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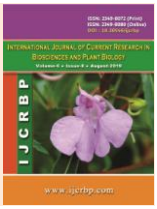

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Screening of PHB biopolymer producing *Bacillus cereus* from municipal sludge waste

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ABSTRACT

Biopolymers are the rarely produced bio products which are used as an alternative for common plastic and some microbes can even digest them. In this study we have confirmed that *Bacillus cereus* can produce such bio polymers. *B. cereus*, isolated from sludge soil sample was standalone which showed substantial PHB production (4.2 ±0.25 g/l) after 48 h of fermentation as examined by spectrophotometric analysis. Time-course (24-96 h) analysis for PHB production indicated that PHB was a growth-associated product and its accumulation significantly increased during the exponential phase and reached maximum after 48 h (5.6 ±0.25 g/l). The maximum PHB yield was achieved after 48-72 h of fermentation (6.5±0.18 g/l). The FT-IR analysis, illustrates bands at 3280.92 and 2924.09 cm^{-1} corresponding to aliphatic ester carbonyl C=O of R COA and C-H stretch, respectively. Other absorption bands at 1633.71 and 1533.09 cm^{-1}, represent the presence of O-H bonding and C-H bond of aliphatic compounds, respectively. The band at 1633.71 cm^{-1} considered as diagnostic signal for PHB. The band found at 1,380.17 cm^{-1} is assigned to symmetric wagging of CH_3 groups. The ^{1}H-NMR spectra showed the presence of three signals, it's the characteristic of the polymer of PHB.

Introduction

Plastics are a ubiquitous part of the modern life, and their molecular structure can be manipulated to meet the requirements of almost any imaginable application including construction, health care, transportation, food packaging, consumer goods and communications. Currently the vast majority of plastics in use are manufactured using a petrochemical route, derived from materials of fossil oil and gas origin. About 4 to 6% of the world's annual petroleum production is used for the production of plastics and more 4% is utilised to power manufacturing processes (British Plastics Federation, 2017).

Plastics are extremely versatile materials and have been essential in the development of society since the 20th century, though they have environmental impact associated not only with their manufacture, but also at their disposal. This is because plastics have a slow degradation rate, making them environmentally persistent and hence presenting a serious pollution issue in land and oceans (Marshall et al., 2013). These alternative types of plastic must retain the desired properties of traditional synthetic plastics and, ideally, should biodegrade completely without leaving any undesirable remains once discarded. Biopolymers can be mainly classified into four groups based on their biodegradable characteristics (Mudgal et al., 2013).

The properties of Polyhydroxybutyrate (PHB) are similar to many synthetic thermoplastic polymers, such as polypropylene, making PHB a promising candidate as a drop in replacement for petroleum-based plastics in wide range of applications such as paper coatings, packaging and manufacturing of plates and bottles (Batcha et al., 2014). Previous research on PHB has focused on making production economical and improving the quality of biopolymers such that they are able to compete with the physical and mechanical properties of petrochemical- derived polymers, allowing biopolymers to be utilised as direct replacements for commonly used synthetic polymers (Batcha et al., 2014).

More than 200 types of microorganisms are able to accumulate PHB, such as photosynthetic bacteria, archaebacteria, and Gram negative and Gram-positive bacteria. *B. cereus* is a Gram-positive bacterium that is a model PHB producing microorganism because it is able to accumulate PHB to a high level of 90 % of dry cell weight (Chee et al., 2015). Unfortunately, *B. cereus* can grow on glucose, the main sugar that is obtained from cellulosic material, which limits the use of this strain for PHB production using sources of non-edible, lingocellulosic biomass. There is potential to use for sludge soil.

Materials and methods

Collection and processing of soil sample

The soil sample was collected from the lake (sludge soil) of Hosur, Krishnagiri district of Tamil Nadu. Collected samples were immediately processed and stored in refrigerator for further process in the PG and Research Centre in Biotechnology of MGR College, Hosur.

