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Research article

Screening of polyhydroxybutyrate producing indigenous bacteria from polluted lake soil

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ABSTRACT

The prime aim of this study was to enumerate predominant bacteria from polluted lake soil samples, which possess polyhydroxybutyrate (PHB) fabricating potential and identify the suitable growth conditions and nutritional factors for PHB fabrication. From several numbers of bacterial cultures, one culture has the competence to yield PHB, and it was endorsed through Sudan Black B stain, Nile red staining, SEM analysis, and growth in PHB selective media. Under the microscopic observation, the fluorescent cells and polymeric granules were observed in the fluorescent microscope and SEM, respectively. This PHB fabricating isolate was recognized as *Bacillus cereus* NDRMN001 through 16S rRNA partial sequence analysis. The structural characteristics of PHB produced by *B. cereus* NDRMN001 were studied through FT-IR, ^{1}H NMR, and ^{13}C NMR analysis. The peak observed at 1759.27 cm^{-1} on FT-IR analysis is corresponding to the signal band of PHB. In ^{1}H NMR peaks were noticed at 1.67, 2.37 to 2.71, and 3.38 to 7.68 which corresponding to –CH3, –CH2, and –CH protons of PHB. About 4 notable peaks were noticed in ^{13}C NMR analysis at 19.62, 68.27, 40.68, and 169.11 ppm which appeared close to the carboxyl group of PHB. About 10% of inoculum, pH 7.5, 2 g L of yeast extract, 20 g L of rice bran, 35 °C, and 2 days of incubation were recognized as optimal growth conditions for *B. cereus* NDRMN001 to produce PHB. The identified *B. cereus* NDRMN001 has the potential to yield 91.48% of PHB as 33.19 g L of PHB from 36.26 g L of culture biomass. The complete results conclude that the *B. cereus* NDRMN001 screened from polluted lake soil has the competence to produce fine quality and quantity of PHB in a short duration of fabrication process under favorable conditions with the utilization of cheap nutritional factors.

1. Introduction

The vast application possibilities of chemical polymers are inevitable substances in the world due to its excellent benefits like being less weight, chemically inert, comfortable, durable, flexible to make desire shape and size, etc (Villalobos et al., 2016; Narayanan et al., 2020a). Thus, these petroleum-based polymers are undisputable from the world as it offered excellent support in various technology and applications for the benefits of humankind (Sholkamy et al., 2015). However, the origin of this polymer is from petroleum; hence it is non-biodegradable, persists in the environment for a prolonged period, and causes severe ecological reduction by unbalancing the environmental conditions (Mahitha and Madhuri, 2015; Narayanan et al., 2020b). Besides that, during fabrication and incineration of petroleum-based polymers creating serious air, water, and soil pollutions (Hong et al., 2019; Mathiyazhagan and Natarajan, 2012). These lead to cause health issues to humans, animals, and entirely collapse the biological systems and cycles. Hence finding a replacement for the petroleum-based polymer is a timely need to avoid and reduce the impacts already caused by traditional polymers due to excess usage and improper disposal to the environment (Nishida et al., 2018b). From the last decades onwards, there has been increasing demand to fabricate and use biodegradable polymeric material from biological (microbes, plants, etc.) and biomolecule sources such as polysaccharides, proteins, and lipids, which have been used as an inexpensive and renewable alternate for traditional polymers (Sabarinathan et al., 2018; Narayanan et al., 2020c). Thus, researchers are searching a

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most comfortable biodegradable polymers from various biological sources which including microorganisms. Fortunately, nature offers an alternative for petroleum-based polymers from natural biological resources (Mathiyazhagan and Natarajan, 2011a).

The researchers are preferred to use and produce biodegradable polymers from microbes, since ease maintenance, controlled fabrication, simple purification, more prospects of genetic modifications with fast biodegradability without collapsing the ecology and environment (Mostafa et al., 2020; Narayanan et al., 2020d). among the various biopolymers, the Polyhydroxybutyrate (PHB) is a preferable one due to high thermoplastic like traditional polymers and possesses most suitable physical, mechanical, immunological properties, which promotes this PHB is suitable alternate for traditional polymers (Khiyami et al., 2011). The progress in the fabrication process, screening novel microbes with PHB producing potential, decomposing mechanisms, genetic modification in the aspect of high quality and quantity PHB fabrication, etc. are evolving every day among the scientific society (Juengert et al., 2018; Mathiyazhagan and Natarajan, 2011b). Among the microbes like bacteria, fungi, yeast, algae, etc. the bacteria-based biopolymer fabrication gain more attention due to it eases controlled, maximum biomass, simple downstream processing, more gene modification possibilities, etc. (Soman et al., 2020a, 2020b; Kumarasamy et al., 2020) More than 300 numbers of bacteria have reported so far as they possess biopolymer producer (Villalobos et al., 2016; Mathiyazhagan and Natarajan, 2011b). Among them, only a few numbers of bacterial cultures such as *Azotobacter vinelandii*, *E. coli*, *Pseudomonas oleovorans*, *Halomonas campaniensis, Alteromonas lipolytica, Aeromonas* sp., *Bacillus megaterium, Methylobacteria* sp, *Herbaspirillum seropedicae*, *Alcaligenes latus*, etc. (Gabr, 2019; Tufail et al., 2017; Shi et al., 2017) are preferably used for PHB and PHA fabrication process. However, the commercialization possibilities of a bacterial based biopolymer are less due to the cost of production since using a purified form of nutritional factors like glucose, lactose, sucrose, etc. Henceforth, this research was framed to isolate PHB producing predominant bacteria from polluted lake soil and determines the suitable growth conditions and cheap carbon and nitrogen sources for PHB fabrication.

