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Optimization of Culture Conditions for Polyhydroxybutyrate (PHB) Production in the Positive Bacterial Isolates of the Garden Soil

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ABSTRACT: Solid plastic waste management issues and global environmental concerns have led to the development of biodegradable plastics which will have similar physical and chemical properties of the conventional plastics. Soil bacteriology possess many different bacteria which possess unique properties. Polyhydroxybutyrates (PHB) are the biological macromolecules produced by numerous soil bacteria and these can be used efficiently as an alternative to conventional plastics. In this research, Polyhydroxybutyrate producing bacteria are isolated from garden soil sample and potent producer identified by Crotonic acid assay. The amount of production of PHB are usually limited due to unbalanced growth conditions and nutrient availability. So these bacteria need optimised condition for maximal production of PHB. Modification of culture conditions, media composition, incubation period, inoculum size, pH, temperature, carbon nitrogen ratio, etc has a notable effect in the production. Slight changes in production conditions alter the bacterial growth in turn making changes in production of PHB. In the present study conducted, the potent strain SM1 was optimised and the optimal conditions were incubation period - 72 hours, inoculum size –2ml, Carbon sources – Glucose, Nitrogen source – Peptone, Carbon: nitrogen ratio – 15:1, Natural source – Rice water, pH –7, Temperature –37°C. Maintaining these optimised conditions during production process, maximum amount of PHB was produced. Further, large scale production can be done in fermenters with these optimised conditions for more production.

Keywords: Polyhydroxybutyrate, Crotonic acid assay, inoculum, fermenters, plastic waste management.

INTRODUCTION

Plastics replace natural materials and have found wide application in human life. They are inert molecules and are extremely durable. Plastic materials are petrochemical based derivatives and exist as environmental hazards since they are not degradable. Plastic material has become an integral part of our lifestyle because of their resistance to degradation (Pabitra *et al*., 2015). These polymers are usually inexpensive and they have a significant environmental impact (Brandl *et al*., 1990).

Instead the usage of Biodegradable plastics protects our environment from the hazards. There are many biodegradable plastics with varied biodegradability range (Getachew and Woldesenbet 2016). Polyhydroxyalkonates are a group of various biopolymers which can be used as bioplastics. Among the PHA s polyhydroxybutyrate gain importance and they are an extensively studied biopolymer.

PHB is a naturally found biological polymer with linear structure. "Polyhydroxybutyrates are macromolecules synthesised by bacteria and are inclusion bodies accumulated as reserve material when the bacteria grow under different stress conditions. More than 75 different bacterial genera have been reported so far. Commonly studied organism accumulating PHB include the following genera of *Bacillus, Azetobacter, Alcaligenes, Rhizobium, Pseudomonas, Rhodospirillum* etc. (Sudesh and Doi 2000; Pabitra *et al.,* 2015). They are completely degraded by biological organisms into carbon-di-oxide and water by depolymerase enzymes found in some of the bacteria (Sakthiselvan *et al*., 2019).

In PHB producing bacteria, PHB synthesis takes place when suitable carbon source is available in excess and cellular growth is made limited due to the lack of other nutrients. Maintaining optimal culture conditions are essential to synthesize considerable amount of PHB. PHB producing bacteria should be provided optimal culture conditions *viz.*, higher concentration of carbon sources, limited amount of nitrogen, phosphorus, trace elements and sulphur (Gabr, 2018).

PHB has a very high demand because they are used as surgical sutures, as tissue scaffolds in tissue engineering and as drug delivery material (Rossi *et al*., 2021). PHB has a very narrow range of thermal processing and at high temperatures they undergo thermal degradation. It may result in the decrease of molecular weight, slight change in colour and mechanical properties. To overcome this problem stabilizers and lending chemicals can be used (Rossi *et al*., 2021; Pabitra *et al*., 2015)

The current study is focused on isolating PHB producing bacteria from garden soil sample, inoculation production and extraction of PHB, Crotonic acid assay for quantification, and optimisation of Size of inoculum, incubation period, pH, temperature, carbon sources, nitrogen sources, carbon nitrogen ratio, natural sources etc. The influence of the latter parameters has marked role in the better yield and quantity of the PHB, thus the optimal levels are identified and studied accordingly.

MATERIALS AND METHODS

Screening and isolation of PHB producing bacteria: The humus soil sample was collected aseptically in a sterile container from the garden area, Nattalam, Kanyakumari District, Tamilnadu. Nattalam is a village located in the Killiyoor Block of Kanniyakumari District.

