ISSN 2709-3662 (Print)
ISSN 2709-3670 (Online)
https://doi.org/10.52587/JAF040201
Journal of Agriculture and Food
2021, Volume 2, No.1, pp. 26-33

Optimized Growth conditions for polyhydroxyalkanoate production by halotolerant bacteria isolated from Karachi mangrove forest

Naima Khan, Nazia Jamil

Abstract

Researchers have been investigating multiple alternatives for synthetic plastic to completely replace polystyrene plastics, one of which is Polyhydroxyalkanoates (PHA). PHA is biodegradable and biocompatible, and it shows elastomeric and thermostable properties comparable to polystyrene. The purpose of this study was to isolate bacterial species TattaPani Kashmir and Karachi Mangrove forest capable of surviving high salinity and poroducing PHA. The isolated bacterial strains were provided with different growth conditions such as pH, Temperature and salinity for optimized growth and PHA production. Ten bacterial strains were selected for this experiment, and it was found that *Bacillus aryabhattai*WK31 produced maximum (61%) PHA at 37℃, 6% NaCl and 6.8 pH. Similarly, strain *Bacillus aryabhattai*S1, Bacillus halotoleranus14SM, and Bacillus aryabhattaiAFSN2 were also able to produce 55, 58 and 61 percent PHA at similar growth condition. Moreover, these bacteria, particularly 14Sm, were able to tolerate salinity as high as 12 g l^{-1}. The importance of optimized growth conditions is unparalleled as bacteria, like other organisms, need ideal conditions to produce a maximum polymer.

Keywords: Biodegradeable, Biocompatible, Mangroves, Salinity
Article History:
Received: 29th May, 2021; **Revised:**7th June, 2021; **Accepted:**16rh June, 2021

Introduction

Institute of Microbiology and Molecular Genetics, University of the Punjab, Quaid e Azam campus 54590, Lahore, Pakistan; Corresponding author: nazia.mmg@pu.edu.pk

PHAs are green plastics and have positive social and environmental effect when compared with conventional polyethene plastics. PHAs are biodegradable – recycled completely into carbon dioxide and water –, and can be produced from no-cost feedstocks. PHAs can be used in synthetic plastics to reduce plastic and organic waste and can act as a sustainable resource in doing so. Moreover, PHAs are biocompatible and do not harm the living systems and environment (Raza, Abid, Banat, & Biodegradation, 2018). PHAs are hydrophobic inclusion bodies that are produced by many bacteria and are made up of various hydroxyalkanoaic acids. Naturally PHAs act as energy sources for bacteria as these are accumulated under carbon excess and nitrogen limited conditions (Javaid et al., 2020).

Optimized growth conditions are very vital to get cost-effective and maximum yield of PHA. Several parameters affect the production of low-cost PHA, and among these, carbon sources and sterilization play a pivotal role (Sabapathy et al., 2020). Bacterial species that can survive constricted growth condition are suitable candidates for PHA production. Halotolerant bacteria can be used for the commercial production of PHA as they can grow in a selective media having high salt concentration and acting as potential competitors for other bacteria (Tan, Wang, Tong, & Chen, 2021). For the past 30 years, PHA has been produced in considerable quantities for many applications research, including medical implants. Due to its diverse and increasing mechanical, biodegradable, and tissue-friendly qualities. Importantly, the byproducts of PHA biodegradation, such as oligomers and monomers, are not harmful to cells and tissues. Some PHA degradation products have also been used in pharmaceutical applications. So far, no study has found any evidence of carcinogenesis caused by PHA or its biodegradation products (Zhang et al., 2018). Bacterial strains that was first identified as PHA producer was genus *Bacilllus* and it has been widely used for the production of PHA due to its wide range of nutrient utilization. In this article, two general – pH, Temperature – and one specific (salinity) growth condition was monitored to get the optimized condition under which the selected bacterial strains give a maximum yield of PHA. The evaluation of growth conditions is very important for the successful polymer production, as microbial bodies work under delicate balance of temperature, pH and nutrients.

Methodology

Sample collection

Several samples of soil and water were collected from TattaPani Kashmir and Karachi mangrove forest.

Sample collection and Isolation of bacteria strain

We collected soil, fine stones, moderate-sized stones and even large-sized stones. Different water samples were also collected. Temperature and pH were also noted to notify indigenous environmental conditions of bacterial flora. Mangroves were collected from the Karachi mangrove forest. Rhizosphere soil was collected, and bacterial strains were isolated. Serial dilutions of collected samples were made, and the spread plate technique was used to isolate bacteria. Twenty-five µl of dilutions made was spread on a nutrient agar plate. Agar plates were incubated at 37°C for 24 hours. Colony-forming unit (CFU) per ml was calculated.