Isolation and screening of PHB producing *Bacillus cereus*

Isolation of PHB producing bacteria was accomplished based on the methods of Khanna and Srivastava (2005) with slight modifications. The microbes were isolated from collected sludge soil sample by standard serial dilution techniques by using saline water. 10^5 and 10^6 dilutions were taken on selective and standard media for screening of PHB producing bacteria. Briefly the dilutions were inoculated 5% w/v on selective media such as *Bacillus cereus* selective agar base enriched with Disodium hydrogen phosphate (2.5g) and Pottasium dihydrogen phosphate (0.25g), Peptone (1g), Mannitol (10g), Sodium chloride (2g), Megnesium sulphate (0.1g), Sodium pyruvate (10g), Bromothymol blue (0.12g) and agar (2g) and incubated at 30° C for 24 h in an shaker incubator. Plates were incubated at 30°C for 2-3 days, and colonies appeared and was exposed to visible light and those showing slight bluish colonies were earmarked as presumptive PHB producers (Thammasittirong et al., 2017).

PHB producing ability of bacterial isolates was examined further by confocal microscopic analysis by using Sudan black, a lipophilic dye (Model Olympus Fluoview Ver 1-7b) as described by Hermawan and Jendrossek (2007). The suspected PHB producing bacterial isolates were first grown in PHB production medium (PPM) which consisted of (g/l^{-1}): MgSO4 7H2O 0.1, K2HPO4 0.5, NH4NO2 0.1, glucose 20.0, malt extract 0.5, yeast extract 1.0, pH 7, for 48 h. Cells were harvested, and immediately stained with 0.1–0.5 volumes of Sudan black solution (0.1–1 mg ml-1 in ethanol). The dark bluish colour results conformed the PHB producing bacteria isolates.

Characterization of selected isolate

Among the bacterial isolates screened for PHB production, the one which generated maximum PHB was selected, and examined based on morphological, physiological, biochemical properties and staining techniques. Biochemical analysis included carbohydrate fermentation tests, Indole, methyl red and Voges-Proskauer test (IMVIC), catalase, Glucose peptone agar (GPA) test, Gelatin test. The conformed bacterial isolates were maintained by frequent sub culturing process. Among various bacterial isolated *B. cereus* was taken for further study.

Production of PHB

All the PHB producing isolates were subjected to submerged fermentation for PHB production. Inoculum was developed by cultivating the cells in production medium under shaking at 30°C for 18 h to attain absorbance (A600) of 0.8-0.9 (approx. 10^8 cfu/ml) and inoculated at 5% (v/v) for PHB

production. Fermentation was executed at 30oC under shaking at 150 rpm. Fermented broth was centrifuged (Eppendorf Centrifuge 5804-R, Germany) at different time intervals (24-96 h) and the resultant biomass was used for PHB extraction (Khanna and Srivastava, 2005).

Extraction and quantification of PHB production

All the Sudan Black B positives isolates were subjected to quantification of PHB production as per the method of Schlegal et. al (1961). The bacterial cells containing the polymer were centrifuged at 10,000 rpm for 10 min and the pellet was washed with equal volume of acetone and ethanol to remove unwanted materials. The pellet was resuspended into 4% of sodium hypochlorite and incubated at room temperature for 30 min. The whole mixture was again centrifuged and the supernatant was discarded. The cell pellet containing PHB was again washed with equal volume of acetone and ethanol. Finally the pellet containing the polymer granules were dissolved in hot chloroform (Arshad et al., 2007). The chloroform was filtered, and to the filtrate 10ml of concentrated hot sulphuric acid was added. The addition of sulphuric acid converts the pellet into crotonic acid which is brown in color. The solution was cooled and the absorbance was read at 400 nm against sulfuric acid as blank.