Serial dilution, plating technique & Sudan black blue staining:

One gram of soil sample was weighed and serial dilution was performed upto 10^{-10} dilution and spread over nutrient agar plates. The plates were incubated at 37°C in an incubator to promote bacterial growth.

Sudan black blue staining:

In petriplates: To screen PHB-producing bacteria in petriplates 0.02% of Sudan black B stain was prepared as described by Parshad *et al.* (2001). Overnight incubated nutrient agar plates were flooded with sudan black blue stain. The plates were kept undisturbed for about 20 minutes. The excess dye was stripped and the plates were washed for about 30 seconds with 80 % Ethanol with constant swirling. Those bacteria capable which appeared bluish black in colour were PHB producers and the white colonies were non PHB producers. The positive isolates were subcultured numerous times to obtain pure colonies. (Kitamura and Doi 1994).

In slides: For further confirmation, the existence of PHB granules in the cytoplasm were checked under microscope. For this 0.3% sudan black blue stain in absolute ethanol was prepared. The stain was filtered with the filter paper to remove the sediments. A Thin smear of bacterial strains were prepared and heat fixed on a clean glass slide. It was then stained for about 20 minutes with the specified dye, and further counter stained for about 10 to 20 seconds with 0.5% aqueous safranin. Then the slides were air dried and observed under a microscope. (Kitamura and Doi 1994).

Nile blue staining: The positive isolates were further screened by Nile Blue A, more specific, rapid and sensitive stain. Carbon rich nutrient agar medium was prepared with excessive addition of glucose. The media was supplemented with 0.5 µg/mL nile blue A. The colonies were inoculated and incubated overnight. The growth of the cells occurred in the presence of Nile blue dye. When irradiated in the presence of UV light, bright orange fluorescence was observed in the colonies. The colonies with bright orange fluorescence were selected as PHB accumulators. (Iman *et al.*, 2018)

Maintenance of pure culture and Biochemical characterisation: Under temperature - controlled condition, the selective strains testing positive for sudan black blue stain were maintained as slants in test tubes

Production and quantification of PHB: The bacterial cultures were inoculated in carbohydrate sufficient medium and incubated for about 72 hours. Extraction of PHB was carried out according to sodium hypochlorite method (Ramsay *et al*., 1994). Following incubation, the cultures were centrifuged at 10,000 rpm for about 10 minutes to obtain the culture filtrate. To the pellet 10ml of sodium hypochlorite was added and mixed well. It was then incubated for about 1 hour in water bath maintained at 50°C. After an hour, it was centrifuged at 5000 rpm for 15 about minutes. Supernatant was discarded and the pellet was washed with distilled water, acetone and ethanol. Finally, 5ml of boiling chloroform was taken and pellet was dissolved in it. It was then poured in a sterile glass plate to evaporate chloroform and kept overnight at 4°C. The next day powdered PHB settled in glass plate and was collected by scratching with a spatula and stored in bottle for further analysis. (Ramsay *et al*., 1994).

Quantification of PHB by Crotonic acid assay: Using the PHB standard the concentration of produced PHB was estimated by crotonic acid assay. The produced PHB powder was taken in a test tube. To that 5ml of concentrated sulphuric acid was poured through the sides of the test tube and kept in boiling water bath for about 40 minutes. Now, the PHB crystals were converted to crotonic acid. The absorbance was measured at 235nm with varied PHB concentration range from 100-1000 µg/ml (Lee and Choi 1999). Based upon the estimation, the potent strain identified and used for optimisation studies.

Optimization:

Size of the inoculum: Inoculum plays an important role in the production. For optimising inoculum size, the cultures are inoculated in five different conical flasks with inoculum size ranging from 1ml, 2ml, 3ml, 4ml, 5ml. After incubating for three days, PHB produced from each flask are subjected to Crotonic acid assay, the optical density values were recorded and analysed through graphical methodology with PHB standard.

Incubation period: The production medium was prepared and 2ml of inoculum was added to it. Each culture flask was incubated at 37°C under different incubation time period *viz.,* 24 hrs, 48 hrs, 72hrs, 96hrs, 120 hrs. After that the PHB was extracted and subjected to Crotonic acid assay to quantify PHB and the optimum incubation period was recorded.