Screening of bacteria strains for PHA production

Polyhydroxyalkanoates detection agar (PDA) (Naeem, Khan, & Jamil, 2018) was used for the Isolation, purification and screening of bacteria for PHA production. The PDA media was supplemented with Nile blue to visualize bacterial colonies under UV as Polymer producing bacteria bind with dye and fluoresce (Gholami et al., 2020). Further confirmation for PHA production was done by staining viable bacterial cells with Sudan Black and visualizing them at 100X magnification(Ali, Jamil, & Technology, 2018).

Evaluation of Optimized, pH, salinity and Temperature

Polyhydroxyalkanoates detection agar (PDA) (Naeem et al., 2018) was used to isolate and purify bacteria. Furthermore, the media was modified by adding NaCl – 0.1M, 0.5M, 1M, 2M and 3 M – to supplement the requirement of halophilic bacteria. Collected samples were diluted and spread on the agar plates and incubated at 37℃ for 24 hours. Optimization of pH was carried out by supplementing bacteria with PDA and 0.5M NaCl at different pH – 4, 6.8, 8 and 10 and incubating at 37℃ for 24 hours. Similarly, Temperature was also optimized by incubating the selected bacteria at 28, 37 and 45℃ for 24 hours.

Extraction of Polyhydroxyalkanoate

The bacterial cells were harvested from 200 ml of culture by centrifugation and lyophilized. The biomass of cells was determined, and then the biomass was subjected to extraction. Eight grams of biomass was suspended in 100 ml of sodium hypochlorite, and biomass was vortexed to ensure removal of clumps, then 100 ml of chloroform was added to the mixture. Biomass containing organic and inorganic solvents was incubated on a shaker at 120 rpm for 90 minutes. Two layers were formed while the organic layer contained PHA, both layers were separated, and the organic layer was dried to get PHA (Munir & Jamil, 2018). The following formula calculated the percentage of PHA:

$$\text{Percentage PHA} = \text{weight of PHA} / \text{Biomass} \times 100$$

Results

Screening of Bacterial Strains for PHA production

Twelve bacterial strains were isolated based on their ability to produce PHA. The PHA producer strain was able to fluoresceNile blue fluorescent dye, as shown in figure 1a. Further confirmation of intracellular PHA granule production was done by Sudan Black staining. The intracellular PHA granules were visible when the cell was viewed under a light microscope using 100X magnification (Figure 1b). Selected bacteria were able to grow in the presence of nile blue as well as nile red fliurescent stains and utilize polyhydroxyalkanoate detection agar for PHA accumulation. After 24 hours of growth almost all selected bacterial strains were showing visible PHA intracellular PHA granules.

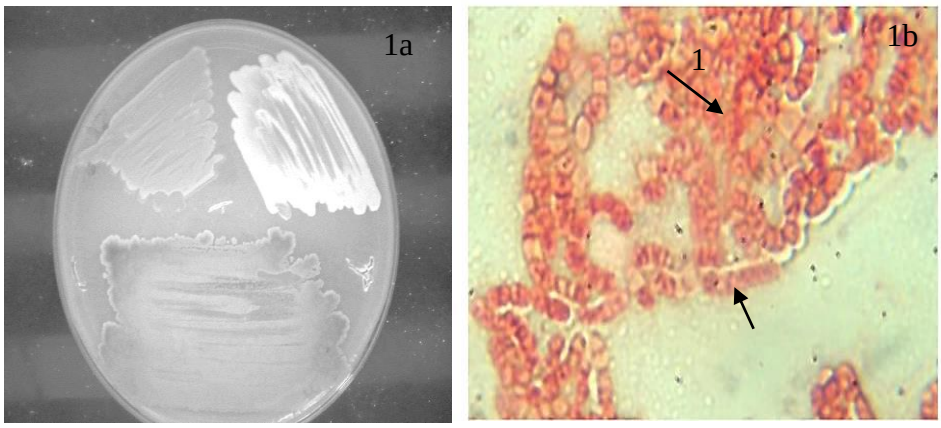

Figure 1. Preliminary screening of PHA producing stain, bacteria strain capable of producing PHA fluoresce when grown in PDA media supplemented with Nile blue A (left), after staining with Sudan black PHA granules can be seen intracellularly as indicated by arrows (right)

Optimization of pH for bacterial Growth

PHA producing bacteria were optimized at different pH values in PDA medium. While optimizatizing pH other growth conditions were kept constant such as temperature at 37°C and NaCl 0.5 M. The pH ranged from 4.8 to 10 and the optimal pH was found to be 6.8 ± 0.2. It was found that all bacteria showed their maximum growth at pH 6.8, least bacterial growth at pH 4 and 10. In contrast, moderate growth was seen at pH 8. Bacterial strain WK31 produced 61% PHA at pH 6.8 and biomass 2.41g/l. Strain 14SM also produced 61 % PHA and 2.4 g/l of biomass at pH 8. It should be noted that this study was conducted at 1M salinity and 37 ºC.

