

Original Research Article

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Effect of Vitamin on Accumulation of PHB by *Zobellella* Species under Submerged Fermentation Process

S. Maity¹, S. Das² and D.P. Samantaray^{1*}¹Department of Microbiology, CBSH, OUAT, Bhubaneswar-3, Odisha-751003, India²Department of Life Science, LEnME, NIT, Rourkela, Odisha - 769008, India

*Corresponding author

ABSTRACT

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In the current context of eco-pollution, the substitute of conventional plastics with bioplastics is a big confront. On account of that, a non-growth associated PHB production study was conducted using *Zobellella* species under submerged fermentation process. As a result, *Zobellella* species showed the ability to accumulate 2.2g/L of PHB at pH 8.0, temperature 37°C, salt concentration 2% in modified growth medium containing vitamins. Then, primary structural characterization of extracted polymer was conducted by FTIR analysis. The high intense peak was obtained at 1720.50 cm⁻¹ corresponding to ester carbonyl (C=O) stretching vibration of PHB. This finding will be paved the way of researchers during selection of suitable vitamins containing inexpensive carbon sources for cost effective PHB production. Thus, extensive research is highly indispensable for bioconversion of vitamin containing raw materials to PHB.

Introduction

In response to risk associated with petrochemical based plastic waste and its effect on the environment, there has been considerable interest on the development and production of an alternative, biodegradable plastic. In the current context of eco-pollution, the substitute of conventional plastics with bioplastics is a big confront. Moreover, their development became indispensable due to the depletion of fossil fuel reserves, high oil prices and increased greenhouse gas emissions. Reports also suggested that burning of bioplastics producing methane, which can be further used as source of energy (Versa and Savita, 2011). Polyhydroxyalkanoates (PHAs) are group of most common biopolymers or bioplastics

found in Gram positive (Kalaivani and Sukumaran 2015; Mohapatra *et al.*, 2016 and Chaudhry *et al.*, 2010) and Gram negative bacteria (Ibrahim and Steinbuchel, 2009; Tortajada *et al.*, 2013 and Poli *et al.*, 2011). PHAs include poly- (*R*)-3-hydroxybutyrate (PHB), a copolymer (PHBV) of (*R*)-3-hydroxybutyrate (3HB) and (*R*)-3-hydroxyvalerate (3HV) and a copolymer (PHBHHx) of 3HB and (*R*)-3-hydroxyhexanoate (3HHx) are produced by various microbes (Xiao and Jiao, 2011; Mohapatra *et al.*, 2014).

These PHAs has a wide range of applications such as plastics materials, medical implants, drug delivery carriers, printing and

photographic materials, nutritional supplements, drugs and fine chemicals (Chen, 2009).

Wide-spread substitution of conventional plastics has been limited due to their high production costs, which hold back its successful commercialization (Waltz, 2008). Thus, additional efforts are needed for making this process economically feasible by analysing inherent mechanism of PHAs accumulation process and improving its productivity. Therefore, exploitation of improved cultivation medium, culture conditions, inexpensive carbon sources and genetically modified microbes can be reduced cost of PHB production (Castilho *et al.*, 2009; Khanna and Srivastava, 2005). Thus, the objective of study is to determine the effect of different vitamins on PHB production using high yielding bacterial isolates. This will be paved the way of lead researchers regarding bioconversion of vitamin containing inexpensive carbon sources for cost effective PHB production.

Materials and Methods

Culture collection

In our preceding work, the Gram-negative *Zobellella* strain was found to produce 2.2 g/L PHB from 3.0 g biomass (CDW) in modified growth medium under optimized growth condition. The preserved (-20°C) bacterial isolate was revived and induced synthesis of PHB granule in their cytosol using modified growth medium (Mohapatra *et al.*, 2015). Then, occurrence of PHB granule in the cytosol was detected by Sudan black (Mohapatra *et al.*, 2015) and Nile red staining (Spiekerman *et al.*, 1999). All the chemicals and culture media used in the study are procured from Merck Millipore and Hi-media Laboratories Private Limited.

