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Evaluation of Cultural Parameters Involved in the Overproduction of Poly-β- Hydroxybutyrate from *Azotobacter vinelandii*

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Poly- β -Hydroxybutyrate (PHB), a Biodegradable Polymer, is accumulated intracellularly in a variety of microorganisms under controlled concentrations of nutrients such as nitrogen, oxygen and mineral ions. In this study, the potency for PHB synthesis was tested for bacteria isolated from the rhizosphere of *Azadirachta indica*. Out of the 9 different isolates, RS6 was selected for further studies. Under the unoptimized condition, RS6 produced 0.20 g/L of PHB in nitrogen deficient broth. Parameters like variation of carbon and nitrogen sources, temperature, pH, incubation period revealed that the highest PHB yield was obtained with glucose (10% w/v) and peptone (1% w/v) as supplements. The optimum pH, temperature and incubation period was found to be 7.0, 35°C and 48 h respectively. Following the optimization of different cultural conditions a 3 fold increase in the PHB production was recorded. Based on the biochemical studies, the isolated strain was identified as Azotobacter vinelandii. The present study provided useful data about important media and physical parameters for PHB production that can be utilized in an industrial perspective in the near future.

Key Words: Biodegradable polymer, PHB synthesis, *Azotobacter vinelandii*.

During the last decade, environmental pollution and exhaustion of non-renewable resources have created interest of human kind in natural materials such as polyhydroxy alkanoates (PHAs), a biodegradable polymer. A biodegradable polymer may be defined as polymer capable of being decomposed by the action of biological agents, under specific environmental conditions.

Chemically, PHAs are polyesters of hydroxylacids, naturally synthesized and accumulated as cytoplasmic inclusions by some microorganisms such as *Alcaligene eutrophes*, *Azotobacter beijerinckia*, *Bacillus megaterium*, *Pseudomonas oleovorans* and various nitrogen fixing microorganisms found in root nodules of legumes when carbon source is in plentiful and other nutrients such as nitrogen, phosphate, oxygen or sulfur are limited.

Conventional plastics of petrochemical origin, take decades to decompose in nature and also produce toxins during the degradation process. Therefore, there is a special interest in the plastics production from materials that can be easily eliminated from our environment[1,2]. The PHAs are also known as bioplastics since they have

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thermoplastic, elastomeric properties and performance characteristics similar to those of conventional plastics. However, bioplastics are easily degraded by the microbial action in the environment. Recently, synthesis of PHAs is seen as an attractive system for the sustained production of large amounts of polymers at low cost[3, 4, 5].

Few forms of PHAs include polyhydroxybutyrate (PHB), poly-β-hydroxyvalerate (PHV) and polyhydroxybutyrate-co-valerate (PHB-V)[2]. Among the members of PHA family, poly β-hydroxy butyrate (PHB) is the most common biodegradable polymer and a promising alternative to synthetic non-degradable plastics.

In the case of PHB, *Azotobacter vinelandii* has shown the ability to produce relatively large amounts of this polymer of high molecular weight on cheap substrates, even allowing for simple extraction processes. The development of fermentation strategies has also shown promising results in terms of improving productivity.

Thus, the present study aims at investigating the potential of *A. vinelandii* as the major source of producing PHB. The study focuses on the formulation of optimized media composition in terms of chemical parameters like various carbon and nitrogen sources and the involvement of physical parameters like pH, temperature and incubation period to achieve more efficient production of PHB from *A. vinelandii*.

MATERIALS AND METHODS

Isolation and screening of PHB-producing bacteria

Soil sample was collected from the rhizosphere of *Azadirachta indica* plant in the institute campus, Bangalore, following aseptic conditions. The soil sample was transported to the laboratory and plated on nutrient agar. The culture plates were incubated at 30°C for 3 to 4 days until the colonies appeared.