Polymer analysis

Fourier transform-infrared spectroscopy (FT-IR) and NMR analysis

PHB production from *B. cereus* was executed under submerged fermentation and PHB produced was subjected for IR spectra recording in the range 4000-400 cm^{-1} at a resolution of 4 cm^{-1} to confirm the functional groups of the extracted polymer as per the method described by Liau et al. (2014). All the experiments were performed in triplicates and all the data is represented here is the Mean +/- SD.

NMR Analysis: ^{1}H Nuclear Magnetic Resonance

The identity of individual monomer unit was confirmed by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. ^{1}H-NMR spectra of PHB sample were recorded in CDCl3 on Bruker ACF 300 spectrophotometer at 300 MHz by using "Tetramethylsilane" as internal standard.

Results and discussion

The collected samples were serially diluted and cultured by spread plate method with the following dilutions10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} with one control. More than 10 bacterial cultures were isolated from lake (sludge soil) of Hosur, Krishnagiri district of Tamil Nadu obtained on nutrient agar medium. The simplest first line screening program for PHB producing bacteria is the use of Sudan black B staining. The isolates which were found positive for PHB granules after Staining with Sudan black B (i.e., showing dark spot inside the pink coloured cells) were further confirmed for their PHB producing potential with specific media, a more specific dye for PHB granules, in which dark blue colour was observed under the microscopy (Fig. 2 and Fig. 3). The PHB produced bacterial culture was further identified by following experimental results.

Isolation of *Bacillus cereus* on selective media

The selective media were prepared and streaked with the suspected colonies in separate plates for each. The isolates grown in only few selective media plates (*Bacillus cereus* selective agar base) and which are confirmed as *Bacillus cereus* (Fig. 1).

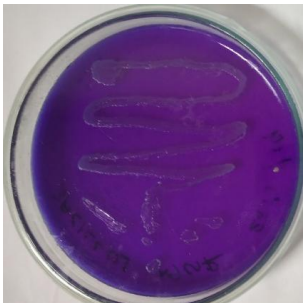

Fig. 1: *Bacillus cereus* pure culture in selective media.

Conformation of PHB producing *Bacillus cereus* by Sudan Black -B staining

The bacteria were initially screened for the PHB production in nutrient broth and the ability to synthesize PHB granules was confirmed using Sudan black. Pure culture of isolated strain was

done by plating method. Presence of PHB granules was identified and confirmed by Sudan Black- B staining and the granules were observed. The isolates were tested for PHB production following the viable colony screening method based on the intensity of staining. The isolates gave the positive result for PHB accumulation through Sudan black staining method scoring. The *Bacillus cereus* was treated with Sudan Black- B positive isolates were subjected to quantitative estimation of PHB production. Black color colonies were taken as positive result.

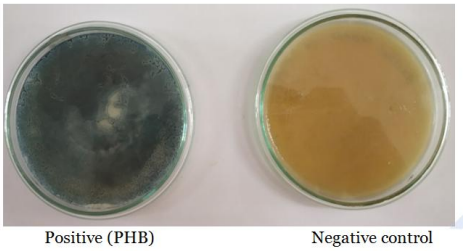

Fig. 2: Sudan Black test.

PHB producing bacterial colonies were showed bluish black color colonies were recorded on Sudan black containing medium. Confocal microscopic analysis of PHB producing bacterial cells indicated appearance of cells filled with red color PHB granules. Alternate staining methods like direct staining of colonies with Sudan black have been used by some researchers for detection of PHB granules (Singh, et al., 2011).

Macroscopic, microscopic and biochemical bacterial analysis and conformation

The isolates were confirmed by macroscopic and microscopic observations. Therefore, obviously bacterial isolate was selected for further studies including its identification by morphological and biochemical means. Macroscopic analysis showed that bacterial colonies were sticky, discrete and creamy white. Microscopic examination established that bacteria were Gram-positive, rod shaped and sporulating (Fig. 2). Besides, isolate was catalase and methyl red positive but negative for VP test, and hydrolysed casein, starch and lipids. PHB from Gram-positive bacteria may be promising for biomedical and tissue engineering applications (Shivakumar, 2012) as Gram-positive bacteria are devoid of lipopolysaccharides and there is no chance of lipopolysaccharides contamination (Fig. 3).

