. It considered the best and common member in a polyhydroxyalkanoate group (PHA) that synthesized completely by bacteria as a carbon energy storage compound. Many different species of bacteria while gram positive or negative have the ability to produce PHB using various sources of carbon. Because the importance of isolating PHB-producing bacteria from different habitats, it is necessary to use rapid method that can screen a wide groups of bacteria in a limited time. There are two main methods for identifying PHB-producing organisms, classical and molecular methods. This review focused on the researches and developments that detect *phaC* gene encodes PHA synthase (key enzyme for PHA synthesis) using polymerase chain reaction (PCR) techniques as a molecular method. Moreover, it illustrated some advantages and disadvantages of both classical and molecular methods.
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1. Introduction;

Petroleum-based plastic is very important material as well one of the major toxic pollutants on the earth (Alarfaj *et al*., 2015). It is used an over the world to produce plastic bags, cups of coffee, bottles, toys, candy wrappers, and many other popular products. It has many features such as light weight, energy saving, can easily be shaped, durable, chemically resistant, easily portable and impermeable to water (Al-Salem *et al*., 2009). On the other hand, petroleum-based plastic is a chemical polymer that non-biodegradable since plastic bottles, for example, needs very long time to decompose (Halden, 2010). Therefore, petroleum-based plastic plays an important role in earth, air and water as a major pollutant (Shaaban *et al*., 2012). For example, either the production or disposal of petroleum-based plastic by burning it lead to accumulating high concentrations of carbon dioxide in the atmosphere that give a rise to greenhouse effect resulting in global warming and *global climate change (*Dana Gopal *et al*., 2014*;* Campos et al., 2014and *Mona et al., 2016*). In addition, petroleum-based plastic not only affects our environment, it also has a wide range of health impact like; endocrine disruption, cancer, damage of nervous system, birth defects, kidney failure, damages in immune system in general, heart diseases, asthma, rashes, nausea and headache. All these symptoms caused by gas evaporation or by emitting substantial amounts of toxic chemicals (*e.g.* dioxins) during the plastic production (Halden, 2010; Chen, 2010). In addition, one of the most important disadvantage of petroleum-based plastic is the large quantity of petroleum that needed for its production, which caused diminishing of it (Dana Gopal *et al*., 2014). Thus, finding new environmentally friendly materials that reduce the hazards of petroleum-based plastic has become in a major concern of governments all over the world.

1. Classification of PHA;

Polyhydroxyalkanoate PHA consisting of three hydroxy fatty acids with different carbon chain lengths. It can be classified into three types based on the number of carbon atoms in the chain. First type, short-chain length PHA (scl-PHA) that means PHA monomers having 5 or less carbon atoms in their chain. Second type, medium-chain length PHA (mcl-PHA) that means PHA monomers having 6 to 14 carbon atoms in their chain. Third type, long-chain length PHA (lcl-PHA) that is uncommon and PHA monomers having more than 14 carbon atoms in their chain. (Kunasundari & Sudesh, 2011; Saharan & Ankita, 2012).

2. Applications of PHA:

There are many beneficial applications of PHA in medical field such as biomedical implant materials or wound dressings, in packaging field such as shampoo bottles or food containers, and in agricultural

field such as fisheries (Chen, 2010; Hazer *et al.,* 2012). In addition, PHA can be used as drug delivery carriers or as drugs itself (Zou *et al,* 2009; Chen, 2009; Kabilan *et al.,* 2012), and as bio fuels (Revellame *et al,* 2012; Montenegro *et al,* 2017). The best and common member in a polyhydroxyalkanoate group (PHA) is Polyhydroxybutyrate (PHB).

3. Polyhydroxybutyrate (PHB):

Polyhydroxybutyrate, which is a PHA member, is a bioplastic or biopolymer that is coming from biological origin (biocompatibility) and can be synthesis by different bacterial species. There are different bacterial species that were isolated or re-constructed for producing PHB, including *Pseudomonas* sp., *Bacillus* sp., *Azotobacter* sp. *Rhodococcus* sp., and *Cupriavidus* sp. (Sangkharak & Prasertsan, 2012). Polyhydroxybutyrate is the only bioplastics that completely synthesized by bacteria (Hassan *et al*., 2016). It is completely biodegradable under natural environment conditions to carbon dioxide and water by microbial enzymatic activities. As a result, it can be used instead of petroleum-based plastic, because the similarity of PHB material properties to synthetic polymers without any impact on health or environment (Chandrashekharaiah, 2005).