PHB production under submerged fermentation

One-stage batch cultivation in shake flasks method was conducted for accumulation of PHB granule in the bacterial cell. The *Zobellella* strain was subjected to PHB production under optimized growth conditions. Briefly, 1L of PHB production medium containing Glutamic acid 3.8 g/L; Malic acid 2.7 g/L; Na₂HPO₄ 1.5 g/L; KH₂PO₄ 2.5 g/L; K₂HPO₄ 2.5g/L; NaCl 20g/L; CaCl₂, 2H₂O 0.2 g/L; MgSO₄ 0.8g/L; MnSO₄ 0.001 g/L; 7H₂O 0.8 g/L; (NH₄)₂SO₄ 3.08 g/L; Glucose 20 g/L was prepared with pH 8.0 and then supplemented with filter (0.45µm) sterilized vitamins solution (Biotin, Thiamine, Niacin, Riboflavin, Folic acid and Patnothenic acid; each 1mg/L) individually and in different combination. Subsequently, submerged fermentation process was carried out with 15% of inoculum (overnight culture containing 1.5x10⁸ cells/ml) at temperature 37°C for 72 h with an agitation rate of 150 rpm.

Extraction and quantitative analysis of PHB

PHB extraction was carried out by sodium hypochlorite digestion and multi-solvent extraction method (Mohapatra *et al.*, 2015). Then, bacterial cells were harvested by centrifugation at 10,000g for 15 min, dried at 50°C to obtain dry cell mass (DCM) and treated with sodium hypochlorite to digest the non PHAs materials (NPM). The mixture was then centrifuged and subsequently washed twice with acetone, methanol and diethyl ether (1:1:1) to remove sodium hypochlorite and debris. Finally, the PHB were dissolved in boiling chloroform to remove all the remaining organic solvents present with PHB pellets and subsequent evaporation by air drying to yield dried form of extracted PHB.

PHB production was also quantified using standard formula.

Characterization of PHB by FTIR Analysis

The functional groups present in the PHB were analyzed by FTIR spectroscopy. PHB sample was mixed with 2% KBr and compressed into translucent sample discs and fixed in the FTIR spectrometer (Perkin-Elmer RX I). Scans were accomplished at spectral range, 4000-400 cm^{-1} ; window material, CsI; 16 scans and resolution 4 cm^{-1} . The detection was conducted with a temperature stabilized, coated FRDTGS detector (Dash *et al.*, 2014).

Results and Discussion

Although wide arrays of bacterial species accumulate PHAs, however the major limitations in the production of PHAs are selection of inexpensive materials and its composition, producer microbes, bioprocess condition, fermentation process and high cost downstream processing. Among all selection of inexpensive material and its composition is the predominant factor affecting PHAs production bioprocess technology. As regards 50% of the production costs of PHAs is depend upon cost of carbon sources and vitamins supplemented in the production medium (Gomma, 2014). In this small piece of research an attempt has been made to study the effect of different vitamin such as biotin, thiamin, niacin, riboflavin, folic acid and pantothenic acid on PHAs accumulation by bacterial isolate.

PHAs production

From the result (Figure 1), it was found that biomass production by the bacterial isolate ranged between 1.20-4.20 g/L in presence of different vitamins. The biomass production was increased with supplementation of thiamin and decreased with folic acid. Thus,

thiamine has higher effect on cellular growth than other vitamin supplemented in the medium. Consequently, PHAs production was found to be 0.062-1.835 g/L and was higher in presence of thiamin and lower with folic acid. The PHAs production was 1.835 g/L and 1.790 g/L in presence of thiamin and biotin respectively. Notably, no significant difference of PHAs production was observed between thiamin and biotin as source of vitamin. However, percentage of PHAs production was 65.42% and 42.93% on supplementation of biotin and thiamin respectively. Hence biotin has a major role on PHAs yield, which is not parallel to biomass production in case of *Zobellella* species. Similarly, different combinatorial effect of vitamin on PHAs production results (Figure 2) indicated that, the biomass and PHAs yield was varied between 2.601-3.847 g/L and 1.401-2.085 g/L respectively. The biomass and PHAs production was increased with supplementation of biotin and thiamin combination and decreased with riboflavin and folic acid. However, higher percentage (68.14%) of PHAs production was obtained on supplementation of multi-vitamin such as biotin and riboflavin in the production medium. From the above data, it is concluded that biotin has a noteworthy role on PHAs production nevertheless after supplementation of biotin, thiamine, niacin, riboflavin, folic acid and pantothenic acid production was increased to 2.2 g/L by *Zobellella* species.