Optimization of pH for PHB production: To standardize the optimum pH at which maximum production occurs, the culture was grown in specialised

media with different pH *viz*., 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and incubated at 37ºC for about 72 hours. After incubation, PHB was extracted using sodium hypochlorite chloroform method and the produced PHB was analysed spectroscopically by Crotonic acid assay. Based on these the optimal pH for PHB production can be found out

Temperature optimization for PHB production: To identify the optimum temperature at which the bacteria produce bulk amounts of PHB, the cultures were grown in specialized medium and incubated at various different temperatures say 25, 30, 32, 35, 37, 40ºC for about 72 hours. After three days of incubation the extraction of PHB was carried out followed by Crotonic acid assay in which absorbance was measured at 635nm. On analysing the maximal PHB, the optimum temperature can be determined.

Optimization of chemicals for PHB production:

Optimization of carbon sources: The different carbon sources like glucose, fructose, mannose, maltose, galactose and sucrose in the concentration of 1% were added to the production media. The specified culture was inoculated into the media with pH 7 and incubated at 37ºC for optimized duration of 72 hours. The produced PHB was then extracted and quantified by Crotonic acid assay. From these recordings, the optimum carbon source was elucidated.

Optimization of Nitrogen Sources: The nitrogen sources include yeast extract, peptone, ammonium sulphate, ammonium chloride and urea were added to the production medium in the concentration of 0.1 g/100 ml. The bacterial culture was inoculated into the medium with pH 7 and incubated at 37 ºC for optimized duration of 72 hours. The PHB produced was then extracted from the medium and subjected to crotonic acid assay & the optimum nitrogen source determined.

Optimising C:N ratio: Balancing of carbon nitrogen ratio plays a key role in the production process. The optimised carbon and nitrogen sources namely Glucose: Peptone are used in this in varied ratios. The ratios used for Glucose: peptone is 10:1, 15:1, 20:1. The culture was inoculated and optimum conditions of pH 7, temperature 37ºC were maintained and incubated for about 72 hours. Further PHB was extracted from each composition and read spectroscopically at 635nm by Crotonic acid assay which indicates the best combination of carbon nitrogen ratio.

Optimisation of Natural sources: The culture is inoculated with different media composition containing different natural sources like Rice water, corn starch, potato starch, wheat bran, molasses. After 72 hrs, the produced PHB was analysed spectroscopically by crotonic acid assay which interprets the best natural additive.

RESULTS AND DISCUSSION

The study was conducted using garden soil sample as the source for bacterial isolation soil bacteriology possess many different bacteria with specialized nature. Among the isolated bacteria, PHB producing bacteria were screened by using sudan blackblue staining. The bluish black colonies (Fig. 1) in petriplates indicating positive results were used for further studies. Five different colonies of bacteria were randomly selected and labelled as SM1, SM2, SM3, SM4 & SM5. Sudan black blue staining in slides (Fig. 2) revealed the bacteria stained black under microscope. Nile blue staining showed PHB accumulators as bright orange fluorescence (Fig. 3). Sodium hypochlorite method was used for extraction of Polyhydroxybutyrate. Further crotonic acid assay reveals the potent producer. Among the five different bacteria, the strain SM1 was found to be the potent producer with good polyhydroxy butyrate yield and used for further studies.

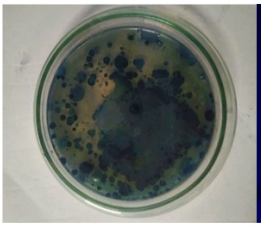

Fig. 1. Sudan black blue staining in petriplates.

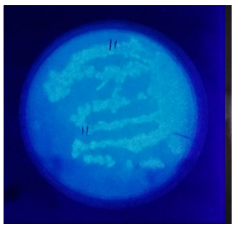

Fig. 2. Nile blue staining (Fluorescence under UV transilluminator).

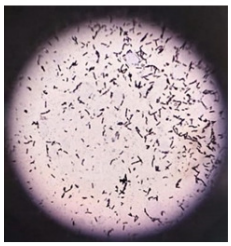

Fig. 3. Sudan black blue staining (viewed under microscope).

In the current study the potent PHB producer was optimised under different conditions *viz.,* size of the inoculum, incubation period, pH, temperature, carbon sources, nitrogen sources, Carbon: nitrogen ratio, utilizing natural sources etc. Optimising the proper condition will result in promising amount of PHB production. Extraction of PHB is done through sodium hypochlorite method. Estimation is performed through crotonic acid assay from which the amount of PHB produced can be calculated at each optimisation.

About 2ml inoculum yielded good amount of PHB (Fig 4a), inoculation with 1ml also provided good yield, but further increase in inoculum size from 3ml to 5ml resulted in poor production. Gradually, as the size of the inoculum increased, the concentration of PHB showed a steady decrease. As the biomass increased, due to nutrient depletion the bacteria used the reserved storage PHB as their energy source. This could be the reason for steady decline in PHB production once the

biomass accumulated (Getachew and Woldesenbet 2016; Vishnuvardhan and Thirumala 2009; Waqas *et al*., 2010).