4. Importance of PHB to Bacteria:

Polyhydroxybutyrate is accumulated under unfavorable bacterial growth conditions as energy and carbon storage materials in the presence of excess carbon source. It stored as granules like water-insoluble polymers in the cytoplasm (Legat *et al*., 2010; Özgen, 2012). The bacteria can enzymatically degrade it when the carbon source in the environment is depleted as a mechanism to overcome stress conditions.

5. Genes responsible for the production of PHB:

In different PHB-producing bacterial species, three sequential enzymatic genes were found to be responsible for synthesizing PHB although the organization of these genes seems to be different among bacterial species. It was foud that *phaA* gene encode 3-ketothiolase, which forms acetoacetly-CoA from acetyl-CoA in the first reaction, while *phaB* gene encode NADPH-dependent acetocaetly-CoA reductase that form 3-HB-CoA in the second reaction and in the final reaction *phaC* gene encodes PHA synthase, which polymerizing and linking 3HB-CoA together by an ester bond to make the final monomers (Enan & Bashandy, 2006; Suriyamongkol *et al.,* 2007; Galehdari *et al.,* 2009; Osman *et al.,*2015; Poltronieri *et al.,* 2016).

Furthermore, depending on the structural and functional properties of PHA synthase enzymes and the organization of gene locus, they can be classified into four different classes. Class I PHA synthases is made up of only one type of subunit encoded by *phaC* and responsible for producing scl-PHA in *Ralstonia eutropha*. In addition, class II PHA synthases made up of only one type of subunit encoded by *phaC* that found in *Pseudomonas aeruginosa*, which is responsible for producing mcl-PHA. On the other hand, class III of PHA synthases made up of two types of subunits encoded by *phaC* and *phaE*, which synthesize scl-PHA in *Allochromatiumvinosum*, while Class IV PHA synthases made up of two types of subunits encoded by *phaC* and *phaR*, which replaced by *phaE* in class III PHA synthases and they have been reported only in *Bacillus* sp. Among all these genes, PHA synthase (*phaC*) is considered as the most important gene for PHB synthesis since it encodes the key enzyme that responsible for the final concentration and content of PHB and it is mostly related to the activity of PHB synthase (Kam, 2009).

6. Classical methods for PHB detection:

The PHB-producing bacteria were effectively detected using different methods. The granules of PHB were detected classically in the bacterial cells using an optical microscope like phase-contrast mode, which present of intracellular refractive granules with the aid of specific dyes (Godbole, 2016). In addition, the light microscope and transmission electron microscopy (TEM) were used to obtain further ultra-structural details of PHB granules by revealing it as white spherical areas inside the cytoplasm (Rohini *et al.,* 2006; Trainer *et al.,* 2010; Ceyhan and Ozdemir, 2011; Mona *et al*., 2016). It is very important to use the staining like sudan black B. as a lipophilic dye for PHB granules detection with light microscope (Singh & Parmar. 2011; Wei *et al.,* 2011; Dhingra & Priya, 2013). Moreover, Nile blue was well known as the most specific stain for PHB granules (Chandrashekharaiah, 2005; Sudesh & Abe, 2010), while the Acridine orange was using as fluorescence staining method in many researches (Sharmila *et al.,* 2011).

Although using the classical methods to PHB-producing bacteria identification gives effective results, each bacterial species need specific growth conditions to produce PHB. Sometimes classical methods fail to detect the production of PHB in some bacterial species that have the genes for PHB production due to the unknown of the optimal growth conditions for PHB production (Shamala *et al*., 2003; Solaiman & Ashby, 2005; Sasidharan *et al*., 2016). On the other hand, the dyes used in PHB screening can react with other lipid inclusion bodies inside the cell where the reagents is not only specific for PHB detection (Enan & Bashandy, 2006). For example; hydrocarbons and ketones including polyhydroxybutyrate (PHB) and fatty acid esters can

be detected by Nile red (Pinzon *et al*., 2011). This means that using classical methods to detect the PHB-producing bacteria is difficult to obtain and time consuming (Montenegro *et al.,* 2017).