2. Materials and methods

2.1. Profile of study area

The Hosur is an industrial area of Krishnagiri district of Tamilnadu, north-western part of India, with a growing population of approximately 5 lakhs in this short radius city. The average climatic conditions such as temperature are a minimum of 17.1 °C to a maximum of 33.6 °C and the average annual rainfall is 84 cm. The peoples residing in this site mostly depend on groundwater for regular usage. The lakes located in and around this city act as a major source for maintaining the groundwater level. Unfortunately, the lake which located within this city regionally termed as SIPCOT Lake (Chandramkudi Lake), is a freshwater lake (latitude: 12. 740371 and longitude: 77.815017) receiving more quantity of pollutants in the mode of direct disposal of municipal & household, and medical wastes and industrial effluents (Figure 1). Hence generally, the bacteria which survive under the polluted environment might possess the potential to synthesis the polymeric granule as an adaptation process to survive in unfavourable conditions (Narayanan et al., 2020a). Based on this perception, the soil sample was collected from this polluted lake for the screening of biopolymer producing predominant bacteria.

2.2. Collection and processing of soil sample

The dust and stones free three (sites) surface soil samples were collected from the polluted lake (Figure 1) in a sterile zip-lock cover using ethanol wiped sterilized spatula, and collected samples were instantly shifted to PG and Research Centre in Biotechnology laboratory and refrigerated at 4 °C for further experiments.

2.3. Enumeration of bacterial culture

The typical serial dilution protocol was followed to screening the predominant cultivable bacterial isolates from stored soil samples after thawed at room temperature. The spread plate method was followed to inoculate approximately 50 μl of 10^{-6} dilutions of collected soil samples, on sterile LB agar plates augmented with 1% of glucose as a basic carbon nutritional supplement, and plates were properly labelled. Triplicate plates were inoculated for each sample and kept under the incubation for 48 h at 37 °C along with sterile control. The predominant and individual colonies were isolated from the master plate and sub cultured for purification by following the quadrant streaking and conserved at 4 °C for further analysis.

2.4. Screening of PHB producing bacterial isolates

Around ten predominant bacterial isolates were selected based on their active growth in LB agar plates and tested those colonies with Sudan Black B stain as per the protocol of Villalobos et al. (2016) to identify the PHB fabricating potential of these isolates. Concisely, 2 mL of Sudan Black B stain (0.05%) was poured above the well-grown colonies on the LB plate and incubate at room temperature for 30 min and splashed with ethanol (60%). Subsequently, the stained culture plates were again incubated for 30 min and noticed the color changes as dark greenish-blue, considered as PHB positive (Nishida et al., 2018a) as primary screening. Out of ten, only one isolate showed positive for primary screening and later which was subjected to the secondary screening on PHB selective media (containing 2.5 g, 25 g, 100 g, 20 g, 1 g, 100 g, 10 g, 1.2 g, 20 g L of H_2KO_4P, HNa_2O_4P, Mannitol, NaCl, $MgSO_4$, $C_3H_3NaO_3$, Peptone, Bromothymol blue, and agar respectively) as per the procedure of Narayanan et al. (2020b). The culture inoculated plates were incubated for three days at 35 °C and bluish colonies were perceived and confirmed that the test isolates possess PHB synthesizing potential (Aarthi and Ramana (2011); Krishnan et al., 2017).

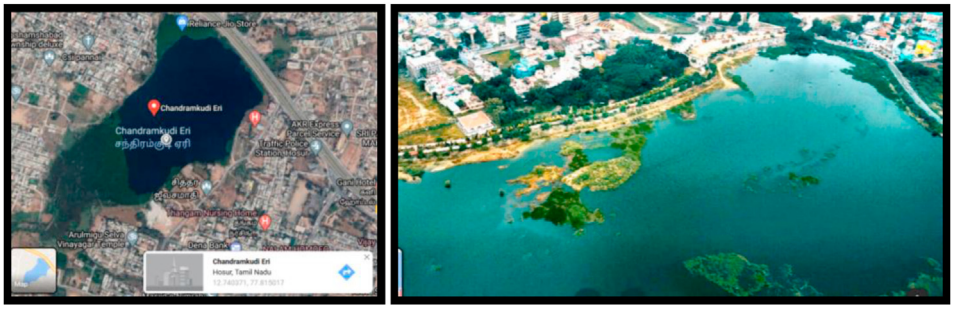

Figure 1. Satellite and live view of the study area.

