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Bacillus sp. was induced to accumulate PHAs granule

PHAs production was carried out by SmF

Extracted PHAs is PHB as confirmed from FTIR

Surface morphology of PHAs film confirmed by FE-SEM

PHAs film was extracted by downstream processing

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Introduction

Academia and industry have provided unique interest towards production and improvement of biodegradable polymer. Among various types of biopolymer, polyhydroxyalkanoates (PHAs) have gained utmost attention due to its biodegradability and biocompatibility (Mohapatra *et al.*, 2017). PHAs are the most common ecofriendly biopolymer synthesized by a wide array of bacteria as energy storage inclusions under nitrogen limiting condition. Literature review states 150 different monomers of PHAs (Li *et al.*, 2016) have been reported from various Gram-positive bacteria such as *Bacillus subtilis*, *B. amyloliquefaciens* DSM7, *B. licheniformis*, *B. cereus* PS10, *B. circulans*, *B. megaterium* Y6, *B. thuringiensis*, *Clostridium* sp., *Corynebacterium* sp., *Nocardia* sp., *Rhodococcus* sp., *Streptomyces* sp., *Staphylococcus* sp. and Gram-negative bacteria, including *Alcaligenes latus*, *Ralstonia eutropha*, *P. putida* KT2440, *P. oleovorans* GPO1 (Tortajada *et al.*, 2013; Sharma and Bajaj, 2015). Nevertheless, *Bacillus* is the predominant genus producing numerous derivatives of PHAs with noteworthy properties and applications. *Bacillus* species produces 90% PHAs from dry cells even in undefined nutrient condition. *Bacillus* species is being used as a model bacteria in both industry and academic research due to its genetic stability and lack of lipopolysaccharides outer layer which makes downstream processing of PHAs easier (Mohapatra *et al.*, 2015, 2017). In addition, they also synthesize various hydrolytic enzymes for utilization of inexpensive carbon sources to produce co-polymers of PHAs under submerged fermentation. Nevertheless, the *Bacillus* species produces spores; however the same can be suppressed under acidic pH and potassium deficiency condition (Mohapatra *et al.*, 2017).

Earlier studies have revealed higher poly-beta-hydroxybutyrate (PHB) production by *Bacillus megaterium* at 15:1 C/N ratio with sucrose and $(\text{NH}_4)_2\text{SO}_4$ as carbon and nitrogen source (Ahn *et al.*, 2015). However, Belal and Farid (2016) reported higher PHB production at 20:1 C/N ratio by *Bacillus cereus*. Thus, it is confirmed that C/N ratio varies from bacteria to bacteria for PHAs production. Process economics of industrial PHAs production study reveal that 50% of its production cost depends on carbon source (Maity *et al.*, 2017). However, proper optimization process makes it possible to reduce the cost of PHAs production, hence, to reduce the cost of production, efforts are needed to study the inherent mechanism of PHAs accumulation. In view of the above, an attempt was made to study the effect of C/N ratio on PHAs production by *Bacillus* species under submerged fermentation process.

Materials and Methods

Culture collection: Energetically stable PHAs producing (ΔG : 535.61 kcal mol⁻¹), pigmented, *Bacillus* sp. C1 (2013) (KF626477) was selected, based on earlier study of Mohapatra *et al.* (2016). The preserved (-20°C) bacterial strain was revived and induced for accumulation of PHAs granule in their cytoplasm using mineral salt medium (MSM). PHAs accumulation by the bacterial

isolate was detected by Sudan Black followed by Nile Red staining.

Optimization of growth parameters by one factor at a time (OFAT) approach: Environmental variation is the key factor that regulates the growth patterns of microbes as well as PHAs production. Several growth parameters such as cultivation medium, pH, temperature, carbon and nitrogen source, carbon and nitrogen concentration and inoculum size were optimized by OFAT approach (Maity *et al.*, 2017). In this approach, two different cultivation medium (growth and mineral salt medium) were prepared, 24 hours fresh inoculum (1.7×10^8 cells ml⁻¹ as per 0.5 McFarland standards) was inoculated and incubated at 37°C for 24 hrs. Bacterial cell biomass was estimated by measuring OD₆₀₀ using UV-Vis spectrophotometer (λ 35-Perkin-Elmer). The cultivation medium depicted that higher cell biomass was kept constant and other parameters including pH (6-10), temperature (23-44°C), carbon (maltose, sucrose, dextrose, glucose, galactose), nitrogen sources (NH_4Cl , NH_4NO_3 , yeast extract, urea, $(\text{NH}_4)_2\text{SO}_4$) in a constant ratio (6:1), carbon (1-5%), nitrogen (1-4%) concentration and inoculum size (5-20% v/v) varied one after another for optimization studies.