Low amount of inoculum can lead to insufficient number of microbial cells and low amount of PHB. On the other hand, a much higher inoculum may cause oxygen limitation, improper mixing as well as fast depletion of nutrients in the culture media (Abusham *et al.,* 2009) and in turn poor PHB yield. When two percentage inoculum was used, *Bacillus sphaericus* NCIM 5149 yielded maximum PHB (Ramadas *et al*., 2010). *Bacillus subtilis* NG220 yielded more with 1% v/v inoculum (Anish *et al*., 2013). Maximum PHA accumulation was noted when 5×104 cells in 100 ml medium was used. High or low amount of inoculum resulted in reduction in PHA production (Kumbhakar *et al*., 2012). The very good approach for PHB production is that very high cell densities should be attained first followed by limiting the conditions which will trigger PHB accumulation (Sindhu *et al*., 2013).

Many reports indicated that PHB production was usually high in the incubation period from 48-72 hours and maximum production observed at 48 hrs (Adwitiya and Prabhu 2009; Gomez *et al.,* 2020; Getachew and Woldesenbet 2016), but after that period there was a steady decrease in production. It means that the organisms are devoid of nutrition and they are moving towards from steady state phase to decline phase. So, harvesting the cells and extraction of PHB during the time interval of 48-72 and exactly at 72 hours would definitely yield good amount of PHB. In *Pseudomonas xiamensis* until 96 hours of incubation, PHB production increased gradually and decreased further (Mostafa *et al*., 2020). During 48 hrs of incubation, *Bacillus pasteuri* and *Micrococcus* showed maximum yield of 36.41% and 34.59% respectively. In *Rhizobium* Sp and *Pseudomonas stutzeri* production of PHB was 81.8% and 83.62% respectively (Mostafa *et al*., 2020). Assessing all these reports it is concluded that PHB production was best achieved during 48-72 hours of incubation. In the current study conducted PHB production constantly increased from 48 hrs to 72 hrs (Fig 4.b) and then it started reducing in declined phase of the organism.

Addition of carbon sources like glucose showed a steady increase in PHB production (Fig. 4c) in the study conducted. Gomez, Getachew reported that when more amount of carbon source is added in the medium there was steady increase in Phb yield, this means that the Bacteria also depend on glucose for their energy production, Adwitiya and Prabhu (2009) reported good amount of PHB yield with glucose as their sole carbon source in the bacterium. The Role of carbon sources are important as they have three different functions in the bacteria viz., biomass production, upholding of cell and polymerization of PHB (Hungund *et al*., 2013). The application of PHB has been recognized in various industrial and biomedical sectors but its commercial use is limited because of high production cost and only carbon source contributes to 50% of the overall production cost. Recent research in PHB production are focused on the identification of renewable and cheaper sources of carbon. According to Sharma and Bijender (2000) *Bacillus cereus* PS 10 seems to possess versatile metabolic potential because it used varied carbon sources for growth and PHB production. On examining the various carbon sources like glycerol and molasses were found to be most effective in PHB production. Glycerol as carbon sources gave a yield of 8.9±0.3 g/L after 72 h, while molasses as carbon source gave 8.6±0.25 g/L after 48 h which was comparable with that of glucose. To lower the PHB production cost, cheap sources such as sucrose (Quillaguaman *et al*., 2007), can be used. Whey was preferred for PHB production. (Ahn *et al*., 2000; Wong and Lee 1998), starch was reported for cheap and effective PHB production (Rhu *et al*., 2003). Soy waste was also used by some researchers which yielded good amount of PHB (Hong *et al.,* 2000).

The various nitrogen sources used in production media are glycine, peptone, urea, yeast extract, ammonium chloride, ammonium sulphate, potassium nitrate etc. The use of Nitrogen sources is usually limited in production media. Under peptone as nitrogen source (Fig. 4d) there was a remarkable increase in PHB production. When inorganic nitrogen sources like ammonium chloride, ammonium sulphate, potassium nitrate are used there is a significant reduction in the production of PHB. Xi *et al*. (2000) determined PHB synthesis was highly dependent on the nitrogen source. In contrast to this observation, in another study, the amount of PHB produced by Ba21S strains in various carbon sources was found to be lower than that of the amount of PHB production in Ml 7 broth medium which was maintained as control (Yuksekdag and Beyatli 2008). Similar results were obtained by Goken *et al*. (2000) who reported that the *Pseudomonas putida* G13 strain was examined for PHB production in different nitrogen sources. The amount of PHB produced by this strain in the medium was lower than the amount of PHB production in nutrient broth medium. PHB synthesis by *Pseudomonas oleovorans* grown was not significantly stimulated by carbon limitation, but *Pseudomonas putida* responded to carbon limitation by greatly increasing the PHB production rate. Similarly, *Halobacterium* and *Pseudomonas sp*. increase PHB production under carbon limitation (Follner *et al*., 1995). PHB synthesis was best in an environment with high carbon to low nitrogen ratio. The carbon: nitrogen ratio of 15:1 (Fig. 4e) yielded remarkable results of PHB production.