2.4.1. Tertiary screening for confirmation of PHB producer by standard microscope and culture identification

Based on the results obtained from secondary screening, tertiary screenings were performed by using Sudan Black B Stained culture smeared slides were observed under a basic ocular microscope by following the protocol of Alarfaj et al. (2015). After the tertiary screening of PHB producing isolate was identified through 16S rRNA partial sequencing analysis (Aarthi and Ramana, 2011).

2.5. Extraction and characterization of PHB

After the tertiary screening, the PHB producing potential isolate was recognized as *Bacillus cereus* NDRMN001 by 16S rRNA analysis. The PHB synthesized by *B. cereus* NDRMN001 was extracted as per the protocol of Sholkamy et al. (2015). Briefly, the biomass containing medium was centrifuged at 8,000–10000 rpm for 8–10 min in a cooling centrifuge. About 1:1 ratio of acetone and ethanol was added to the pellet and rinsed to breaking the cell wall to release PHB granules and spun for 5 min at 8000 rpm, and the supernatant was discarded. The attained pellet was liquefied with 4% of NaOCI and incubated at room temperature for 30 min and later spun for 8 min at 8000 rpm, and the supernatant was discarded. The attained pellet was washed with a 2:1 proportion of acetone and ethanol. The final polymeric particles were liquefied with chloroform and sieved by Whatman No. 1 filter paper and 10 mL of conc. H_2SO_4 was added to filtrate for 1 min; it converts the polymeric granules into crotonic acid, then reading was taken by spectrophotometer at 235 nm, and conc. H_2SO_4 was used as standard and the graph was plotted for PHB quantification.

2.5.1. Fourier transform-infrared spectroscopy (FT-IR) analysis

The extracted PHB was categorized through FT-IR investigation by following the protocol of Sholkamy et al. (2015) with some modifications. Briefly, 1 mg of crude PHB extract was liquefied in 7 mL of chloroform in a test tube, and 1 drop of this mixture was coated on FT-IR KBr disk (PerkinElmer, spectrum 3™ Tri-Range FT-IR). The spectrum range of solvent vaporized PHB extract was read in an IR double beam spectrophotometer with a resolution of 4 cm^{-1} and 400-4000 cm^{-1} of vacuum pressure.

2.5.2. ^{1}H NMR and ^{13}C NMR analysis

^{1}H-NMR analysed the dynamic of component, individual monomer, and molecular organization of PHB extract by following the methodology of Sholkamy et al. (2015). The spectra of the sample were noted in DMSO on Bruker ACF300 spectrophotometer at 300 MHz and Tetramethyl saline used as an internal reference with phase and frequency resolution >0.1 deg and >0.1 Hz correspondingly. The structural information of PHB extracted from the isolate was analyzed by ^{13}C NMR as a final confirmation by following the methodology of Vrabel et al. (2013) and using a 400 MHz NMR spectrometer with the MAS frequency of 170 MHz and 10 kHz, respectively, at 30 °C.

2.6. Optimization of PHB synthesize

The suitable growth conditions of *B. cereus* NDRMN001 for PHB synthesize were optimized by following the procedure of Krishnan et al. (2017) and Mostafa et al. (2020) with some modifications. Hence, the appropriate growth (one at a time) conditions such as percentage (2.5, 5, 7.5,10, 12.5, and 15%) of inoculum (2×10^8 CFU mL), 20 g L^{-1} of carbon sources (sugar molasses, rice bran, and sago molasses), 2 g L of nitrogen sources (peptone, $NaNO_3$, and yeast extract), physical factors like temperature (25; 30; 35; 40; and 45 °C), pH (5.5; 6.5; 7.5; , 8.5; and 9.5), and incubation time (12; 24; 36; 48; 60, and 72 h) were assessed. The simple PHB production medium was used with the amendment of test carbon and nitrogen sources with different physical parameters. Triplicates were performed with each parameter to get reproducible results and incubated on a shaker incubator for three days with 150 rpm, and the quantity of PHB and cell biomass produced under optimized conditions were studied. The growth kinetics of *B. cereus* NDRMN001 in PHB synthesize was studied by following the protocol of Soman et al. (2020b). Briefly, the growth of *B. cereus* (600 nm) and PHB synthesis (400 nm) were analyzed in a spectrophotometer with respective wavelengths in a day interval basis.

2.7. Statistical analysis

For accuracy and reproducibility, the statistical analyses such as mean, standard error, and one-way ANOVA were analyzed through SPSS.13.0.

3. Results and discussion

The synthesis and accumulation of PHB in microbes are related as a response to the environmental stress for successful survival on that stress site (Juengert et al., 2018). Since in this study, the PHB producing isolate was isolated from a polluted lake soil sample, that lake has been continuously receiving unprocessed wastes and effluents from residents, corporations, hospitals, etc (Mathiyazhagan and Natarajan, 2013). Thus, the perception of microbes survive in this site might develop adaptation evolution, which includes synthesizing polymeric granules to survive in this stressful environment; the samples were taken from this polluted site.