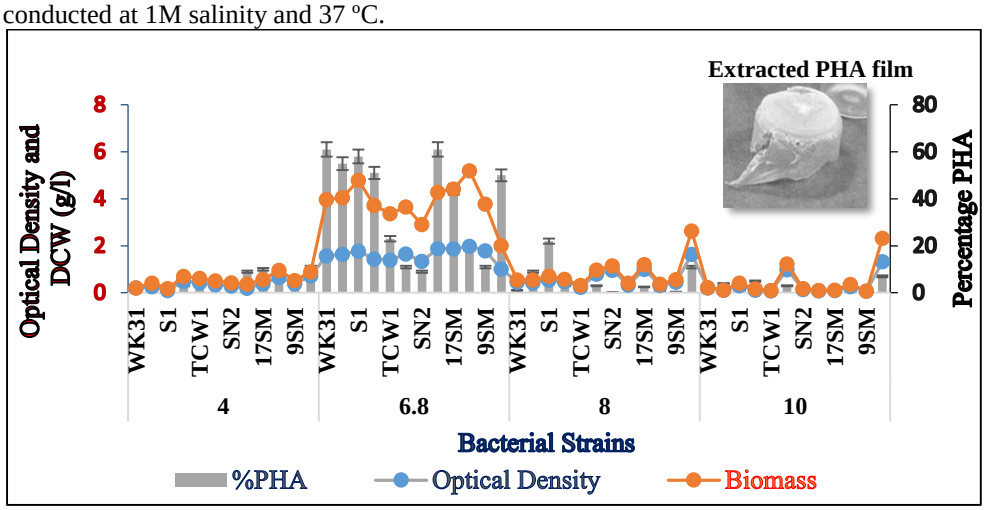

Figure 2. Optimization of PHA production and growth kinetics at different pH

Optimized salinity for bacteria growth and polymer production

Isolated bacterial strains could tolerate high salinity, and bacterial strains isolated from Khewra Salt Mines showed maximum salt tolerance. Bacterial strain WK31 and 14SM tolerated salt concentration as high as 3M (18g/l). Maximum PHA production by these strains was noted at 1M NaCl; however, at 3M salinity, both strains also produced above 55 % PHA. only a few bacterial strains were able to grow in the presence of 2 and 3 M NaCl including WK31, 14SM, and 17SM. While other strains such as NK19 although able to tolerate 1M NaCl, couldn't grow in the presence of high salinity.

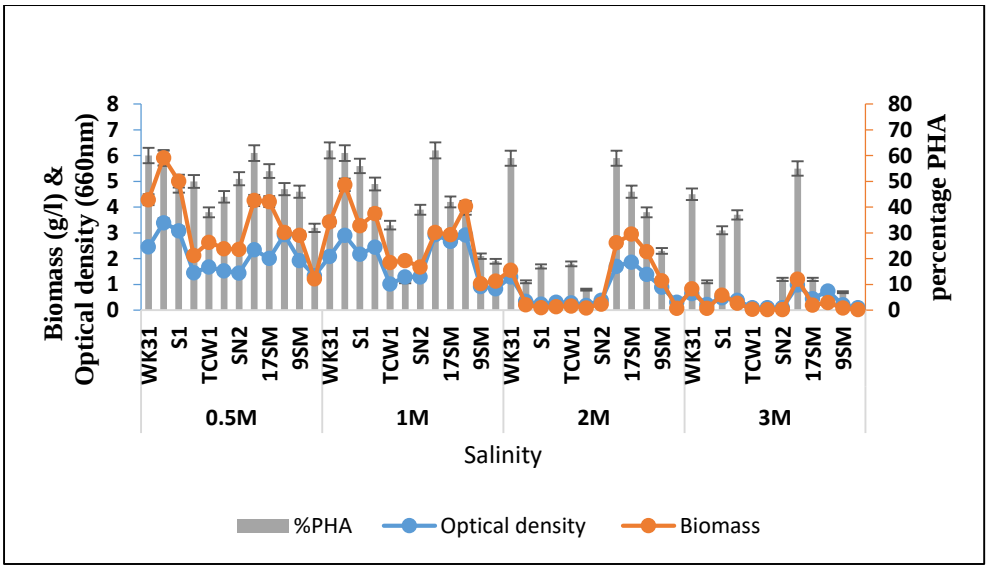

Figure 2. Optimization of PHA production and growth kinetics at different levels of salinity