Natural Carbon sources like rice water (Fig. 4f) had a great impact in PHB production with high yield while compared to other natural sources. Carbon sources like walnut shell powder and almond shell powder are being reported for the time, and showed promising results for PHB production by B. cereus PS 10. (Sharma and Bijender 2000) Molasses which is a common industrial by-product from sugar industries is rich in nutrients, minerals and at the same time it is cheap, available in plentiful amounts and could be exploited for production of variety of industrial products (Follner *et al*., 1995). However, molasses may have various growth inhibitory compounds also. Molasses was used as a carbon source for PHB production from *Alcaligenes* sp. and presence of growth factors like organic acids, vitamins and

minerals in molasses may enhance PHB yield (Tripathi and Srivastava 2010). PHB producing organism PFN29 was cultured in modified minimal medium, pH 7.0 for 72 h at 30°C and with different carbon sources. The results of the analysis showed that the PHB concentration was 2.63 ± 0.06 g/l and were maximal when glucose was used as carbon source (Sriyapai *et al.,* 2022). The carbon source was always provided in excess to allow maximum PHB accumulation in the biomass.

For the strain *P. xiamensis* optimum pH was found to be 7.5. Acidic and alkaline pH were not found to be suitable for PHB production (Mostafa *et al.*, 2020). *E. coli, Bacillus subtilis, Ralstonia eutropha,* cells reported maximum PHB production at pH 7. Effect of pH on biopolymer production increased with increase in pH from 5.0 to 9.0 and then slowly declined with further increase in pH of the fermentation medium. Maximum biopolymer production was found 3.27 g/L at pH 7.0. (Ramya *et al*., 2017), reported maximum Poly hydroxybutyrate yield at pH 7.0. (Tripathi and Srivastava 2011), observed higher PHB production at pH 6.54 through submerged mode of fermentation with *Alkaligenes* sp. (Ranganadhareddy *et al*., 2017) mentioned maximum PHB yield at pH 7 by using the strain *Bacillus sp* 88D through submerged fermentation process. Alterations in PHB yield was noted with the different pH and at pH 7 there was a good amount of PHB production (Fig. 4.g). The results conclude that acidic and alkaline pH are not suitable for PHB production.

Polyhydroxybutyrate production increases with an increase in temperature up to 37 °C (Fig. 4h) and later slows down at elevated temperature in production process. Thus, 37 °C was found optimum temperature for Polyhydroxybutyrate creation by *A. nosocomialis* RR20 strain (KY913802) (Chen *et al*., 1991). Researchers reported increase in PHB production by *Bacillus* sp. JMa5 strain when the incubation temperature was maintained at 37°C. Studies revealed that high concentration of PHB was obtained (2138.5 ±174mg/L and 29.5 ± 4.5%) from *Bacillus cereus* SH-02 (OM992297) around 37°C when compared to other temperatures (Hamdy *et al*., 2022). Tokiwa and Ugwu (2007) observed that at higher temperature (>40 °C), PHB yield reduced due to the activity of PHB depolymerase enzyme which is in correlation with present findings.