3.1. Screening of PHB producer

From several numbers of isolates, one isolate was recognized as competence to produce PHB granules, multiple stages of the screening process proved it. In the primary screening by Sudan Black B staining on test isolate grown petri dish results revealed that after the pouring of Sudan Black B stain into the petri dish, the color of colonies changed as dark greenish-blue color (Figure 2). This sign is considered as positive for PHB accumulations in test isolates (Mostafa et al., 2020). Similarly, Kalaivani and Sukumaran (2013), isolated the novel strains with biopolymers producing potential by the initial screening of Sudan Black B staining. The PHB selective media was used in this study as secondary screening, the results showed that growth of slight bluish colonies (Figure 2), which strengthen that the isolated strain has the PHB producing potential according to Juengert et al. (2018). Mahitha and Madhuri (2015) have also reported that the colonies grown in PHB selective media with bluish color can be considered as the test strain that has the competence to synthesize and accumulate PHB.

Finally, the microscopic observations were performed to confirm the PHB producing competence of test isolate. The Sudan Black B stained (Figure 3) isolate showed slight reddish dark color with vesicles like granules (look like a hole) under the standard microscopic observation (Mahitha and Madhuri, 2015; Kalaivani and Sukumaran, 2013). Similarly, Mostafa et al. (2020), isolated the PHB accumulating *Erythrobacter aquimaris* from the Mangrove rhizosphere region; they confirmed the PHB accumulation competence of this strain by microscopical observation with Sudan Black B stain, Acridine orange stain, etc. as confirmation test for PHB isolation. The Sudan Black-B has a strong attraction with lipid interacted biopolymer, which has significance in the screening of PHB producers. Further, the microscopic results partially revealed that the intracellular lipid and biopolymer content associated with the cytoplasmic materials and structural essentials of the isolate (Moorkoth and Nampoothiri, 2016). These previous reports strengthen the findings of the present study that the isolate has the potential to produce PHB.

The PHB producing test isolate was identified as *B. cereus* NDRMN001 through 16S rRNA sequence analysis, and the phylogenetic tree was analyzed and found the genetic similarity with the same genus and species (Figure 4). The genomic similarity of the test isolate was showed with 0.0001. Based on the phylogenetic analysis, hereditary origin of the test isolate was confirmed as *B. cereus*. Similarly, Mahitha and Madhuri

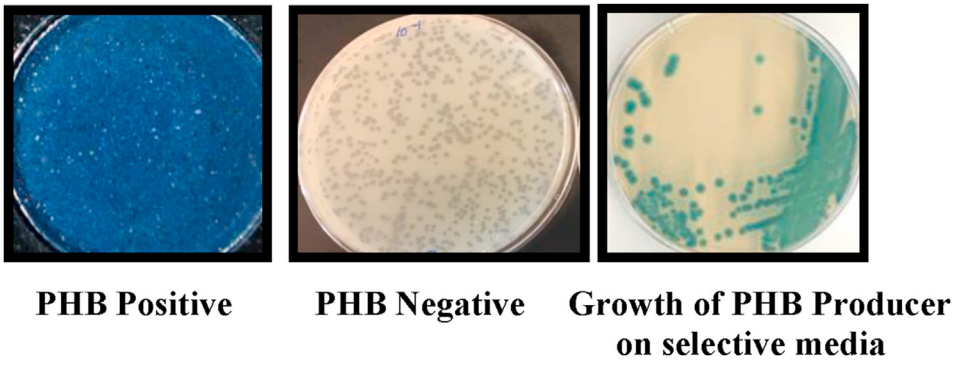

Figure 2. Initial screening of PHB producer by Sudan Black B staining and selective plate method.

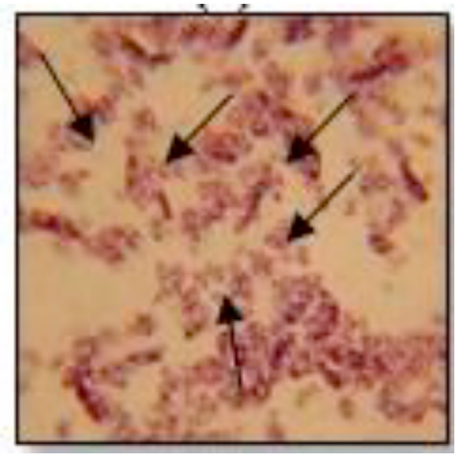

Figure 3. Microscopic observations of Sudan Black B stained PHB producer. Arrow marks indicate the presence of PHB molecules.

(2015) used molecular tools like 16S rRNA analysis to identify the novel bacterial species with PHB producing competence from polluted sites.

3.2. PHB characterization

The PHB from the *B. cereus* NDRMN001 was successfully extracted and analyzed their structural characteristics through FT-IR, ^{1}H NMR, and ^{13}C NMR analysis.