Following the development of the colonies, the plates were flooded with 8 ml Sudan black solution (0.2% Sudan black B in 96% ethanol) for 10 minutes. The dye was then decanted and plates were gently rinsed by addition of 10 ml of 100 % ethanol. Bluish-black colonies were considered positive and selected for PHB production studies[6]. The selected isolates were streaked on nutrient agar and incubated until pure cultures were obtained.

Inoculum preparation

The inoculum for the individual selected isolates was prepared in 250-mL Erlenmeyer flasks containing sterile nutrient broth. All the flasks were incubated at 37°C for 24 h on a rotary shaker at 120 rpm. Individual culture broth was centrifuged at 8000 rpm for 15 min, washed twice, and suspended in physiological saline and stored under refrigeration for future use.

Selection of the isolate producing highest PHB in unoptimized broth medium

The cell suspension of the selected isolates demonstrating the PHB production were individually grown in nitrogen-deficient media (composition in g/L: glucose, 10; $MgSO_4$, 0.2; NaCl, 0.1; KH_2PO_4, 0.5; and yeast extract, 2.5, pH 7.0 ±0.2) and incubated under shaking condition at 30°C for 24 h. Following incubation the broth was centrifuged at 5000 rpm for 15 min at 4°C to collect the cell pellet. For the extraction of PHB, the bacterial pellets were lysed in sodium hypochlorite at 37°C for 2 h and centrifuged at 10,000 rpm. The residues were washed twice with water, acetone, ethanol, and diethyl ether respectively. The residues left behind were extracted with boiling chloroform and filtered through Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, England). The chloroform extracts were evaporated to dryness, and Law and Slepecky method[7] for the quantification of PHB was followed using a UV-VIS spectrophotometer (SANYO Gallenkamp, Germany). The production of PHB was expressed as PHB yield (g/L).

Characterization of the selected isolate

The selected isolate was identified based on its morphological, physiological and biochemical characteristics. The morphological characteristics were identified by culturing the isolate on nutrient agar plates and studying the shape, size, colour, opacity, texture, elevation, spreading nature and margin of the colonies. The physiological characteristics were identified by gram's staining and motility test. The biochemical characterization of the isolate was performed by indole test, methyl red test, Vogesproskauer test, oxidase test, gelatin liquefaction test, lecithinase

production test, starch hydrolysis test and nitrate test. Bergey's Manual of Determinative of Bacteriology (7th Edition) was used as a reference to identify the isolates.

Optimization of Cultural Conditions

The nitrogen-deficient medium was supplemented with different carbon sources (10% w/v: maltose, lactose, sucrose, xylose, fructose and glucose) and organic and inorganic nitrogen sources (1% w/v: peptone, tryptone, yeast extract, beef extract, ammonium sulphate, urea and sodium nitrate). Various physical parameters such as pH (4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5 and 8), effects of temperature (10, 25, 30, 35, 40, 45, 50, 60, 70 and 80 °C) and incubation period (24, 48, 72, 96, 120, and 144 h) were optimized by conventional methods for maximal PHB production.

Statistical Analysis

All the optimization studies were conducted in triplicate and the data were analyzed using single factor analysis of variance (ANOVA). All the data are graphically presented as the mean ± S.D. of triplicates (n = 3). ANOVA was performed using Microsoft Excel 2007. *P* values < 0.05 were considered significant with a confidence limit of 95%.

RESULTS AND DISCUSSION

Several bacteria produce PHB, a polymer of the polyhydroxyalcanoates family of polyesters under unbalanced growth conditions. This polymer is accumulated as a result of depletion of nitrogen, phosphorous or oxygen and an elevated level of a carbon source present in the environment[8]. It is known that the PHB accumulated serves as a store of carbon and energy and as an electron sink into which excess reducing power can be channeled[9]. The PHB biosynthesis is a NADPH-dependent pathway [10].