No comprehensible evident is available in the public domain regarding increase in PHAs production by *Zobellella* species on supplementation of vitamin. In addition, vitamins including thiamin, riboflavin and biotin are essential growth factors for different bacterial isolates (Du *et al.*, 2011; Preciado *et al.*, 2015). Thus, vitamin and amino acids can be supplemented for efficient PHAs production (Varsha and Savitha, 2011). In contrast to our observation, biotin is also

able to switch the carbon flux of acetyl-CoA towards biosynthesis of PHB by *Corynebacterium glutamicum* thereby increases production (Jin *et al.*, 2009). However, vitamin was no effect on PHB production by *Pseudomonas pseudomallei* and *Rhodobacter sphaeroides* 14F

(Chowdhury *et al.*, 2005; Sangkharak and Prasertsan 2008). In our observation the PHAs production was increased with supplementation of vitamins such as biotin, thiamin and riboflavin, which suggesting selection of vitamin containing raw material for cost effective production.

Fig.1 Effect of vitamins on cell biomass and PHAs production

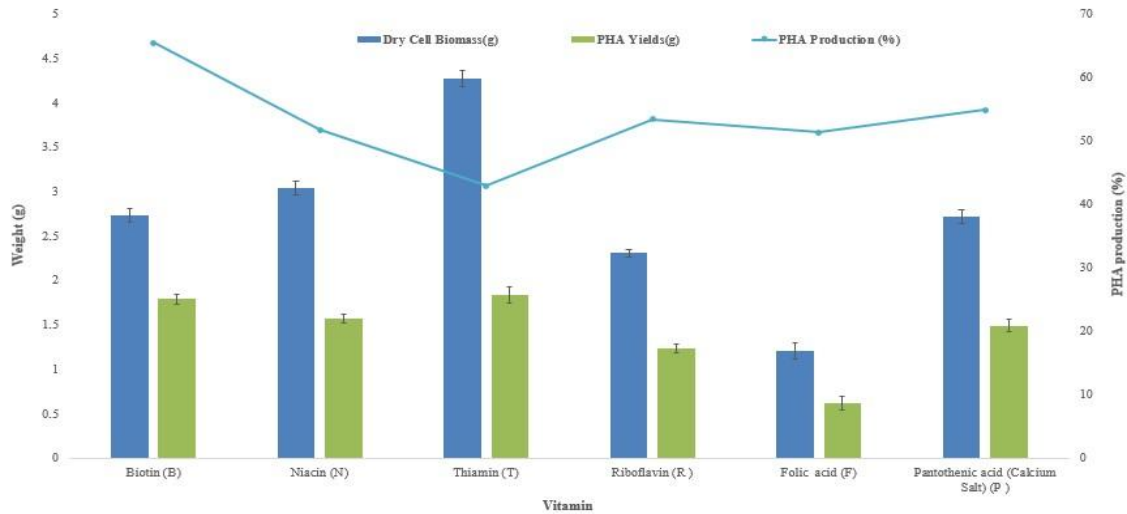


Fig.2 Effect of vitamins (combination) on cell biomass and PHAs production

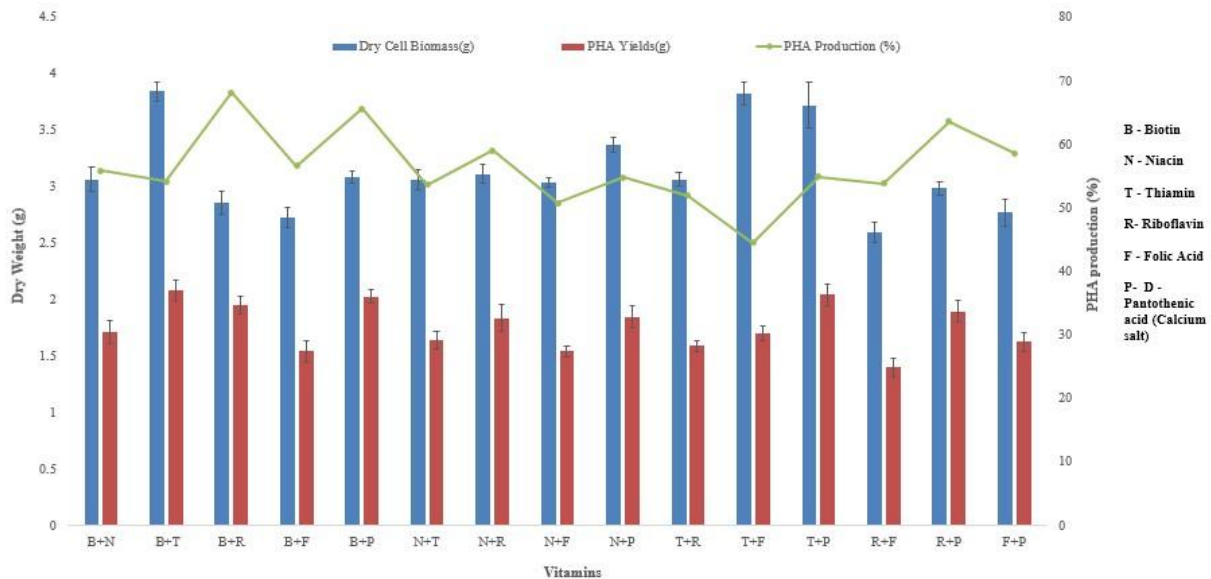
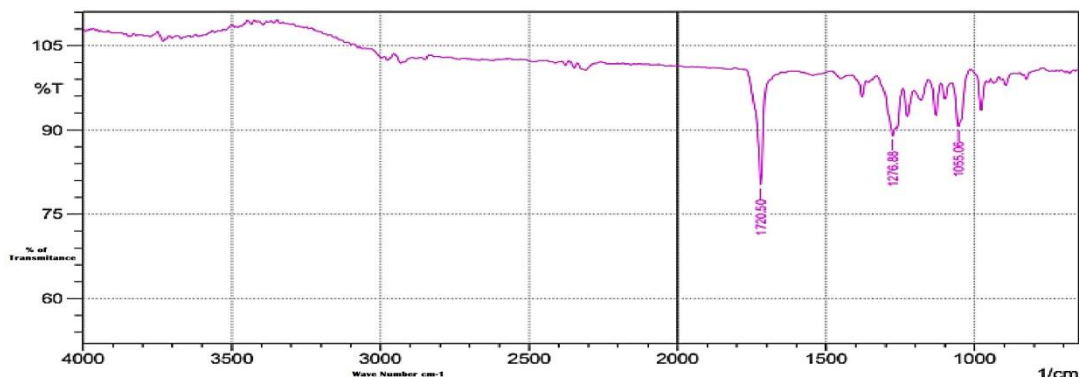


Fig.3 FTIR analysis of PHAs extracted from *Zobellella* sp.



FTIR spectral analysis of extracted PHAs