7. Molecular method for PHB detection:

Molecular methods especially polymerase chain reaction (PCR) techniques, whether normal PCR or colony PCR proved to be effective for the detection of PHB-producing bacteria because of its speed results, efficient detection and their simplicity of processing (Lane & Benton, 2015). The PCR technique can solve all mentioned disadvantages of classical methods. Moreover, PCR technique was previously used in many researches for accurate and rapid detection of PHB-producing bacteria (Montenegro *et al.,* 2017; Nehra *et al.,* 2015; Yang *et al.,* 2013).

8. *PhaC* gene as the most important indicator for PHB detection:

Many researchers agree that the most important gene among PHA production genes is *phaC* since it encodes the key enzyme for PHA synthesis. PHA synthase involve in the polymerization of PHB, which is very important in controlling the biosynthesis of PHB polymer (Bhubalan*et al*., 2011). Different primers are used for *phaC* detection in deferent bacteria (Table 1). It was stated that single and double mutations in *phaA* and *phaAB* were not effected on the synthesizing of PHA, whereas single mutation in *phaC* were effected and prevent the PHB production (Kranz *et al*., 1997). This means that *phaC* is the most important gene for PHB synthesis. In addition, it is very notable that detection of the *phaC* gene that responsible for encoding PHB synthase in the bacterial genome and can be considered as a confirmation of PHB production in the PHB-producing bacteria (Nehra *et al.,* 2015). Moreover, Zhang*et al*.- (2001) found that PCR cloning technique can be used as a rapid and accurate identification tool for the different organization types of PHA synthase genes, when they used the complete PHA synthase genes from *Pseudomonas pseudoalcaligenes* HBQ06 (*phaC1*) and *Pseudomonas nitroreducens* 0802 (*phaC2*), then they successfully cloned PCR product and expressed it in *Escherichia coli*. Furthermore, twenty-three different bacterial isolates from soil were screened using PCR with two primer pairs designed from *Bacillus megaterium* to detect PHB synthase gene. Twelve isolates gives positive results with identical amplicons to *B. megaterium* like *B. sphaericus, B. brevis B. circulans, and B. licheniformis*, which means that PCR is an efficient method to detect PHB synthase gene in PHB-producing species. (Shamala *et al.,* 2003). Even more, the highly conserved sequences regions between *PseudomonasphaC1* and *phaC2* genes that code for type II PHA synthases were detected using PCR protocol to identify *P. corrugata* 388 as a medium-chain-length (mcl)-PHA (Solaiman et al., 2000). In addition, colony PCR technique, amplified regions of interest gene was used as an accurate method for the detection of PHB-accumulating bacteria isolated from the environment to detect *R. eutropha phaC* synthase gene. Thirty-eight PHB positive strains were rapidly isolated. This protocol is suitable for the screening of large numbers of environmental isolates. The PHB-producing bacteria colonies can be directly isolated from environmental samples by PCR with no need for further culturing or DNA extraction (Sheu *et al.,* 2000). Sujatha *et al*. (2005) used three PCR primer pairs to detect successfully PHB synthase genes in thirty-five PHB-accumulating isolates of *Pseudomonas*, either by medium chain length PCR or colony PCR. Moreover, Enan & Bashandy (2006) proved that using polymerase chain reaction (PCR) technique to detect PHB synthase gene is a rapid and sensitive technique when they cloned PHB synthase gene directly from *Aeromonas hydrophila* genome. In addition, Gao with his co-researchers used quantitative RT-PCR as a rapid technique to determine *phaC* gene and to prove that the PHB production ability in *E. coli* was significantly notable when bacteria harboring *phbA*, *phbB*, and different *phaC2* $_{Ps}$ from PHB- producing bacteria called *Pseudomonas stutzeri* 1317. Then, they found that the enhancement of synthase gene expression was completely responsible of the increased PHB content (Gao *et al.,* 2012). In the second place, Yang *et al*. (2013) could isolate different PHB-producing bacteria from activated sludge using PHB synthase gene primers that detected by PCR technique. They analyzed DNA sequencing and found 80 *phaC* genes, 76 related to the Class I PHB synthase, and only four related to the Class II PHA synthase gene. Even more, Nayak *et al.* (2013) designed primers for specific amplification region (*phbC*) in *B. megaterium* for the polyhydroxyalkanoate synthase *phbC* gene and used PCR as rapid and specific technique for the identification of PHB-producing Bacillales members especially *B.megaterium*. Also, Nehra *et al.* (2015) used PCR protocol to detect PHB synthase gene using specific primers and found that *Bacillus anthracis* (IBB) and *Bacillus subtilis* (ITG), isolated from industrial effluent discharge places. They found to be exhibited amplification fragments for *phaC* gene, which proved the presence of PHB producing genes in the isolates. Further, Tufail *et al.* (2017) detected the PHB synthase gene (*phaC*) among six bacterial strains using different carbon sources including; waste frying oils, diesel, canola oil and glucose and, he found that maximum PHB accumulation (53.2% w/w), was achieved using waste frying oils by *P. aeruginosa* (KF270353). On the other hand, a