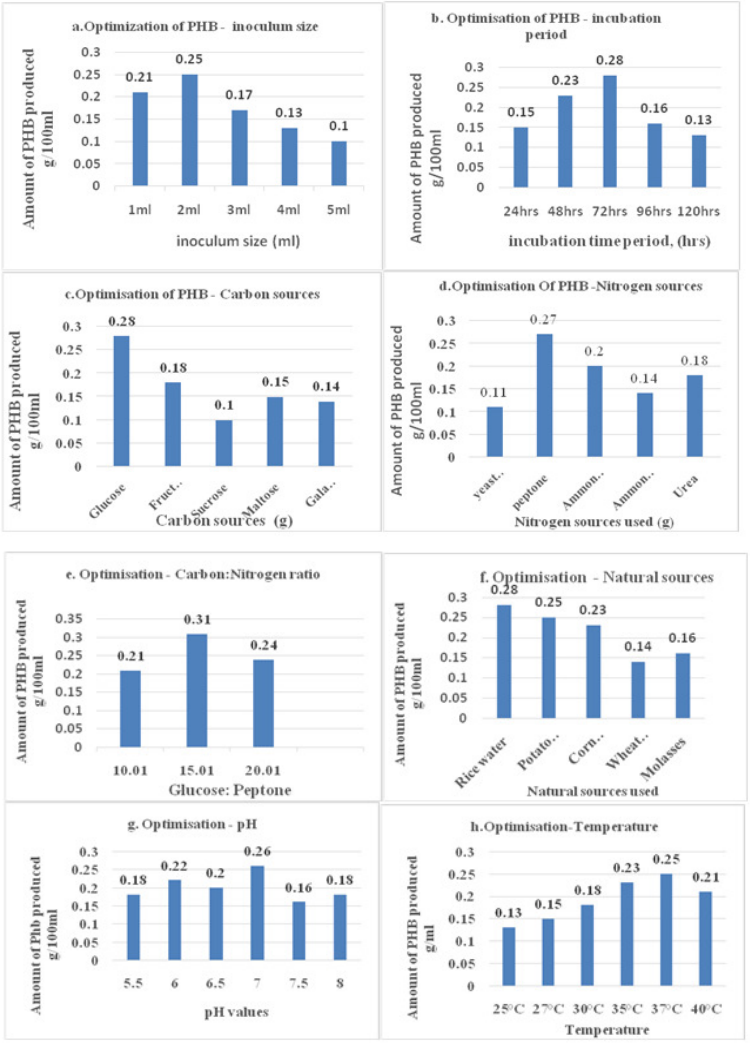

Fig. 4. Optimisation of polyhydroxybutyrate based on a. inoculum size, b. Incubation period, c. Carbon sources, d. Nitrogen sources, e. Carbon: nitrogen ratio, f. Natural sources, g. pH, h. Temperature.

CONCLUSIONS

The optimised conditions, 2ml inoculum yielded 0.25g/100ml, incubation period 72 hrs – 0.28g/100ml, carbon source glucose – 0.28 g/100ml, nitrogen source peptone – 0.27g/100ml, glucose: peptone (15:1) – 0.31g/100ml, Rice water – 0.28 g/100ml, pH 7-0.26g/100ml, Temperature 37ºC – 0.25 g/100ml. By incorporating the optimised conditions, higher production of polyhydroxybutyrate was achieved.

FUTURE SCOPE

By following the optimised conditions, the production can be further increased by culturing the bacteria in large scale in fermenters. Polyhyroxybutrate produced in considerable amount can be extracted, purified and developed into bioplastics. The produced bioplastic will be environment friendly and thus will be a solution to plastic pollution.

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Competing Interests. None.

REFERENCES

Abusham, R. A., Raja, N. Rahman, R. Abu, B. and Mahiran, B. (2009).Optimization of physical factors affecting the production of thermo-stable organic solvent tolerant protease from a newly isolated halo tolerant *Bacillus subtilis* strain. *Microbial cell factories, 8*,1-9.

Adwitiya, R. and Prabhu, N. (2009). Optimization of Process Parameters for Maximum Poly (-beta-) hydroxybutyrate (PHB) Production by *Bacillus thuringiensis* IAM 12077. *Polish journal of microbiology*, 149-54.

Ahn, W. S., Park, S. J, and Lee, S.Y. (2000). Production of Poly (3-hydroxybutyrate) by fedbatch culture of recombinant *Escherichia coli* with a highly concentrated whey soy ion. *Applied Environmental Microbiology*, *66,* 3624-3627.

Anish, K., Gulab, S. and Neeraj, K. (2013). Isolation and Screening of Polyhydroxyalkanoates Producing Bacteria from Pulp, Paper, and Cardboard Industry Wastes. *International Journal of Biomaterials*, *4*, 160-175.

Brandl, H., Gros, R. A. Lenz, R.W. and Fuller, R. C. (1990). Plastics from bacteria and for bacteria: Poly(B-hydroxyalkanoates) as natural, biocompatible, and biodegradable polyesters. *Advances in Biochemical Engineering/Biotechnology*, *41*, 77–93.

Chen, G. Q., Konig, K. H. and Lafferty, R. M. (I991). Occurrence of poly-D (-)-3-hydroxyalkanoates in the genus *B. megaterium*. FEMS Microbiology Letters, *84*,174-176.