3.2.1. FT-IR, ^{1}H NMR, and ^{13}C NMR analysis

The FT-IR analysis (Figure 5A) results revealed that the presence of active groups in crude PHB molecules extracted from *B. cereus* NDRMN001, by noted the bands at 3282.68 cm^{-1} corresponding to aliphatic ester carbonyl C=O and 2712.21 cm^{-1} analogous to C–H stretch of biopolymers (Castro-Mayorga et al., 2018). The supplementary bands were recorded at 1759.27 cm^{-1} and 1528.16 $cm^{-1,}$ which confirmed the existence of O–H and C–H bonds related to aliphatic compounds individually (Figure 5A). The diagnostic signal band for this crude PHB was perceived at 1759.27 $cm^{-1,}$ and it was almost similar to the typical PHB. Similarly, Shi et al. (2017) and Hong et al. (2019) reported that the band noticed at 1724 cm^{-1} and 1728 cm^{-1} which were corresponded to PHB extracted from *Alteromonas lipolytica* and *Vibrio proteolyticus,* respectively. The variations among the banding pattern of the present study with previous reports are related to the quality of PHB and the metabolic impacts of microbes on the substrate utilized for PHB fabrication (Kalia et al., 2019; Narayanan et al., 2020e). The banding and peak pattern of PHB was varied due to the microbial metabolism activity. The band acquired at 1448.34 cm^{-1} is committed to stretching CH_3, and additional methyl groups and bands recorded at 500–1300 cm^{-1} were related to the stretching of the C–O bond of the ester group (Castro-Mayorga et al., 2018; Narayanan et al., 2020e).

The structural details of *B. cereus* produced PHB was possibly analyzed by ^{1}H NMR. In the present study, the first peak started at 1.67, corresponding to -CH3 proton, and multiplet peaks started from 2.37 to 2.71 states about the presence of -CH2 (methyl) proton and the peaks at 3.38, 5.28, 5.31, and 7. 68 ppm are might be the triplet peaks corresponds to the –CH proton (Figure 5B). These peaks were directly confirmed the test molecule derived from *B. cereus* NDRMN001 was PHB (Figure 5B). These results were perfectly interrelated with the report of Sabarinathan et al. (2018), who had found the initial peak at 1.267 followed subsequent peats from 2.63 to 2.44 and a final peak at 5.29, which corresponded to $–CH_3$, $–CH_2$, and –CH proton belongs to PHB molecule produced by *P. plecoglossicida* ^{1}H NMR analysis. Similarly, Alarfaj et al. (2015) reported that the doublet $–CH_3$ proton corresponding peak was found at 1.25, and peaks from 2.46-2.57 corresponding to-CH_2 proton and found the triplet peak at 5.23 of ^{1}H NMR analysis. Mohandas et al. (2017) and Sathiyanarayanan et al. (2013) obtained three peaks at 1.21:

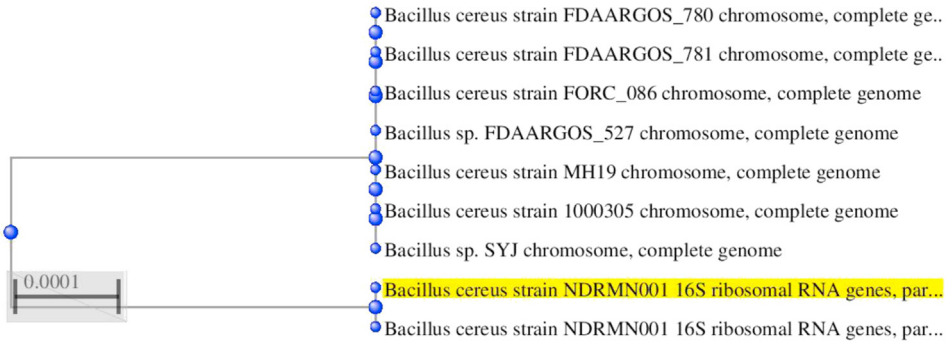

Figure 4. Phylogenetic tree of *Bacillus cereus* NDRMN001 (From NCBI). Phylogenetic distance tree based on 16S rRNA partial sequences displaying the evolutionary origin of *B. cereus* NDRMN001 within a lineage shared by the genera *Bacillus* is 0.0001.