The precursor of PHB biosynthesis is acetyl coenzyme-A (acetyl-CoA), a product of catabolization of the carbohydrate through the Entner–Doudoroff pathway[11, 12]. The increase in the NADH level, under oxygen limitation and excess of carbon source inhibits the citrate synthase and isocitrate dehydrogenase activities of the tricarboxylic acid cycle, thus resulting in the increase acetyl- CoA levels, and in turn the PHB synthesis[9].

Of all the 17 soil isolates tested for their reaction with Sudan black stain, 9 isolates (RS2, RS4-6, RS11-13, RS15 and RS17) proved to produce PHB since the colonies retained the colour of the Sudan black stain when washed with alcohol. When inoculated in the nitrogen-deficient medium, isolate RS6 accumulated the highest yield of PHB (0.20 g/L) as compared to the other isolates (Fig. 1) and was selected for further studies.

The economics of biodegradable polymer production is dependent on several factors which include substrate cost and the ability to produce biodegradable polymers from inexpensive or renewable substrates[13]. The type of carbon source used has a huge influence on PHB productivity, because intercellular accumulation of PHB appeared to be carbon source specific. The carbon source must always be provided in excess to allow

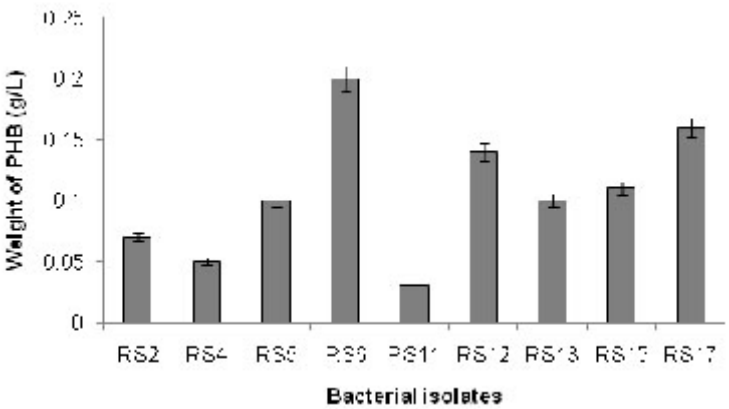

Fig. 1. Different levels of PHB production by the bacterial isolates. Data represent mean ± S.D. (n=3); P < 0.05

for maximum PHB accumulation in the biomass.

The organism was quite flexible in utilizing different carbon and nitrogen sources for the biopolymer accumulation. The highest level of PHB accumulation was observed in nitrogen deficient medium supplemented with glucose (PHB accumulation 0.41 g/L), followed by lactose, xylose, fructose and maltose (Fig. 2). Our study is in perfect coordination with the results found by a previous study where 2% glucose acted as the best carbon source in the production of PHB by *Azotobacter chroococcum*[14]. PHB production by *Bacillus subtilis* 25 and *Bacillus megaterium* 12 strains revealed that the highest level of PHB accumulation was observed in the medium with glucose as carbon sources in *B. subtilis* 25 (19.51%) and *B. megaterium* 12 (19.49%)[15].

Maximum accumulation of PHB by *Methylobacterium* SPV-49 was observed with glucose as the carbon source. Methanol and sugars such as sucrose and lactose also induced PHB accumulation[16].

37 isolates and mutants of *A. chroococcum* when checked for the PHB production using Sudan black B staining method demonstrated that with 2% glucose and 15 mM/L ammonium acetate, PHB production was found to be maximum at 36 and 48 hours of growth under submerged cultivation and under stationary cultivation respectively. It was also observed that PHB production was higher on sucrose and commercial sugar as compared to glucose and mannitol[17]. Likewise, it was reported that *Bacillus* sp. JMa5 strain accumulated 25-35% (w/w) PHB during sucrose fermentation[18].