The FTIR data indicated (Figure 3) different characteristic peak at 1055.06 cm^{-1} and 1276.88 cm^{-1} corresponding to C-N stretch and C-C, C-H, O-H stretch respectively. On the other hand, the high intense peak was obtained at 1720.50 cm^{-1} conferred to ester carbonyl (C=O) stretching vibration of PHB. Thus, IR spectrum depicts correct insight for the chemical configuration of PHB, which is the most common homopolymer of PHAs. This result is also similar to the IR spectrum obtained at 1720.50 cm^{-1} corresponding to (C=O) ester carbonyl group, characteristics of PHB (Shah, 2012; Sharma and Bajaj, 2015).

The present study was designed to determine the effect of different vitamins on PHB production using high yielding bacterial isolates. As a result, *Zobellella* species showed the ability to accumulate 2.2g/L of PHB in growth medium with vitamins which can increase the cost of production. However, this finding will be paved the way of researchers during selection of suitable inexpensive carbon sources for PHB production. Raw materials (cane molasses, beet molasses, banana peel and fish waste) containing different vitamin such as biotin, folic acid, pantothenic acid, riboflavin,

thiamine and niacin may be used for cost effective PHB production. Thus, extensive research is highly indispensable for bioconversion of vitamin containing raw materials for PHB production.

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