multiplex PCR was used in addition to the normal PCR and colony PCR to detect the *phaC* gene. A multiplex PCR means using multiple primers in a single PCR experiment to amplify more than one target sequence, which leads to obtain a correct positives and large range of results (Montenegro *et al.,* 2017). In addition, Castroverde *et al*. (2006) proved that using three combination of primers in a single PCR is efficient for the identifying 30% of pathogenic bacteria isolated from soil which give fragments of the expected size. Besides, Tzu & Semblante (2012) found that using multiplex PCR was more efficient than testing primers separately when isolating PHB-producing bacteria from wastewater and activated sludge. This technique increased the detection accuracy up to 90% of PHA synthases classes II and I. In addition, Montenegro *et al.* (2017) designed ten degenerate primers from multiple alignments of *phaC* gene sequences in 218 species and they used a multiplex PCR with the combination of two oligos *phaCF*3/*phaCR*1 to screen PHB-producing bacteria and found that the target amplicon is found in 30% of the tested bacteria.

Although the advantages of using PCR technique to detect PHB synthase gene in the PHB-producing bacteria, this technique is still not common in the scientific researches. This may refers to the sequences of *phaC* genes among all classes of PHA synthases are not similar, so the primers which used in PCR that can cover all the PHA classes are limited (Yang *et al.,* 2013). Many primers have proposed from different bacterial species to cover either *phaC* of Classe I, II or IV (Sheu *et al*., 2000; Solaiman *et al*., 2000; Zhang *et al.*, 2001; Montenegro *et al.,*2017). Thus, only certain range of bacterial species can covered using the primers for *phaC* detection, which become useful tool to explore the diversity of *phaC* genes in the natural habitat and screening of new PHB-producing isolates (Yang *et al.,* 2013; Montenegro *et al.,* 2017).

Conclusion;

This review represents that using polymerase chain reaction (PCR) techniques especially a multiplex PCR are effective molecular methods for rapid and accurate identification of PHB- producing bacteria. *Pha*C gene encodes the key enzyme for PHB synthesis, so it was targeted to be amplified using the PCR techniques as the most important tool when isolating and identifying the PHB-producing bacteria from different environments.

Table (1); List of the primers used for PCR amplification of targeted *phaC* gene in PHB-producing bacteria in different studies.