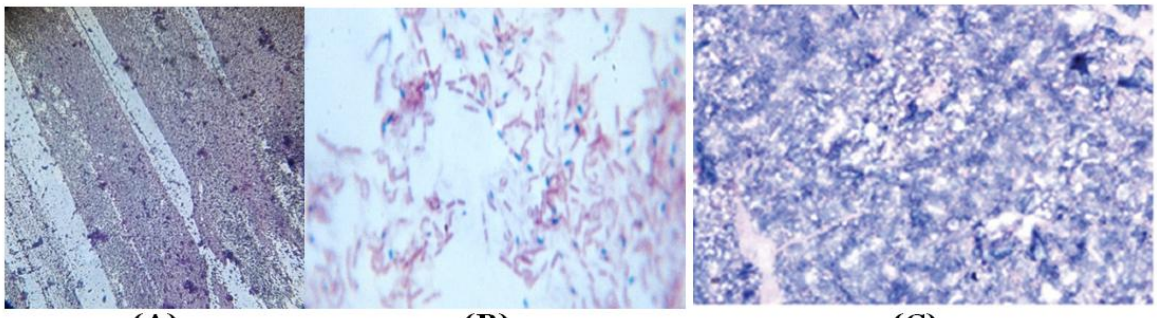

Fig. 3: Staining results. (A) Gram staining; (B) Endospore staining; (C) Sudan black B staining.

Biochemical characterization of *Bacillus* isolates

Biochemical characteristics of isolate was studied on the support of different biochemical test like Glucose Peptone agar assay, and Citrate utilization test, indole test, etc. The cultures were confirmed as *Bacillus cereus* by the standard biochemical result.

Production of PHB

After initial screening, *B. cereus* isolate was subjected to submerged fermentation in the PHB production medium. *B. cereus*, isolated from sludge soil sample was standalone which showed substantial PHB production (4.2 g/l ±0.25) after 48 h of fermentation as examined by spectro-photometric analysis. Time-course (24-96 h) analysis for PHB production indicated that PHB was a growth-associated product and its accumulation significantly increased during the exponential phase and reached maximum after 48 h (5.6 ±0.25 g/l). The maximum PHB yield was achieved after 48-72 h of fermentation (6.5±0.18

g/l). After 72 h, a decrease in the level of cell dry weight coincided with a small decrease in PHB content.

During the adverse conditions i.e., with glucose the PHB was used by the cell as an internal reserve of carbon and energy. Furthermore, increase in viscosity of the medium may also lead to unfavorable conditions such as production of extracellular metabolites, presence of an intracellular PHB depolymerase, depletion of essential nutrients in the medium or self-utilization of PHB by bacteria due to nutrient depletion and cells, consumption of PHB as a carbon source (Madison and Huisman, 1999; Ramadas et al., 2009). Fermentation time for maximum PHB production varies among different bacteria and depends largely upon cultural/environmental conditions employed during fermentation and genetic make-up of the organism.

It is desirable that PHB production be attempted by using inexpensive agricultural or other residues as carbon source to substantially reduce the cost of PHB production considering carbon source as the major cost determining factor for PHB production (Valappil et al., 2007). PHB production has been reported from several bacteria by using waste materials or agricultural residues as inexpensive carbon sources (Van-Thuoc et al., 2008; Yezza et al., 2006). Furthermore, readily available low-price carbon sources may have high amounts of nutrients, such as amino acids and peptides, which contributes to improved cell growth and metabolite biosynthesis and pave the way for resourceful and cost-effective production of PHB. PHB being the reserved food polymer produced during time of starvation is degraded to provide carbon and energy when external carbon source is exhausted (Shivakumar, 2012). However, *B. megaterium* showed maximum PHB accumulation when glucose was used as carbon source (Hori et al., 2002). *Bacillus* species has been reported to accumulate PHB up to 90% of cell dry weight by utilizing soy molasses oligosaccharides like raffinose without the need for nutrient limitation (Full et al., 2006; Das et al., 2017).