Of the various nitrogen sources tested, peptone supported maximum PHB accumulation (0.45 g/L), followed by beef extract, tryptone, ammonium sulphate, ammonium chloride, sodium nitrate and urea (Fig. 3). Such enhancement of PHB

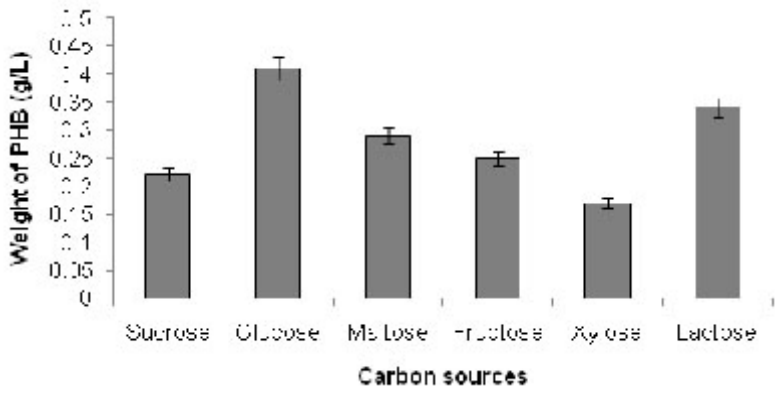

Fig. 2. Effect of additional carbon source on PHB production. Data represent mean ± S.D. (n=3); P < 0.05

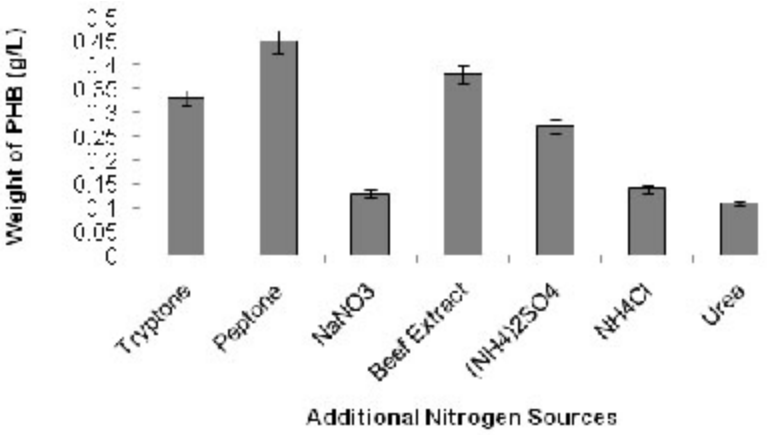

Fig. 3. Effect of additional nitrogen source on PHB production. Data represent mean ± S.D. (n=3); P < 0.05

accumulation may result from the presence of amino acids and peptides in peptone which leads to the channeling of the carbon source into PHB accumulation. Similarly, the highest level of PHB was observed in the medium with protease peptone as nitrogen source in *B. subtilis* 25 (78.69%) and in *B. megaterium* 12 (77%) [19, 20]. PHB production by *A. vinelandii* UWD strain was found to be better in a variety of commercially available complex nitrogen sources like fish peptone, proteose peptone, yeast extract, casitone, phytone and tryptone[21]. Researchers investigating the effect of different nitrogen and carbon sources and PHB production in two strains of *Rhizobium* sp. noted that the strains produced less PHB in yeast extract mannitol (YEM) broth media with different carbon (glucose, sucrose, arabinose) and nitrogen (L-cysteine, L-glycine, DL-tryptophan, proteose peptone, potassium nitrate) sources, while the highest level of PHB accumulation was observed in the media with L-cysteine and L-glycine[22]. Similar enhancement of PHB accumulation was reported in *A. vinelandii*, *A. chroococcum*, *A. beijerinckii* and *E. coli* when the organisms were grown in media containing organic nitrogen sources[21, 23, 24, 25, 26, 27].