Primer type	Primer Sequence	Expected amplicon size (bp)	Positive Isolates reaction	References
F 1 R 1 R 2	5'-AACTCCTGGGCTTGAAGACA-3' 5'-TCGCAATATGATCACGGCTA-3' 5'-ACGGTCCACCAACGTTACAT-3'	590 bp 380 bp	*Bacillus sphaericus, Bacillus circulans, Bacillus brevis*, *Bacillus licheniformis*	Shamala *et al.,* 2003
F 1 R 1	5'-ATTCGTAACGGAATGGGAAAAG-3' 5'-ATTAGAACGCTCTTCAAGCAAT-3'	1070 bp	*Bacillus anthracis*, *Bacillus subtilis*	Nehra *et al.,* 2015
F 2 R 2	5'-ATGACTACATTCGTAACGGAATGG-3' 5'-TTAATTAGAACGCTCTTCAAGCCA-3'	1089 bp		
F 1 R 1	5'-CGTGCAAGAGTGGGAAAAAT-3' 5'-TCGCAATATGATCACGGCTA-3'	900 bp	*Bacillus megaterium*	Nayak*et al.* 2013
F 1 R 1	5'-AACGGCGATTCCACCAATCT-3' 5'-TCCAGGGGACGATGTGATCT-3'	181 bp	*Microbacteriumparaoxydans*	Osman *et al.,*2015
F 1 R 1	5′-ATCAACAARTWCTACRTCYTSGACCT-3′ 5′-AGGTAGTTGTYGACSMMRTAGKTCCA-3′	500 bp	*Acinetobacter sp.* and *Pseudomonas sp.*	Yang *et al.,* 2013
F 1 R 1 F 2 R 2	5′-CCAC/TGACAGCGGCCTGTTCACCTG-3′ 5′-GTCGTCGTCA/GCCGGCCAGCACCAG-3′ 5′-CTGGTGCTGGCCGGC/TGACGACGAC-3′ 5′-TCGACGATCAGGTGCAGGAACAGCC-3′	3000 bp 2800 bp	*Pseudomonas pseudoalcaligenes, Pseudomonas nitroreducens*	Zhang *et al.,* 2001
F 1 R 1	5′-CGTAATTGGGGCCCATGCAG-3′ 5′-AGCCGCCGCCGAAGCTTCCGATGGC-3′	1704 bp	*Chromobacterium sp.*	Kam, 2009
F 1 R 1	5′-CCGCCSTGGATCAACAAGT-3′ 5′-GTGCCGCCGAYGCAGTAGCC-3′	239 bp	*Bacillus pumilus; B. thurigiensis; B. megaterium;.*	Montenegro *et al,* 2017
F 1 R 1	5′-ATGGATCAAGCCCCCTCTTT-3′ 5′-TCAGCCTTTCACGTAACGG-3′	1704 bp	*Azotobacter vinelandii*	Galehdari*et al,* 2009