Polymer breakdown, so that the PHB is utilized at the rate almost equal to the rate of its synthesis (Singh et al., 2011). *Bacillus sphaericus* NCIM 5149 showed pH 7.5 as the most apt for PHB production (Ramdas et al., 2009) while for *Bacillus* species pH of 6-7 the most effective for PHB accumulation (Singh et al., 2011). Current study showed that inoculum at 5%, (v/v) was best for maximum PHB yield (5.6 ±0.15 g/l).

Extraction and purification of PHB

After the 30mins incubation of isolates in ice cold methanol, a slimey layer of PHB (poly-hydroxybyturate) were observed, and the layer were collected separetely in to a watch glass and dried at 60°C for 12 h (Fig. 4).

Fig. 4: Watch glass with dried PHB.

Higher or lower inoculum level resulted in yield reduction of PHB from *B. cereus* IR spectrum of the extracted polymer, illustrates bands at 3280.92 and 2924.09 cm^{-1} corresponding to aliphatic ester carbonyl C=O of RCOA and C-H stretch, respectively. Other absorption bands at 1633.71 and 1533.09 cm^{-1}, represent the presence of O-H bonding and C-H bond of aliphatic compounds, respectively (Fig. 5). The band at 1633.71 cm^{-1} considered as diagnostic signal for PHB. The band found at 1,380.17 cm^{-1} is assigned to symmetric wagging of CH3 groups. The remaining bands located at 1000 ~ 1300 cm^{-1} correspond to the stretching of the C–O bond of the ester group. The results described above are congruent with the findings reported previously. The most prominent marker (ester carbonyl) band for PHB was at 1740 cm^{-1} and 1724.03 cm^{-1} for pure PHB (Panda et al., 2010). In pure PHB granule, asymmetrical deformation of C-H bond in CH2 groups and CH3 groups C=O bond stretching and C-O ester bond are represented by wave numbers 1460, 1379, 1726 and 1150 cm-1, respectively (Tripathi et al., 2013; Panda et al., 2018).

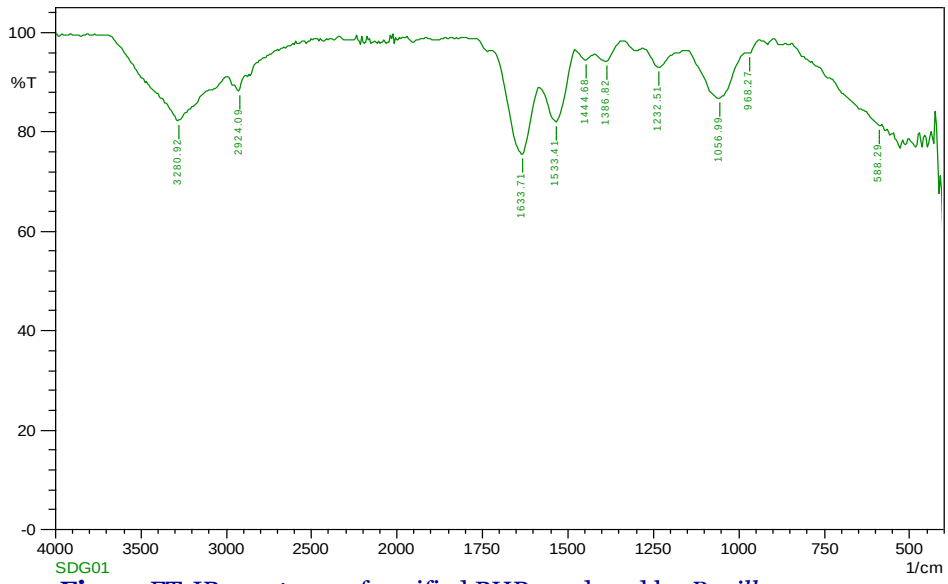

Fig. 5: FT-IR spectrum of purified PHB produced by *Bacillus cereus*.