Elsayed, N. S., Aboshanab, K.M. Aboulwafa, M. M. and Hassouna, N. A. (2013). Optimization of bioplastic (poly-β-hydroxybutyrate) production by a promising 98 *Azomonas macrocytogenes* bacterial isolate P173, *African Journal Microbiological Resources, 7,* 5025-5035.

Follner, C. G., Babel, W. and Steinbuchel, A. (1995). Isolation and purification of granule associated proteins relevant for poly (3-hydroxybutyric acid) biosynthesis from methylotrophic bacteria relying on the serine pathway. *Canadian Journal of Microbiology, 41,* 124-130.

Gabr, G. A. (2018). Isolation and Identification of Bacterial Strains Able to Biopolymer Polyhydroxybutyrate (Phb) Production from Soil of Al-Kharj Probes, Saudi Arabia. *Journal of Pharmaceutical Research International*, 21(6), 1–11.

Getachew, A., and Woldesenbet, F. (2016). Production of biodegradable plastic by polyhydroxybutyrate (PHB) accumulating bacteria using low cost agricultural waste material. *BMC Resource Notes*, *9*(1), 509–517.

Goken, T., Stube, P. and Sinskey J. (2000). Isolation, purification and characterization of biodegradable biopolymer producing bacteria *Pseudomonas cepacia* G13 strain. *Journal of Bacterial*, *99*, 82-89.

Gomez, C., Javier. Velasco, B. Rodrigo and Marin, P. (2020). Fed-batch production and characterization of polyhydroxybutyrate by *Bacillus megaterium* LVN01 from residual glycerol. *DYNA*, *87*, 111-120.

Hamdy, S. M., Danial, A. W. Gad El-Rab, S. M. F. (2022). Production and optimization of bioplastic (Polyhydroxybutyrate) from *Bacillus cereus* strain SH-02 using response surface methodology. *BMC Microbiology, 22,* 183-189.

Hong, K., Leung, Y. C. Kwok, S. Y. Law, K. H. Lo, W. H, Chua, H. and Yu, P.H. (2000). Construction of recombinant *Escherichia coli* strains for polyhydroxybutyrate production using soy waste as nutrient. *Applied Biochemical Biotechnology,* 381-390.

Hungund, B., Shyama, V. S. Patwardhan, P. and Saleh, A. M. (2013). Production of polyhydroxyalkanoate from *Paenibacillus durus* BV-1 isolated from oil mill soil. *Journal of Microbiology Biotechnology, 5,* 013-017.

Iman, H., Alaa, A. Ameena, G. Amal, A. Sana, K. and Azhar, S. (2018). Isolation and characterization of *pseudomonas putida* producing bioplastic (polyhydroxy alkanoate) from vegetable oil wastes. *Pakistan Journal of Biotechnology, 15,* 469-473.

Kitamura, S., and Doi, Y. (1994). Staining method of poly (3-hydroxyalkanoic acids) producing bacteria by nile blue. *Biotechnological Technology*, *8*, 345–350.

Kumbhakar, S., Singh, P. K Vidyarthi A. S. (2012). Screening of root nodule bacteria forthe production of polyhydroxyalkanoate (PHA) and the study of parameters influencing the PHA accumulation. African Journalof Biotechnology, 11, 7934- 7946.

Lee, S. Y., Choi, and J. Wong, H. (1999). Recent advances in polyhydroxyalkanoate production by bacterial fermentation: mini-review. *International Journal of Biological Macromolecules, 25,* 31–36.

Mostafa, Y. S., Alrumman, S. A. and Alamri, S. A. (2020). Bioplastic (poly-3-hydroxybutyrate) production by the marine bacterium *Pseudodonghicola xiamenensis* through date syrup valorization and structural assessment of the biopolymer. *Science Reports*, *10,* 8815-8824.

Pabitra, B., Pradhan, S. and Dash, H. R. (2015). Optimization and characterization of polyhydroxybutyrate, a biodegradable plastic by *Bacillus spp. Bioscience Biotechnology Biochemistry*, *79*(9), 1454-63.

Parshad, J., Suneja, S. Kukreja, K. and Lakshminarayana, K. (2001). Poly-3-hydroxybutyrate production by *Azotobacter chroococcum. Folia Microbiologica*, *46,* 315–20.

Sharma, P. and Bijender, K. (2015). Production and characterization of poly-3-hydroxybutyrate from *Bacillus cereus* PS 10. *International Journal of Biological Macromolecules*, *81,* 241-248.