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References

1. Alarfaj, A. A., Arshad, M., Sholkamy, E. N., & Munusamy, M. A. (2015). Extraction and Characterization of Polyhydroxybutyrates (PHB) from Bacillus thuringiensis KSADL127 Isolated from Mangrove Environments of Saudi Arabia. Brazilian Archives of Biology and Technology, 58(5), 781-788.
2. Al-Salem, S. M., Lettieri, P., & Baeyens, J. (2009). Recycling and recovery routes of plastic solid waste (PSW): A review. Waste management, 29(10), 2625-2643.
3. Bhubalan, K., Chuah, J., Shozui, F., Brigham, C, Taguchi, S., Sinskey, A. and Sudesh, K. (2011). Characterization of the highly active polyhydroxyalkanoate synthase of Chromobacterium sp. strain USM2. Appl Environ Microbiol., 77 (9): 2926-2933.
4. Campos MI, Figueiredo TVB, Sausa LS, Druzian JI. The influence of crude glycerin and nitrogen concentrations on the production of PHA by Cupriavidus necator using a response surface methodology and its characterizations. Industrial Crops and Products, 2014; 52: 338–346.
5. Castroverde, C.D.M.; San Luis, B.B.; Monsalud, R.G.; Hedreyda, C.T. (2006). Differential detection of vibrios pathogenic to shrimp by multiplex PCR. J. Gen. Appl. Microbiol. 2006, 52, 273–280.
6. Ceyhan, N. and Ozdemir, G. (2011). Poly-[3hydroxybutyrate (PFfB) production from domestic wastewater using Enterobacter aero genes 12Bi strain. Aff J Microbiol Res., 5 (6): 690-702.
7. Chandrashekharaiah, P. (2005). Isolation, Screening and Selection of Efficient Poly-Hydroxybutyrate (Phb) Synthesizing Bacteria. M. Sc. Thesis Submitted To The University of Agricultural Sciences, Dharwad, India. 84 pages.
8. Chen, G. (2009). A microbial polyhydroxyalkanoates (PHA) based bio-and materials industry. Chem Soc Rev., 38 (8): 2434-2446.
9. Chen, G. (2010). Plastics from Bacteria: Natural Functions and Applications. Chen, George Guo-Qiang, 1st Edition, 450 p.
10. Dhingra, H. and Priya, K. (2013). Physiological and molecular identification of polyhydroxybutyrates (PEPB) producing micro-organisms isolated from root nodules of leguminous plants. Afr J Microbiol Res., 7 (30): 3961-3967.
11. Enan, M. R., & Bashandy, S. A. (2006). PCR cloning of polyhydroxybutyrate synthase gene (phbC) from Aeromonas hydrophila. Arab gulf journal of scientific research, 24(1), 1.
12. Gao, X., Yuan, X. X., Shi, Z. Y., Guo, Y. Y., Shen, X. W., Chen, J. C., & Chen, G. Q. (2012). Production of copolyesters of 3-hydroxybutyrate and medium-chain-length 3-hydroxyalkanoates by E. coli containing an optimized PHA synthase gene. Microbial cell factories, 11(1), 130.
13. Godbole, S. (2016). Methods for identification, quantification and characterization of polyhydroxyalkanoates. International Journal of Bioassays, 5(04), 4977-4983.
14. Halden, R. (2010). Plastics and health risks. Annual review of public health, 31 (5): 179-194.
15. Hassan MA, Bakhiet EK, Ali SG, Hussien HR. Production and characterization of polyhydroxybutyrate (PHB) produced by Bacillus sp. isolated from Egypt. J App Pharm Sci, 2016; 6 (04): 046-051.
16. Hazer, D., Kihfay, E. and Hazer, B. (2012). Poly (3-hydroxyalkanoate) s: Diversification and biomedical applications: A state of the art review. Materials Sci and Engin., 32 (4): 637-647.
17. Kabilan, S., Ayyasamy, M., Jayavel, S. and Paramasamy, G (2012). Pseudomonas sp. as a Source of Medium Chain Length Polyhydroxyalkanoates for Controlled Drug Delivery: Perspective. Inter J Microbiol. 2012, 10 pages.
18. Kam, Y. C. (2009). Cloning And Characterization Of An Unusual Polyhydroxyalkanoate Acid Synthase (phaC) Gene From a Chromobacterium Sp. Isolated Locally (Doctoral dissertation, Universiti Sains Malaysia).
19. Kranz, R. G., Gabbert, K. K., Locke, T. A., & Madigan, M. T. (1997). Polyhydroxyalkanoate production in Rhodobacter capsulatus: genes, mutants, expression, and physiology. Applied and environmental microbiology, 63(8), 3003-3009.
20. Kunasundari, B., & Sudesh, K. (2011). Isolation and recovery of microbial polyhydroxyalkanoates. Express Polymer Letters, 5(7).
21. Lane, C. E., & Benton, M. G. (2015). Detection of the enzymatically-active