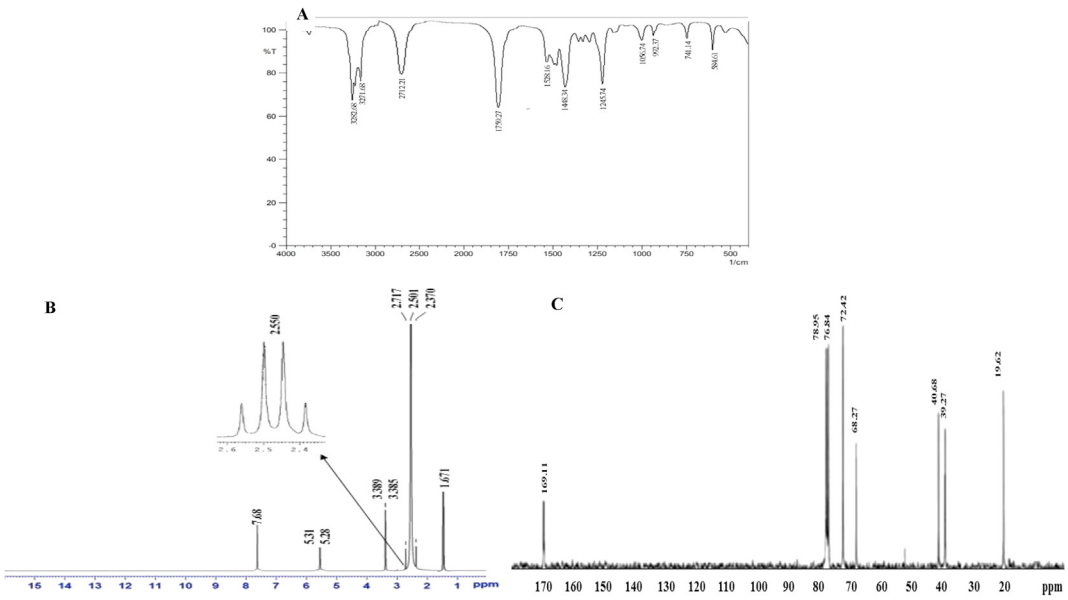

Figure 5. FT-IR (A), 1H-NMR (B) and 13C-NMR (C) analysis of PHB produced by *B. cereus*. The DMSO solvent peak inserted in A.

1.23, 2.56: 2.5, & 5.2: 5.2 ppm of ^{1}H NMR spectrum corresponding to methyl, methylene, and methane of PHB derived from *Vibrio harveyi* and *B. subtillis*. These reports supported the findings of the present study on *B. cereus* as it can produce PHB polymers.

The presence of carbon moieties of PHB produced by *B. cereus* NDRMN001 was studied by ^{13}C NMR analysis. Figure 5C revealed the presence of 4 significant peaks (19.62, 68.27, 40.68, and 169.11 ppm), which seemed close to the carboxyl group of PHB (Mostafa et al., 2020), and the DMSO peak was recorded at 39.27 ppm. These obtained results were interrelated with the results of Alarfaj et al. (2015), who had stated 4 dominant peaks (19.18, 40.15, 67.25, & 169.21 ppm) which corresponding to the carbon moieties of PHB produced by *B. thuringiensis* KSADL127 in ^{13}C NMR analysis using CDCL3 (76.77–77.41 ppm). Similarly, Sabarinathan et al. (2018), found 4 peaks indicating the presence of 4 carbon moieties of PHB derived from *P. plecoglossicida* in ^{13}C NMR. The remaining peaks (72.42, 76.84, and 78.95 ppm) obtained in ^{13}C NMR analysis might be impurities present in the extracted PHB or intermediate of PHB biosynthesis (Figure 5C). Since the Acetyl-CoA-acetyltransferase enzyme which involved in the condensation of 2 Acetyl-CoA components and produced Acetoacetyl-CoA, later, it reduced as (R)-3-hydroxybutyrate-CoA through acetoacetyl-CoA-reductase, and then the polymerization of (R)-3-hydroxybutyrate-CoA to PHB by the PHB synthase (Juengert et al., 2018).

3.3. Optimization- PHB production

The optimal growth requirements, which including physical and nutritional factors for test isolate *B. cereus* NDRMN001 were studied and attained results, were depicted in Figure 6 (A to F).

3.3.1. Inoculum (%)

The effective microbial production process mostly relays on the percentage of inoculum used and types of raw material used as nutritional sources. Hence, the PHB fabrication through bacteria is partially related to the concentration of inoculum. In this study, various percentages such as 2.5–15% of inoculum were studied, among them 7.5 % of inoculum produced (53.58%) 16.2 g L of PHB from 30.23 g L of *B. cereus* NDRMN001 biomass (Figure 6A). The obtained value was statistically significant at $P < 0.005$ than other percentages of inoculum. The low concentration of inoculum might take more days to reach a steady-state to produce PHB and the higher rate might rapidly utilize the essential nutritional contents, thus the yield of PHB was reduced at both lower and higher concentrations of inoculum (Nishida et al., 2018a). Hence, the *B. cereus* NDRMN001 isolated from polluted soil can produce 53.58% of PHB by using 7.5% inoculum as an optimal range.

3.3.2. Carbon and nitrogen sources

The primary and essential nutritional factors like carbon and nitrogen sources are the most significant factors which determine the quality and quantity of the microbial production process (Narayanan et al., 2020b). The major drawback in commercializing the microbial polymer is cost; thus the product is derived from costly nutritional materials like glucose, sucrose, etc. for microbial growth. In this study, the cheapest agriculture residues, such as sugarcane molasses, sago molasses, and rice bran, were used for PHB fabrication. Among these three affordable carbon sources, *B. cereus* NDRMN001 preferably utilized rice bran as the sole carbon source and yielded 75.24% of PHB (39.58 g L of PHB from 52.6 g L of biomass) than other carbon sources (Figure 6B). The obtained value was statistically significant at $P < 0.001$ than other carbon sources. Khiyami et al. (2011) reported that Date molasses provide the best carbon supplement for *Bacillus* sp. To produce more quantity of PHB (52.8). Interestingly, a similar kind of study was performed by Gabr (2019) with various carbon sources and reported that the palm molasses act as the most suitable source for PHB fabrication (58.3%) by *Bacillus* sp.

