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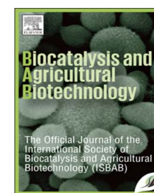

# Developing a green and sustainable process for enhanced PHB production by *Azohydromonas australica*

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A B S T R A C T

Reports suggest that *Azohydromonas australica* DSM 1124 is an eminent Poly 3-Hydroxy Butyrate (PHB) producer capable of utilizing different carbon sources with insufficient lactose assimilation. The main idea behind the present study was the adaptation of *A. australica* to a defined optimal lactose concentration through lactose enrichment technique and thereby enhancement in PHB production. Herein; statistical medium optimization was done using synthetic lactose as carbon substrate and microorganism was adapted on lactose containing medium (up to 2% w/v). Results clearly showed that a maximum PHB of 2.07 ± 0.2 g $L^{-1}$ was obtained from 4.71 ± 0.3 g $L^{-1}$ of dried cell biomass with 8.5 fold increase in PHB production by an adapted strain as against wild type strain after optimization in 72 h. The process could be scaled up in 7 L bioreactor with nearly same amount of PHB produced. The utilization of lactose by *A. australica* would pave the way to use cheap carbon substrate such as cheese whey in order to reduce the overall cost and to make the process cost-effective.

## 1. Introduction

Globally plastic materials became an indispensable part of life because of many desirable properties and extensively used plastics do not naturally degrade to a large amount when released into the environment (Webb et al., 2013). Annual plastic production has increased dramatically from 1.5 million tonnes in the 1950s to approximately 280 million tonnes in 2011 (Wright et al., 2013). Problems associated with global environment and solid waste management have generated interest in the development of biodegradable plastic, which must still retain the desired physical and chemical properties of conventional synthetic plastic. Poly- β -hydroxyalkanoates (PHAs) have been considered one of the promising biodegradable plastics, which accumulates in various gram positive and gram negative bacteria under the condition of limiting nutritional elements such as N, P, S, O or Mg and in the presence of excess carbon source (Khanna and Srivastava, 2005). Of all the PHAs, poly 3-hydroxybutyrate (PHB) has attracted considerable interest for biodegradable and biocompatible plastics. PHB is most commonly studied in microorganisms belonging to the genera *Alcaligenes*, *Azotobacter*, *Bacillus*, and *Pseudomonas* (Shivakumar, 2011).

PHB obtained from renewable resources have attracted an increasing amount of attention over the last two decades, predominantly due to environmental concerns and having finite petroleum resources. In this respect, several inexpensive carbon substrates such as molasses, whey, cellulose, plant oils and hydrolysates of starch (corn and tapioca) have proven to be an excellent substrate for the bacteria utilizing them so as to produce PHA (Yu et al., 2006).

Cheese whey, a by-product of dairy industry, is an attractive raw material for PHA production. However, the inability of most PHA producing bacteria to utilize lactose has restricted the use of whey as a potential raw material. Very few reports are available for PHB production from lactose and whey (Koller et al., 2008; Povolo et al., 2010; Pais et al., 2014). Till date, there are no adequate studies reported in *A. australica* using lactose as carbon substrate. With this in view, the current study was carried out in order to produce PHB by *A. australica* using lactose as carbon substrate and to reduce the overall cost of substrate so as to economize the process. Researchers had reported PHB production in presence of lactose and whey by various microorganisms. Young et al. (1994) noted 2.75g$L^{-1}$ of PHB with %PHB content of 56.12%w/w using lactose by *Pseudomonas cepacia* ATCC 17759. Yellore and Desai (1998) noted PHB production of 2.6g$L^{-1}$ and 0.4g$L^{-1}$ while working with *Methylobacterium* sp. ZP24 using whey supernatant and whole whey as carbon source supplemented with nitrogen source. On the other hand, whole whey without the supplementation of nitrogen source resulted in an enhanced PHB yield of 1.1g$L^{-1}$. Povolo and Casella (2003) reported PHB produced alongwith a %PHB content from whey permeate by *Sinorhizobium meliloti* 41 (0.017 g/l; 3.51%w/w) and *Hydrogenophaga pseudoflava* DSM1034 (0.017 g/l; 4.53%w/w). The thermophilic bacterium, *Thermus thermophilus* HB8 (DSM 579) was