Effect of C/N ratio and time period on PHAs production: PHAs production under submerged fermentation was conducted through one stage batch cultivation shake flask technique using optimized growth condition (Ishak *et al.*, 2015). Briefly, 1 liter culture medium was prepared with carbon and nitrogen source. The carbon concentration was kept constant and different C/N ratio 2:0.1, 2:0.2, 2:0.3, 2:0.4 and 2:0.5 (nitrogen deficient condition) were prepared by regulating the amount of nitrogen concentration. Inoculum was then added to the medium and incubated at 37°C for 48 hrs. Bacterial cells were harvested by centrifugation and PHAs was recovered by sodium hypochlorite digestion-multi-solvent (acetone, methanol, diethyl-ether and chloroform) extraction method. PHAs production was estimated by the following formulae

$$\text{PHAs (\%)} = \frac{\text{Weight of PHAs} \times 100}{\text{Dry cell weight}}$$

Similarly, PHAs production was also carried out under all optimized condition by varying the time period (24, 48, 72 and 96 hrs).

Fourier transform infrared (FTIR) spectroscopic analysis: To determine the functional groups of extracted PHAs, FTIR analysis was conducted. The extracted PHAs (2 mg) was placed on Attenuated Total Reflectance (ATR) diamond based plate and IR-spectrum was recorded using a single beam spectrometer (Perkin-Elmer RX I) between 4000 and 400 cm⁻¹ wavelength. FTIR analysis was also conducted with standard PHB.

Field-emission scanning electron microscopic imaging of PHAs film: Surface morphology of PHAs film was studied by field emission imaging. Extracted PHAs film was mounted on

Fig. 1: (A) Black-blue coloured cells under Sudan Black and (B) fluorescent yellow coloured cells under Nile Red staining indicates PHAs accumulation by *Bacillus* sp.

aluminum stumps for FE-SEM. The film was then rinsed in buffer and coated with gold in a sputter coater (Polaron SC7620) and observed under FE-SEM (QUANTA 200 FEG from FEI Netherlands) at an accelerating voltage of 15 kV.

Statistical analysis: The results obtained from optimization of growth parameters and PHAs production were analyzed using one-way ANOVA, where $p < 0.05$ was considered to be significant statistically using statistical package for social science (SPSS 20) software.

Results and Discussion

Generally, PHAs accumulation by microbes takes place under environmental stress condition like elevated carbon and inadequate nitrogen, phosphorus, magnesium, potassium, sulphur and oxygen concentration. In particular, PHAs accumulation by bacteria *Cupriavidus necator*, *Bacillus cereus* E6 and *Bacillus thuringiensis* IAM 12077 is favoured under nitrogen deficient conditions (Ahn et al., 2015; Belal and Farid, 2016 and Gowda and Shivakumar, 2014). In this context, the preserved PHB producing *Bacillus* sp. C1 (2013) (KF626477) was revived and accumulation of PHAs granule in their cytoplasm was confirmed by Sudan Black and Nile Red staining (Fig. 1 A, B). Bacterial strain is energetically stable (ΔG : 535.61 kcal mol⁻¹) (Mohapatra et al., 2016), hence, PHB biogenesis was not suppressed after four years of preservation at low temperature (-20°C). Several environmental parameters play a vital role on cell biomass production and accumulation of PHAs by bacteria. Growth optimization investigation revealed that higher cell

biomass production was obtained in mineral salt medium at pH 9.0, 37°C, 2% dextrose as carbon source, 1% (NH₄)₂SO₄ as nitrogen source and 15% inoculum size were optimum at $P < 0.05$ significance level (Fig. 2 A-H). Correspondingly, previous studies detected higher cell biomass production by *Bacillus subtilis* (MTCC 9763), *Bacillus thuringiensis* SBC4 and *Bacillus* sp. ISTVK1, *Bacillus cereus* and *Bacillus* sp. (Muralidharan et al., 2015; Odeniyi and Adeola, 2017; Morya et al., 2018; Belal and Farid, 2016; Mohapatra et al., 2014) under similar growth condition. As *Bacillus* species are predominant microflora of soil with faster growth rate and can synthesize various hydrolytic enzymes such as amylase and cellulase, therefore, higher cell biomass production by many *Bacillus* species were observed under similar growth condition (Mohapatra et al., 2017).