polyhydroxyalkanoate synthase subunit gene, phaC, in cyanobacteria via colony PCR. Molecular and cellular probes, 29(6), 454-460.
22. Legat, A., Gruber, C, Zangger, K, Wanner, G and Stan-Lotter, H. (2010). Identification of polyhydroxyalkanoates in Halococcus and other haloarchaeal species. Appl Microbiol Biotechnol., 87 (3): 1119-1127.
23. Mona Albureikan, Magda Aly, Haddad El Rabey (2016). Cloning and Sequencing of Poly-β-hydroxybutyrate (PHB) Synthesis genes. LAP LAMBERT Academic Publishing. (September 7, 2016). ISBN-10: 3659945560, ISBN-13: 978-3659945564.
24. Montenegro, E. M. D. S., Delabary, G. S., Silva, M. A. C. D., Andreote, F. D., & Lima, A. O. D. S. (2017). Molecular Diagnostic for Prospecting Polyhydroxyalkanoate-Producing Bacteria. Bioengineering, 4(2), 52.
25. Nair, S. S., Reddy, H., & Ganjewala, D. (2008). Screening and characterization of biopolymers polyhydroxybutyrate producing bacteria. Advance Biotech, 7(4), 13-7.
26. Nayak, P. K., Mohanty, A. K., Gaonkar, T., Kumar, A., Bhosle, S. N., & Garg, S. (2013). Rapid identification of polyhydroxyalkanoate accumulating members of Bacillales using internal primers for phaC gene of Bacillus megaterium. ISRN Bacteriology, 2013.
27. Nehra, K., Chhabra, N., Sidhu, P. K., Lathwal, P., & Rana, J. S. (2015). Molecular identification and characterization of Poly-β-hydroxybutyrate (PHB) producing bacteria isolated from contaminated soils.Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 17, No. (4): 2015: 281-290.
28. Osman, Y. A., Elrazak, A. A., Khater, W., Nashy, E. S., & Mohamadeen, A. (2015). Molecular Characterization of a Poly-β-Hydroxybutyrate-Producing Microbacterium Isolate. International Journal of Applied Sciences and Biotechnology, 3(2), 143-150..
29. Özgen, C. (2012). Hydrogen and poly-beta hydroxyl butyric acid production and expression analyses of related genes in Rhodobacter capsulatus at defferent acetate concentration (Doctoral dissertation, Middle East technical University).
30. Pinzon NM, Aukema KG, Gralnick JA, Wackett LP. 2011. Nile red detection of bacterial hydrocarbons and ketones in a high-throughput format. mBio 2(4): e00109-11. doi:10.1128/mBio.00109-11.
31. Poltronieri, P., Mezzolla, V., & D'Urso, O. F. (2016). PHB production in biofermentors assisted through biosensor applications. In Multidisciplinary Digital Publishing Institute Proceedings (Vol. 1, No. 2, p. 4).
32. Dana Gopal, N. M., Phebe, P., Kumar, E. S., & Vani, B. K. K. (2014). Impact of Plastic Leading Environmental Pollution. Journal of Chemical and Pharmaceutical Sciences ISSN, 974, 2115.
33. Reddy, C. S. K., Ghai, R., & Kalia, V. (2003). Polyhydroxyalkanoates: an overview. Bioresource technology, 87(2), 137-146.
34. Rehm, B. and A. Steinbuchel. (1999). Biochemical and genetic analysis of PHA synthases and other proteins required for PHA synthesis. International Journal of Biological Macromolecules. 25(1-3): p. 3-19.
35. Rehm, B. (2003). Polyester synthases: natural catalysts for plastics. Biochemical Journal,. 376: p. 15-33.
36. Revellame, E., Hernandez, R, French, W., Holmes, W., Benson, T., Pham, F. and Callahan, R. (2012). Fipid storage compounds in raw activated sludge microorganisms for biofuels and oleochemicals production. RSC Advances, 2(5), 2015-2031.
37. Rohini, D., Phadnis, S. and Rawal, S. (2006). Synthesis and characterization of poly-beta-hydroxybutyrate from Bacillus thuringiensis R 1. Indian J Biotechnol, 5 (3): 276-283.
38. Saharan, B. S., & Ankita, S. D. (2012). Bioplastics-for sustainable development: a review. Int J Microbial Res Technol, 1, 11-23.
39. Sangkharak, K., & Prasertsan, P. (2012). Screening and identification of polyhydroxyalkanoates producing bacteria and biochemical characterization of their possible application. The Journal of general and applied microbiology, 58(3), 173-182.
40. Sasidharan, R.S.; Bhat, S.G.; Chandrasekaran, M. Amplification and sequence analysis of phaC gene of polyhydroxybutyrate producing Vibrio azureus BTKB33 isolated from marine sediments. Ann. Microbiol. 2016, 66, 299–306.
41. Shaaban, M. T., Attia, M., Turky, A. S., & Mowafy, E. I. (2012). Production of some biopolymers by some selective Egyptian soil bacterial isolates. J. Appl. Sci. Res, 8(1), 94-105.
42. Shamala, T.R.; Chandrashekar, A.; Vijayendra, S.V.; Kshama, L. (2003). Identification of polyhydroxyalkanoate (PHA)-producing Bacillus spp. using the polymerase chain reaction (PCR). J. Appl. Microbiol. 2003, 94, 369–374.
43. Sharmila, T., Meenakshi, S., Kandhymathy, K., Bharathidasan, R., Mahalingam, R. and Panneerselvam, A. (2011). Screening and characterisation of polyhydroxybutyrate producing bacteria from sugar industry effluents. World J Sci Technol, 1 (9): 22-27.