The nitrogen is another most significant factor for the sufficient growth of bacterial, and it might support the metabolism process in bacteria (Likitha et al., 2018). In this study, peptone, yeast extract, and sodium nitrate were used to find a suitable nitrogen source for *B. cereus* NDRMN001. Significant cell growth and PHB production was noticed on yeast extract containing media, and it supports the PHB fabrication as 51.01% (14.6 g L of PHB from 28.62 g L of *B. cereus* NDRMN001) than other nitrogen sources (Figure 6C). The obtained value was statistically significant at $P < 0.001$ than other nitrogen sources. The quality and the quantity of PHB produced by bacteria is directly related to the available form (readymade form or complexed form) of nitrogen sources (Patel et al., 2017). Under the complex and limited availability form of nitrogen source could enhance the PHB production with fine quality (Getachew and Woldesenbet 2016; Kourmentza et al., 2017).

3.3.3. Temperature and pH

Apart from the nutritional factors, the temperature and pH are also the essential factor to determine the quality and quantity of PHB and any other microbial production process (Kourmentza et al., 2017). In this

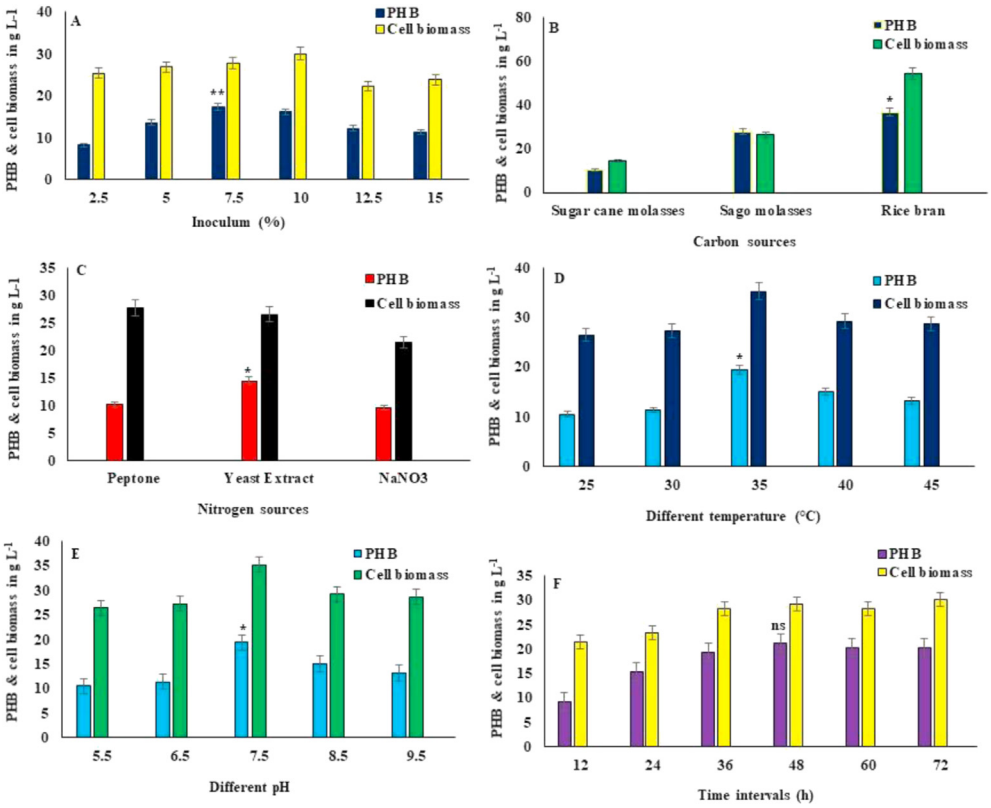

Figure 6. Optimized growth parameters for PHB synthesis by *B. cereus* NDRMN001. The mentioned values are mean and standard error (±SE) of triplicates. *: Significant at $P < 0.001$, **: Significant at $P < 0.005$, **ns**: not significant.

study, among various temperature (25, 30, 35, 40, and 45 °C) and pH (5.5, 6.5, 7.5, 8.5, and 9.5), the *B. cereus* NDRMN001 effectively grown and produced PHB and at 35 °C (60.21%) and pH 7.5 (58.74%) than other temperature and pH (Fig. 6D & E). The obtained value was statistically significant at $P < 0.001$ than different temperatures and pH. The attained results were perfectly correlated with the findings of Mostafa et al. (2020), who had been reported that the suitable temperature for *E. aquimaris* to produce PHB was 35 °C. Similarly, Mahitha and Madhuri (2015) and Kalaivani and Sukumaran (2013) they were found and reported the 30 °C for *Nacardiopsis* sp and *Vibrio harveyi* and 50 °C for *S. thermophilus* as the most convenient temperature for effective PHB fabrication. The attained suitable pH condition for this study has entirely interconnected with the results of Sasidharan et al. (2015), they stated that the optimal pH condition for PHB fabrication by bacteria was pH 7–8. Since the neutral pH could enhance the bioavailability of the nutritional contents and chelate enzymes activity (β-ketothiolase, acetoacetyl-CoA-reductase, PHB synthase, etc.), which involved in the PHB synthesis in bacteria (Bhagowati et al., 2015).