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found to be able to utilize lactose from whey-based media, using both glucose and galactose, for the biosynthesis of PHAs under nitrogen limiting conditions with 0.57 $gL^{-1}$ of PHB produced and a PHB content of 35.6% w/w (Pantazaki et al., 2009). *Azohydromonas lata* DSM 1123 was reported to produce poly-3(HB-*co*-12.7% − 3HV) from whey hydrolysate with a yield of 1.66$gL^{-1}$and %PHB content of 18.04%w/w (Baei et al., 2010). Recombinant *Cupriavidus necator* mRePT accumulates 2.4$gL^{-1}$ PHB with 30%w/w of PHB content using hydrolyzed whey permeate under shake flasks (Povolo et al., 2010).

In the present study, PHB production was examined on lactose as carbon source in the basal medium (Wang and Lee, 1997), so as to increase the PHB content per cell dry mass. Here, the bacterium, *A. australica* was adapted through lactose enrichment technique in the selected optimal medium. The adapted strain of *A. australica* developed was examined for the selection of an optimal nitrogen source. Furthermore; the selected medium recipe was statistically optimized by Plackett Burman and Response Surface Methodology. The growth kinetics was also studied using optimized media recipe in bioreactor (7 L) under controlled conditions in order to investigate persistent yield of PHB production and growth by *A. australica*.

## 2. Materials and methods

### 2.1. Microorganism

The culture *Azohydromonas australica* DSM 1124 was procured from German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). The culture was maintained on nutrient agar plates at 5 °C and sub cultured monthly.

### 2.2. Medium

#### 2.2.1. Seed culture and initial shake flask study

A loopfull of bacterial culture from nutrient agar plates were inoculated in the 50 ml of nutrient broth containing 1% lactose in 250 ml Erlenmeyer flasks. The bacterial culture was incubated for 48 h at 33 °C, 200 rpm (Gahlawat and Srivastava, 2012). The culture was initially characterized using Gram staining technique and biochemical tests carried out using Hi Assorted Biochemical kit (Hi media).

The mineral salt medium consisted of: $KH_2PO_4$ 1.5 g $L^{-1}$; $Na_2HPO_4 \cdot 12H_2O$, 9.0 g $L^{-1}$; $MgSO_4 \cdot 7H_2O$, 0.2 g $L^{-1}$; $CaCl_2 \cdot 2H_2O$, 0.01 g $L^{-1}$; $(NH_4)_2SO_4$, 2.0 g $L^{-1}$; citric acid,0.1 g $L^{-1}$, supplemented with 1 ml of trace elements solution ($FeSO_4 \cdot 7H_2O$, 20 g $L^{-1}$; $MnCl_2 \cdot 4H_2O$, 0.03 g $L^{-1}$; $H_3BO_4$, 0.3 g $L^{-1}$; $CuSO_4 \cdot 5H_2O$, 0.01 g $L^{-1}$; $CoCl_2 \cdot 6H_2O$, 0.2 g $L^{-1}$ $(NH_4)_6Mo_7O_{24} \cdot H_2O$, 0.03 g $L^{-1}$; $ZnSO_4 \cdot 7H_2O$, 0.03 g $L^{-1}$; $NiSO_4 \cdot 7H_2O$,0.03 g $L^{-1}$). Lactose (20 g $L^{-1}$) was supplemented as a carbon source. Chemically synthesized D-Lactose Monohydrate (Himedia Labs, Mumbai, India) was obtained from Bio Activator, Delhi, India. The pH of medium was adjusted to 7.0 with 2N NaOH/ 2N HCl. Medium was sterilized at 121 °C on 15 psi pressure.