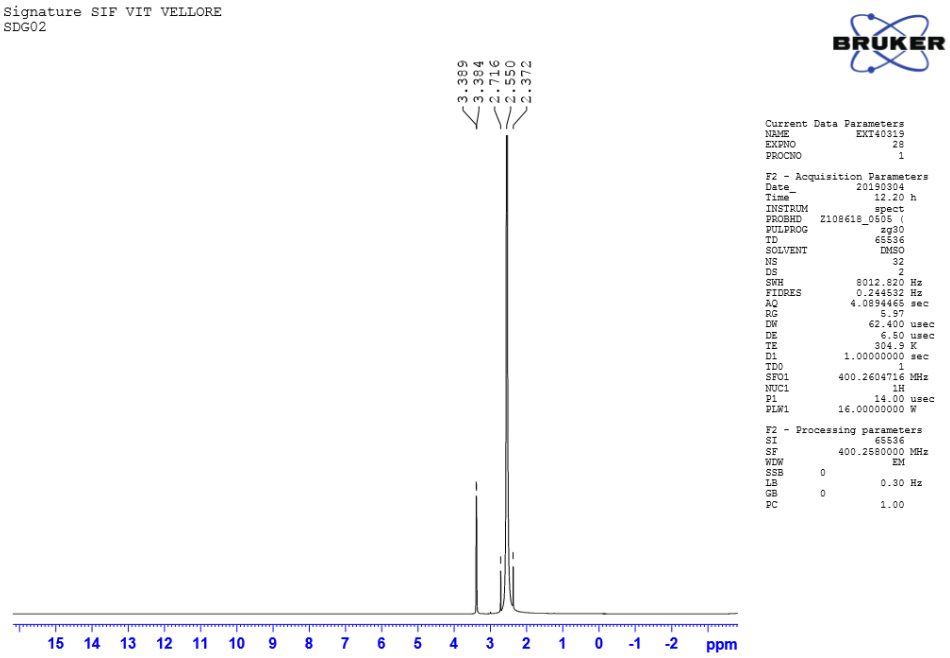

Fig. 6: ^{1}H NMR signals of PHB produced by *Bacillus cereus*.

The present study concludes that *Bacillus cereus* has got enormous enzymatic/metabolic potential to utilize glucose sources for growth and PHB production. Presence of glucose supported maximum PHB production by *B. cereus* Polymer got remarkable characters as per industrial view-point. The chemical nature of extracted polymers from *Bacillus cereus* was confirmed by ^{1}H-NMR spectroscopy. The ^{1}H-NMR spectra (Fig. 6) showed the presence of three signals, characteristic of the polymer of PHB. The ^{1}H-NMR spectra of polymer extracted from isolate showed a doublets at 2.372 ppm, corresponding to the methyl group (–CH3), and two multiplets at 2.55 and 3.389 ppm corresponding to methylene group (–CH2–) and methyne (–CH–) group, respectively. In this study with reference PHB standard, the nature of polymer produced by the isolate was confirmed.

Conclusion

The *Bacillus cereus* isolated from sludge soil of Hosur lake area was able to efficiently utilize the glucose as nutrient source for PHB production. The first line of impression of this study concludes that the glucose could directly serve as an inexpensive nutrient source for production of biodegradable plastic. Thus, this study may solve the problem of high production cost of biodegradable bioplastic and help in the conservation of petroleum products which were used in the commercial production of plastic production.

Acknowledgement

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Conflict of interest statement

Authors declare that they have no conflict of interest.

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