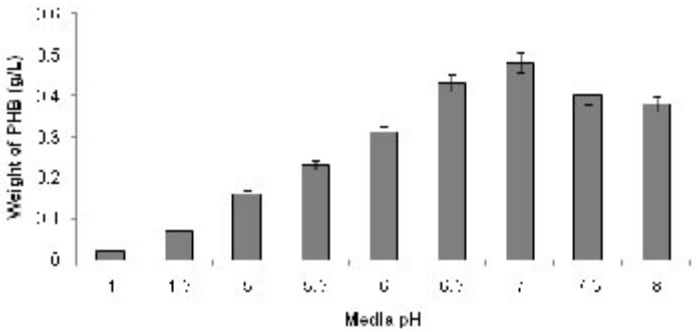

Fig. 4. Effect of media pH on PHB production. Data represent mean ± S.D. (n=3); P < 0.05

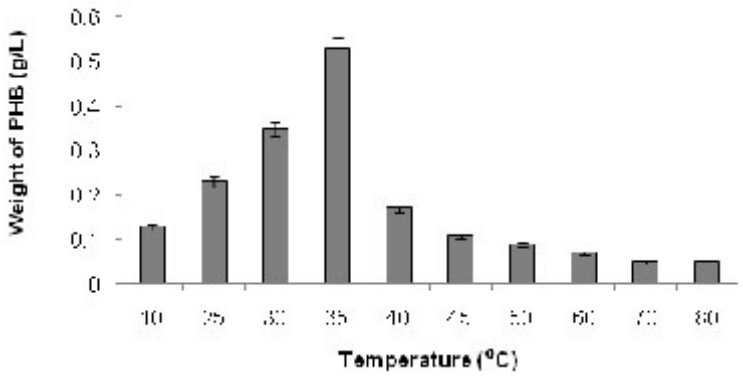

Fig. 5. Effect of incubation temperature on PHB production. Data represents mean ± S.D. (n=3); P < 0.05

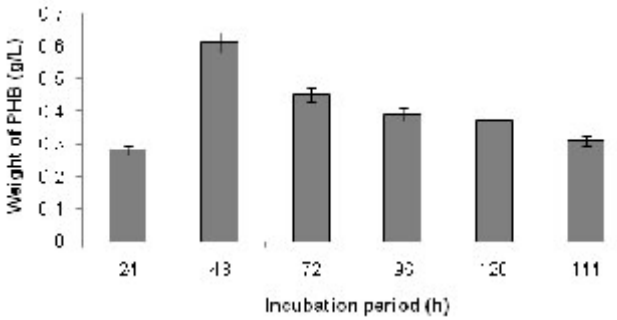

Fig. 6. Effect of incubation period on PHB production. Data represent mean ± S.D. (n=3); P < 0.05

pH affects both bacterial growth and biopolymer production. When bacterial cells are exposed to pH beyond their optimum range, maintenance energy is used for pH control. This reduces the energy available for biopolymer production, thus the bacterial ability to produce the biopolymer is reduced. The media pH also affects the permeability of the bacterial cell membrane thus affecting the biochemical activities of the cell required for biopolymer production[28]. Our study revealed that neutral pH was best suited for the accumulation of PHB by *A. vinelandii*. At pH 7.0 the highest accumulation of the polymer (0.48 g/L) was recorded. As the pH shifted from the neutrality towards the acidic range there was a steep decrease in the polymer accumulation. A decrease in the PHB accumulation was also observed when the media pH was in the alkaline range (Fig. 4).

Bacillus sp. growing in PHB glucose and YEM medium when kept at different pH, i.e, 6.5, 7.0 and 7.5 showed the highest percentage PHB yield in media with pH 6.5 (i.e. 82% in PHB medium and 49% in YEM medium) followed by pH 7.0 (63% in PHB medium and 40% in YEM medium) and 7.5 (65% in PHB medium and 39% in YEM medium) [29]. Literature reveals that pH value ranging from 6.5-7.5 is optimum for PHB production[30]. In an earlier study, PHB synthesis had a pH optimum around pH 6.4 and that the lack of polymer accumulation at higher pH values could be best explained by an effect on the degradative enzymes of polymer breakdown. Thus, PHB was utilized at a rate almost equal to the rate of its synthesis[31].