The deviations among these various reports are related to the microbes originated sites. Generally, the bacteria isolated from the various polluted environment might have the competence to produce PHB, since the unfavorable conditions, including temperature and pH enhance the PHB accumulation in bacteria (Audic et al., 2014). Since the temperature and pH determine the enzymatic activity involved in PHB production.

3.3.4. Incubation time

Generally, when microbes attaining sufficient incubation time to complete their lifespan, then only we could get valuable microbial products from microbes. Hence in this study, the most suitable incubation time was assessed for *B. cereus* NDRMN001 to produce the maximum yield of PHB. The attained results revealed that the *B. cereus* NDRMN001 have more yield at 48 h of incubation than other incubation time. Nevertheless, the acquired value was not statistically significant (Figure 6C). Under this optimal incubation time (48 h), *B. cereus* NDRMN001 yielded 70.98% of PHB (20.12 g L of PHB from 28.34 g L of biomass). This *B. cereus* NDRMN001 might reach the middle of the log phase and begun of stationary phase during the 48 h of incubation. During this period, the metabolic process in the cell could be very active in producing more quantity of PHB with necessary essential growth factors (Nishida et al., 2018b). The result of this parameter was co related entirely with the findings of Belal (2013) and Lathwal et al. (2015); they had been reported that the 48 h incubation was effective for PHB production by *Pseudomonas* sp, *Bacillus* sp, and *Rhizobium alti.* In some cases, 40 h and 24 h of incubations were also considered as the suitable incubation period for *B. subtilis* (Sathiyanarayanan et al., 2013) and *B. megaterium* (Dhangdhariya et al., 2015) respectively.

3.4. Production of PHB under optimized condition

The PHB fabrication competence of this *B. cereus* NDRMN001 under the optimized conditions (7.5% of inoculum, pH 7.5, 35 °C, rice bran, yeast extract, and 48 h of incubation) was performed. The obtained results revealed that under the optimized conditions, *B. cereus* NDRMN001 produced 91.48% of PHB (33.19 g L of PHB from 36.26 g L of cell biomass). The obtained value was statistically significant at $P < 0.001$.

The growth kinetics study revealed that the fabrication of PHB by *B. cereus* was steadily raising along with the cell biomass increasing (OD: 0.71) up to 6th day of the fabrication process, later on, 7th day onwards, the growth of *B. cereus* NDRMN001 was getting reduced it might reach the decline phase and the fabrication of PHB was also ceased and stayed constant with the OD of 0.65. The bacterial growth rate could be gradually raised through strongly consuming the available nutrients and simultaneously accumulate the PHB. While the nutrients in the medium get declined, the growth, and microbial product synthesis could be instantly reduced or halted (Kourmentza et al., 2017). The attained results were moderately connected with the reports of Mostafa et al.

(2020), who had achieved the maximum yield of PHB from *Erythrobacter aquimaris* under the optimal conditions. Similarly, the *B. subtilis* isolated from the mangrove root region showed better PHB production under standardized growth factors (Sathiyanarayanan et al., 2013). Since, the suitable growth conditions could enhance and maintain the balanced metabolic reaction within the cell, thus increasing the microbial products and biomass reasonably.

4. Conclusions

The bacteria isolated from polluted soil possesses the PHB producing competence, and it was qualitatively confirmed by Sudan Black B, Nile red staining, selective media method, and microscopic observations. The PHB producing isolate was identified as *B. cereus* NDRMN001 using 16S rRNA analysis. The PHB extracted from *B. cereus* NDRMN001 was categorized using FR-IR, ^{1}H NMR, and ^{13}C NMR analysis. The PHB corresponding band was recorded at 1759.27 cm^{-1} on FT-IR analysis, and it validates that the component extracted from *B. cereus* NDRMN001 containing PHB molecules. In the structural elucidation analysis of PHB by ^{1}H NMR, the peaks related to -CH3 proton, -CH2 (methyl) proton, and –CH proton were spotted at 1.67, 2.37–2.71, and 3.38–7. 6 ppm respectively. Further, the optimal growth requirements of *B. cereus* NDRMN001 for PHB fabrication were optimized as 7.5% of inoculum, with pH 7.5, the temperature at 35 °C, rice bran (carbon source), yeast extract (nitrogen source), and 48 h of incubation. About 91.48% of PHB was produced by *B. cereus* NDRMN001 under suitable growth conditions. The complete outcome of this study concludes that the *B. cereus* NDRMN001 has the competence to fabricate PHB with a commercialization perspective by consuming cheap nutritional sources with the most appropriate growth circumstances. The PHB production and about the commercialization possibilities studies are under progress.

Declarations

Author contribution statement

Mathiyazhagan Narayanan, Gajendiran Kandasamy: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Sabariswaran Kandasamy, Keerthana Gnanavel: Contributed reagents, materials, analysis tools or data.

Suresh Kumarasamy, Muthusamy Ranganathan: Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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