Quillaguaman, J., Munoz, M. Mattiasson, B. and Hatti-Kaul, R. (2007). Optimizing conditions for poly (beta-hydroxybutyrate) production by *Halomonas boliviensis* LCI in batch culture with sucrose as carbon source. *Applied Microbiology*, *74*, 981-986.

Ramya, R. Sangeethadevi, A. Manikandan, V. and Rajesh, K. (2017). Standardisation of biopolymer production from seaweed associative bacteria, *International Journal of Biological Macromolecules*, *102*, 550–564.

Ramadas, N. V., Soccol, C. R. and Pandey, A. (2010). A statistical approach for optimization of polyhydroxybutyrate production by *Bacillus sphaericus* NCIM 5149 under submerged fermentation using central composite design. *Applied Biochemistry Biotechnology*, 162, 996-1007

Ramsay,J.A., Berger, E. and Voyer, R. (1994). Extraction of poly-3-hydroxybutyrate using chlorinated solvents.*Biotechnological Techniques*, *8,* 589–594.

Ranganadha Reddy, A., Bharath Kumar, R. and Vidyaprabhakar, K. (2017). Isolation and identification of PolyHydroxy Butyrate (PHB) producing bacteria from sewage sample, *Research Journal of Pharmacy and Technology*, *10*(4), 01-05.

Rhu, D. H., Lee, W. H. BCim, J. Y. and Choi, E. (2003). Polyhydroxyalkanoate (PHA) production from waste. *Water Science Technology*, *48*, 221-228.

Rossi., Marta, M. Sara, A. Neda, A. and Marco, P. (2022). A Polyhydroxybutyrate (PHB)-Biochar Reactor for the Adsorption and Biodegradation of Trichloroethylene. *Design and Startup Phase Bioengineering*, *5*, 192-197.

Sakthiselvan, P. and Madhumathi, R. (2019). Optimization on Microbial Production of Polyhydroxybutyrate (PHB): A Review. *International Journal of Research Analysis Reviews, 6*, 243–251.

Sindhu, R., Silviya, N. Binod, P. and Pandey, A. (2013). Pentose-rich hydrolysate from acid pre-treated rice straw as a carbon source for the production of poly-3-hydroxybutyrate. *Biochemical Engineering Journal*, *78*, 67– 72.

Sriyapai, T., Chuarung, T. and Kimbara, K. (2022). Production and optimization of polyhydroxyalkanoates (PHAs) from *Paraburkholderia* sp. PFN 29 under submerged fermentation. *Electronic Journal of Biotechnology*, 56, 1-11.

Sudesh, K. and Doi, Y. (2000). Synthesis, structure and properties of polyhydroxyalkanoates: Biological polyesters. *Progress in Polymer Science*, 25, 1503-1555.

Tokiwa, Y. and Ugwu, C. U. (2007). Biotechnological production of (R)-3- hydroxybutyric acid monomer. *Journal of Biotechnology, 132,* 264-272.

Tripathi, A. D. and Srivastava, S. K. (2011). Novel approach for optimization of fermentative condition for polyhydroxybutyrate (PHB) production by *Alcaligenes sp*. using Taguchi (DOE) methodology. *African Journal of Biotechnology, 10,* 7219-7224.

Vishnuvardhan, S. and Thirumala, M. (2009). Production of PHB and P (3HB-co-3HV) biopolymers by *Bacillus egaterium* strain OU303A isolated from municipal sewage sludge. *World Journal Microbial Biotechnology*, *25*, 391-397.

Waqas, N., Chaudhry, N. Jamil, L. Ali, M. Hashim, A. and Shahida, H. (2010). Screening for Polyhydroxyalkanoates (PHA) producing bacteria strains and comparison of PHA production from various inexpensive carbon sources. *Annals of microbiology*, *10,* 1007-1013.

Wong, H. H. and Lee, S. Y. (1998). Poly-(3-hydroxybutyrate) production from whey by high-density cultivation of recombinant *Escherichia coli*. *Applied Microbiology Biotechnology*, 30-45.

Xi, J., Wu, Q. Yan, Y. Zhang, R. Yu. PHF. Cheung, M. Zhang, R. and Chen, G. Q. (2000). Hyper production of polyesters consisting of medium chain length hydroxyalkanoate monomers by strain *Pseudomonas stutzeri* 1317. *Antonie van Leeuwenhoek*, *78*, 43-49

Yuksekdag, Z. N. and Yavuz, B. (2008). Production of Poly-|3- hydroxybutyrate (PHB) in different media by *Streptococcus thermophilus* Ba21S strain. *Journal of Applied Biological Sciences*, *2*(2), 07-10.

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