### 2.3. Study of growth kinetics of the culture

For the study of growth kinetics, nutrient broth containing 1% lactose was used to raise seed culture. The 4% inoculum was transferred into the mineral salt medium in a 250 ml Erlenmeyer flask containing 50 ml of the medium described above. The organism was initially cultivated at 200 rpm, 33 °C for 96 h. Initial pH of medium was adjusted to 7.0. For PHB production, 100 ml of media containing 20 g $L^{-1}$ lactose was taken in a 500 ml flask and inoculated with 4 ml of inoculum. The flasks were kept under similar conditions mentioned as above. Samples were withdrawn at regular intervals up to 96 h and were analyzed for biomass, PHB and residual nutrients.

### 2.4. Lactose enrichment technique

To enhance PHB production and cell growth, microbial adaptation was carried out. Herein, the culture was grown in nutrient broth (NB) having 1% lactose for 48 h at 33 °C, 200 rpm. The grown culture (from 1% lactose conc.) was then subsequently transferred to their respective fresh production medium with mineral salts containing 1% lactose concentration. The bacterial culture was maintained at defined lactose concentration in the respective optimized medium for a desired period under defined culture conditions and was repeated at the defined lactose concentration upto two-three successive cycles followed by successive transfer to a higher lactose concentration. This technique was carried out upto the concentration of 2.5% w/v. The strain developed after lactose enrichment technique was evaluated for cell growth and PHB production.

### 2.5. Effect of nitrogen source

Different nitrogen (organic and inorganic) sources were evaluated for cell growth and PHB production in the aforesaid medium with the same percent equivalent of nitrogen. The different nitrogen sources evaluated were: Corn steep liquor (CSL), urea, yeast extract, ammonium sulphate [$(NH_4)_2SO_4$], and ammonium chloride. A negative control was also set up which was devoid of any nitrogen source. After incubation at 33 °C, 200 rpm for 72 h; biomass and PHB were estimated in the fermentation broth.

### 2.6. Lactose stability

Initially, lactose was added separately through filter sterilization and through the chromatographic profile (HPLC), 1 ml of the basal medium (sterilized at 121 °C) containing filter sterilized lactose was loaded/injected into HPLC to determine the change in lactose concentration. Similarly, the second set of experiment was carried out wherein, lactose was added in the medium and then autoclaved. The chromatographic profiling of autoclaved lactose containing medium was carried out. The lactose stability has been determined through the amount of lactose present after medium sterilization by chromatographic analysis was calculated through the plot prepared between the pure lactose standard (Sigma grade) of defined concentration versus lactose area (obtained through HPLC).

### 2.7. Analytical methods

Cell growth was monitored by measuring the absorbance of the fermentation broth at 600 nm on a Specoll 1200 (Analytik Jena, Germany) after suitable dilution with distilled water. Cell mass concentration was determined by standard plot between $OD_{600}$ nm and dry cell mass. The dry cell mass was calculated through the formula, wherein, difference between the final weight of eppendorf [weight of dried cell mass + weight of dried eppendorf (2 ml)] and the initial weight of dried eppendorf (2 ml) multiplied by 1000 and divided by the sample volume (ml) inorder to get the yield of dried cell mass. Herein, the fermentation/culture broth of requisite time period was centrifuged and further dried at 60 °C till constant weight was achieved. The dried cell pellet was weighed on weighing balance upto three readings and the average of three is considered as the final cell weight and is expressed in $mgL^{-1}$. The supernatant obtained by centrifugation of the culture broth at 10,000 rpm for 10 min at 4 °C was used for residual lactose analysis. Residual lactose was estimated by a dinitrosalicylic acid (DNS) method (Miller, 1959). The content of residual ammonia nitrogen was determined according to a Kjeldahl method (Horwitz, 1980). Residual phosphate in the supernatant was determined by the Murphy and Riley method (Murphy and Riley, 1962). PHB content in the dried cells was determined by gas chromatography (GC 2010 Shimadzu Co., Japan) with benzoic acid as an internal standard

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