44. Sheu, D. S., Wang, Y. T., & Lee, C. Y. (2000). Rapid detection of polyhydroxyalkanoate-accumulating bacteria isolated from the environment by colony PCR. Microbiology, 146(8), 2019-2025.
45. Singh, P. and Parmar, N. (2011). Isolation and characterization of two novel polyhydroxybutyrate (PUB)-producing bacteria. Afr J Biotechnol., 10 (24): 4907-4919.
46. Solaiman, D. K., & Ashby, R. D. (2005). Rapid genetic characterization of poly (hydroxyalkanoate) synthase and its applications. Biomacromolecules, 6 (2), 532-537.
47. Solaiman, D. K., Ashby, R. D., & Foglia, T. A. (2000). Rapid and specific identification of medium-chain-length polyhydroxyalkanoate synthase gene by polymerase chain reaction. Applied microbiology and biotechnology, 53(6), 690-694.
48. Sudesh, K. and Abe, H. (2010). Practical Guide to Microbial Polyhydroxyalkanoates. Smithers Rapra Technology. Published by Smithers Rapra Technology. ISBN: 978-1-84735-117-3.
49. Sujatha, K., Mahalakshmi, A., & Shenbagarathai, R. (2005). A study on accumulation of PHB in native Pseudomonas isolates LDC-5 and LDC-25.
50. Thirumala, M. and Reddy, V. (2012). Production of PHA by recombinant organisms. Int J Life Sc Bt Pharm Res., 1 (2): 2250-3137.
51. Trainer, M., Capstick, D., Zachertowska, A., Lam, K., Clark, S. and Charles T., (2010). Identification and characterization of the intracellular poly-3-hydroxybutyrate depolymerase enzyme phaZ of Sinorhizobium meliloti. BMC Microbiol., 10 (1): 92.
52. Tufail, S., Munir, S., & Jamil, N. (2017). Variation analysis of bacterial polyhydroxyalkanoates production using saturated and unsaturated hydrocarbons. Brazilian Journal of Microbiology.4 8 (4): 629–636.
53. Tzu, H.Y.; Semblante, G.U. (2012). Detection of polyhydroxyalkanoate-accumulating bacteria from domestic wastewater treatment plant using highly sensitive PCR primers. J. Microbiol. Biotechnol. 2012, 22, 1141–1147.
54. Wei, Y., Chen, W., Huang, C, Wu, H, Sun, Y., Lo, C. and Janarthanan, O. (2011). Screening and evaluation of polyhydroxybutyrate-producing strains from indigenous isolate Cupriavidus taiwanensis strains. Inter J Molec Scl, 12: (1).
55. Yang, C., Zhang, W., Liu, R., Zhang, C., Gong, T., Li, Q., & Song, C. (2013). Analysis of polyhydroxyalkanoate (PHA) synthase gene and PHA-producing bacteria in activated sludge that produces PHA containing 3-hydroxydodecanoate. FEMS microbiology letters, 346(1), 56-64.
56. Zhang, G., Hang, X., Green, P., Ho, K. P., & Chen, G. Q. (2001). PCR cloning of type II polyhydroxyalkanoate biosynthesis genes from two Pseudomonas strains. FEMS microbiology letters, 198(2), 165-170.
57. Zou, X., Li, H, Wang, S., leski M., Yao, Y, Yang, X., Huang, Q. and Chen, G (2009). The effect of 3-hydroxybutyrate methyl ester on learning and memory in mice. Biomaterials. 30 (8): 1532-1541.

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