Optimization of temperature for bacterial growth

Bacteria were also grown at different temperatures, and it was found that bacterial isolates had better growth at 28 ºC. At the same time, AFSN2 and WK31 were able to show maximum growth at 37 ºC. as shown in figure 3; bacteria were able to grow at 28℃ as well. It should be mentioned here that the bacterial strains were isolated from moderate or mild temperature areas; therefore, the bacteria were able to grow at temperatures as low as 20℃.

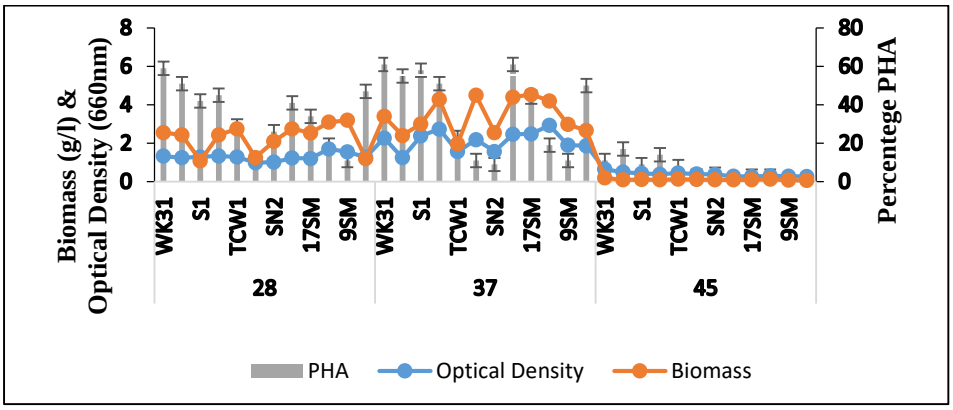

Figure 3. Optimization of PHA production and growth kinetics at different temperatures

Discussion

Various habitats have been explored for PHA producing strains as different environments offer variable growth conditions and bacteria adapt to these conditions. Many attempts have been made to produce PHA from low-cost waste substrates, especially food processing waste, to reduce the price of PHA. Lipid-based products, such as waste frying/cooking oil (Obruca, Snajdar, Svoboda, Marova, & biotechnology, 2013) and palm kernel oil (Loo et al., 2005), in particular (Ye et al., 2018). Optimization of growth conditions for maximum product yield is very vital in microbiological studies. Various growth parameters were tested for optimal production of PHA. While optimizing pH, maximum biomass and PHA production was observed at 6.8 pH. However, NK19 produced 11% PHA at pH 10 and 8 while showing considerable growth.

Bacterial strains, WK31, 7SM, 9SM, 14SM, could tolerate salinity even at 13g l^{-1}. Strain 14SM as isolated from salt mines could tolerate nearly 18 g l^{-1} of salt and produced 55% PHA at high salinity. Bacterial strain AFSN2, S1 and S9 were able to tolerate salt concentrations up to 6g/l; these bacterial strains could grow at higher salt concentration; however, the growth was not very considerable. Bacteria that are able to grow in saline environments cannot survive in low salt concentrations their proteomes are very acidic and most of the proteins degrade in low salt (Oren, 2008). All bacterial strains were able to grow at 20℃ and 37℃. The reason for growth at 28℃could is that the isolated bacterial strains were mild and low-temperature areas. Therefore, for further experimentation, the room temperature was used, which ranged from 25℃ to 38℃. Due to several unique characteristics, such as a high salinity requirement that prevents microbial contamination, a high intracellular osmotic pressure that allows for easy cell lysis for PHA recovery, and the ability to use a wide range of low-cost substrates, halophiles are thought to be a promising cell factory for PHA synthesis. The use of halophiles has made it possible to accomplish large-scale manufacturing at minimal cost by optimizing fermentation settings. Some bacteria survive high saline environment by excluding the salt from their cytoplasm or produce solutes that do not interfare with the metabolic activities of the cell (Chen, Lu, Shyu, & Lin, 2017). Further research into halophiles has revealed diverse and

even novel PHA synthesis routes in various halophilic organisms, which has a significant impact on PHA type (Mitra, Xu, Xiang, & Han, 2020).