The effect of different pH on PHB production in two strains of *R. meliloti* revealed that the strains showed different growth rates and there was a decrease in PHB content in the medium with an acidic pH[32].

Temperature has a profound role to play in the production of PHB. According to our study it was found that 35°C yielded the highest PHB accumulation (0.53 g/L) (Fig. 5). Our study is in perfect accordance with the study conducted by previous workers[33], who found out that the optimum results were obtained at 35°C when using whey broth as fermentation medium for *Azotobacter* strain. Although *A. chroococum* could produce biopolymer at 25°C incubation, maximum yields were obtained from fermentation carried out at 35°C.

Media optimization for PHB production from *Bacillus* sp. revealed that when the *Bacillus* culture was inoculated in PHB glucose and YEM medium and incubated at different temperature viz. 25°C, 30°C and 37°C, the isolate in PHB glucose medium yielded highest PHB at 37°C and 30°C (63% and 62% respectively) followed by 25°C (45%). The effect of temperature on PHB production in YEM medium was same as in PHB glucose medium; however, the percentage yield was lower than PHB medium (41% at 37°C and 30°C, 40% at 25°C)[29]. The results of PHB yield at different temperature conditions are in accordance with[30], who used 33°C for optimum PHB synthesis in fermentation condition and concluded that the temperature range from 30-37°C is optimum for PHB synthesis.

Bacteria which synthesize PHA can be divided in to two groups. The first group, accumulating PHA during the stationary phase, requires limitations of N, P, Mg and an excess of carbon sources. The second group, accumulating PHA during the growth phase, includes *Alicaligens latus,* a mutant strain of *A. vinelandii, A. beijerinckii* or recombinant strains of *E. coli* bearing the PHA operon of *Ralstonia eutropha*[19].

In our study, the accumulation of the PHB was influenced by the period of incubation, where the highest accumulation (0.61 g/L) was detected at 48 h. Beyond 48 hrs there resulted a constant decrease in the PHB accumulation (Fig. 6). The most probable reason for such decrease in PHB yield may be the unfavourable conditions developing in the medium as a result of the increase in medium viscosity due to exopolysaccharide production. Such a condition resulted in oxygen transfer limitation and hence a decrease in PHB synthesis. Likewise, beyond 48 hrs the organism might have entered the stationary phase where the number of new cell formation was equal to the number of cell death. Under such a condition, PHB instead of being accumulated was utilized by the organism as a source of carbon.

The results obtained in our study are in agreement with earlier studies where the production of PHB by the *Rhizobium* spp. 2426 strain was detected between 24 h and 120 h in YEM medium with L-cysteine and at the end of 48 h, *Rhizobium* spp. 2426 produced very satisfying results in terms of PHB yield (74.03%, 0.285 g/L)

The yield decreased to 36.10% at 72 h and 21.72% at 120 h[22]. On the contrary, when the time course of aerobic growth and PHB accumulation in *Bacillus thuringiensis* IAM 12077 was measured at different time intervals after the transfer into the nitrogen deficient medium, cell mass increased by 16 h from 0.633 g/L to 1.4 g/L and maintained steadily. PHB production also increased gradually, attained its maximum at 24 h of cultivation (from 0.066 g/L to 0.683 g/L), with a gradual increase in PHB yield and accumulation with maximum at 24 h (0.683 g/L, 47 %) after which the PHB yield gradually decreased by 48 h[19].

CONCLUSION

In conclusion, media and cultural parameters have a profound effect on the yield of PHB produced by *Azotobacter vinelandii*. Incorporation of glucose and peptone supported the maximum yield. A neutral to slightly alkaline pH along with a temperature around 35°C facilitates the highest yield. Taking into account the current findings, there is no doubt that *A. vinelandii* has a remarkable potency for the production of PHB. But in order to enhance the productivity and polymeric properties of the PHB, it is necessary to screen different mutant strains of *A. vinelandii* and to develop recombinant strains and evaluate their performance at laboratory bioreactor level and at greater scale bioprocess.

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