In an earlier study, carried out by Mohapatra et al. (2014) the selected bacterial strain was found to produce 0.909 g l⁻¹ PHAs at a fixed carbon to nitrogen ratio (6:1). However, among all factors, PHAs accumulation by many bacteria, including species of *Bacillus*, is regulated by C/N ratio. Consequently, in this research different C/N ratio were maintained using the best carbon and nitrogen sources dextrose/ammonium sulphate as well as their effect on PHAs production were evaluated. The resultant effect of C/N ratio at a fixed dextrose concentration (20 g l⁻¹) on PHAs accumulation is shown in Fig. 3A. The *Bacillus* sp. was found to produce 15.5, 29.3, 38.7, 49.2 and 36.8 % of PHAs at 2:0.1, 2:0.2, 2:0.3, 2:0.4 and 2:0.5 C/N ratio, however, the PHAs production trend increased from 2:0.1 to 2:0.4 C/N ratio and reduced at 2:0.5 C/N ratio. Amongst different C/N ratio tested, 2:0.4 was found optimum for higher (1.09 g l⁻¹ or 49.2%) PHAs

Fig. 2: Effect of culture medium (A); pH (B); Temperature (C); Carbon sources (D); Nitrogen sources (E); Dextrose concentration (F); Ammonium sulphate concentration (G) and Inoculum size (H) on growth of PHAs producing *Bacillus* sp.

Fig. 3: PHAs yield percentage and concentration at different (A) C/N ratio and (B) time period.

Fig. 4: (A) FTIR high intense absorption band at 1720.18 cm^{-1} illustrated C=O functional group of PHB and (B) Surface morphology (rough) of PHB film under FE-SEM imaging.

production. Thus, under higher degrees of nitrogen deficient condition, PHAs production by bacteria increased to 0.181 g l^{-1} as compared to previous results. Preceding studies also reported higher PHAs production (1.4 , 4.6 and 2.6 g l^{-1}) under limited nitrogen condition ($6:1$, $20:1$ and $8:1$) from *Bacillus* sp. PJC48, *Bacillus cereus* and *Bacillus thuringiensis* IAM 12077, respectively (Gu et al., 2018; Belal and Farid, 2016; Gowda and Shivakumar, 2014). The increase in nitrogen deficient condition resulted in higher PHAs yield. These unbalanced nutrient status (C/N ratio) creates a selective pressure which increases the

proton concentration gradient across bacterial cell membrane. The increase in proton gradient inhibits the first two enzymes of TCA cycle and leads to higher PHAs synthesis (Mohapatra et al., 2017). Then, the PHAs production was carried out under optimized C/N ratio for 72 hrs. The result (Fig. 3B) indicated that the PHAs production increased (33.7 - 49.2%) from 24 to 48 hrs and then decreased (30.3%) up to 72 hrs. Moreover, *Bacillus* sp. C1 (2013) (KF626477) produced 1.09 g l^{-1} (49.2%) of PHAs from 2.218 g (DCW) cell biomass at 48 hrs. Belal and Farid (2016) also observed higher PHAs production (66.7%) from *Bacillus cereus*

at 48 hrs. Moreover, *Bacillus subtilis* NG05, *Bacillus thuringiensis* IAM 12077, *Bacillus subtilis* 25 and *Bacillus megaterium* 12 produced 50.1, 47.1, 18.03 and 14.79% of PHAs at 72, 24 and 45 hrs, respectively (Singh et al., 2012; Pal et al., 2009; Yuksekdağ et al., 2004). Over all, the PHAs production decreased after 48 or 72 hrs due to depletion of nutrients, especially carbon source in the production medium. Under nutrient depletion condition, some PHAs producing bacteria including species of *Bacillus* depolymerized PHAs to D-hydroxy-butyrate and then metabolized to acetoacetate and acetoacetyl-CoA to provide energy to the cell (Mohapatra et al., 2017; Belal and Farid, 2016; Singh et al., 2012).

The FTIR spectrum of extracted PHAs showed intense absorption bands at 1720.18 cm^{-1} resembles to ester carbonyl functional group of PHB (the most common homopolymer of PHAs), which is comparable to standard PHB (1722.26 cm^{-1}). Many other stretches such as CH_2 and CH_3 and $-\text{CH}_3$ were also detected in the extracted PHB (Fig. 4A). Correspondingly, Maheshwari et al. (2018), Murlidharan and Radha (2015) and Swornakumari et al. (2018) observed similar intense absorption band at 1720 , 1634.7 and 1647.5 cm^{-1} depicting chemical structure of PHB, which is the most common homopolymer of PHAs. The surface of PHB film was rough and fairly regular as confirmed from FE- SEM imaging (Fig. 4B). Rough surface of the PHB film enhances attachment of microbes, leading to biodegradation of polymer to CO_2 and water in oxic and methane in anoxic condition (Varsha and Savitha, 2011; Boyandin et al., 2012). It can be concluded that *Bacillus* sp. C1 (2013) (KF626477) produced 1.09 g l^{-1} of PHB, the most common homopolymer of PHAs under higher nitrogen deficient condition and rough surface of PHB film improves its biodegradability. However, further research is highly indispensable by selecting bacteria of different genera in this regard.

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