Conclusion

Cost management is one hindrance that makes commercial production of PHA challenging. Halotolerant bacteria can be investigated for the large-scale production of PHA as it decreases the cost by limiting the need for sterilization. High salt concentration in the growth medium allows halotolerant bacteria to grow in the medium without competing with non-salt tolerant bacteria for nutrients. In this study, growth conditions were optimized for PHA production by halotolerant bacteria. We isolated screened five bacterial species able to tolerate high salt concentration and produce PHA. The selected bacterial strains tolerated upto 3M NaCl, the optimum pH was recorded to be 6.8 and temperature 37°C ± 5

References

Ali, I., & Jamil, N. (2018). Biosynthesis and characterization of poly3-hydroxyalkanote (PHA) from newly isolated bacterium Bacillus sp. AZR-1. *Iranian Journal of Science and Technology, Transactions A: Science*, *42*(2), 371–378.

Chen, Y. H., Lu, C. W., Shyu, Y. T., & Lin, S. S. (2017). Revealing the saline adaptation strategies of the halophilic bacterium Halomonas beimenensis through high-throughput omics and transposon mutagenesis approaches. *Scientific reports*, *7*(1), 1–15.

Gholami, A., Ghasemi, Y., Kazemi, A., Abootalebi, S. N., Irajie, C., Ireji, A., Mohkam, M. J. J. o. E. T. T. (2020). Bacterial Strain Isolated from High-Salt Environments Can Produce Large Amounts of New Polyhydroxyalkanoate (PHA). *8*(4), 1268–1273.

Javaid, H., Nawaz, A., Riaz, N., Mukhtar, H., Ul-Haq, I., Shah, K. A., Rasool, A. (2020). Biosynthesis of Polyhydroxyalkanoates (PHAs) by the Valorization of Biomass and Synthetic Waste. *Molecules*, *25*(23), 5539.

Loo, C.-Y., Lee, W.-H., Tsuge, T., Doi, Y., & Sudesh, K. J. B. l. (2005). Biosynthesis and characterization of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) from palm oil products in a Wautersia eutropha mutant. *27*(18), 1405–1410.

Mitra, R., Xu, T., Xiang, H., & Han, J. J. M. c. f. (2020). Current developments on polyhydroxyalkanoates synthesis by using halophiles as a promising cell factory. *19*, 1–30.

Munir, S., & Jamil, N. J. J. o. b. m. (2018). Polyhydroxyalkanoates (PHA) production in bacterial co-culture using glucose and volatile fatty acids as carbon source. *58*(3), 247–254.

Naeem, T., Khan, N., & Jamil, N. J. F. i. B. (2018). Synthesis of scl-poly (3-hydroxyalkanoates) by Bacillus cereus found in freshwater, from monosaccharides and disaccharides. *13*(1), 63–69.

Obruca, S., Snajdar, O., Svoboda, Z., Marova, I. J. W. J. o. m., & biotechnology. (2013). Application of random mutagenesis to enhance the production of polyhydroxyalkanoates by Cupriavidus necator H16 on waste frying oil. *29*(12), 2417-2428.

Oren, A. (2008). Microbial life at high salt concentrations: phylogenetic and metabolic diversity. *Saline systems*, *4*(1), 1–13.

Raza, Z. A., Abid, S., Banat, I. M. J. I. B., & Biodegradation. (2018). Polyhydroxyalkanoates: Characteristics, production, recent developments and applications. *126*, 45–56.

Sabapathy, P. C., Devaraj, S., Meixner, K., Anburajan, P., Kathirvel, P., Ravikumar, Y., . . . Qi, X. J. B. t. (2020). Recent developments in Polyhydroxyalkanoates (PHAs) production–A review. *306*, 123132.

Tan, D., Wang, Y., Tong, Y., & Chen, G.-Q. J. T. i. B. (2021). Grand Challenges for Industrializing Polyhydroxyalkanoates (PHAs).

Ye, J., Huang, W., Wang, D., Chen, F., Yin, J., Li, T., . . . Chen, G. Q. J. B. j. (2018). Pilot scale-up of poly (3-hydroxybutyrate-co-4-hydroxybutyrate) production by Halomonas bluephagenesis via cell growth adapted optimization process. *13*(5), 1800074.

Zhang, J., Shishatskaya, E. I., Volova, T. G., da Silva, L. F., Chen, G.-Q. J. M. S., & C, E. (2018). Polyhydroxyalkanoates (PHA) for therapeutic applications. *86*, 144–150.

Citation of Article

Khan, N., Jamil, N. (2021). Optimized Growth conditions for polyhydroxyalkanoate production by halotolerant bacteria isolated from Karachi mangrove forest. *Journal of Agriculture and Food*2(1), 26–33. https://doi.org/10.52587/JAF040201.