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Production of poly-3-hydroxybutyrate (P3HB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) P(3HB-co-3HV) from synthetic wastewater using *Hydrogenophaga palleronii*

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HIGHLIGHTS

- P3HB and P(3HB-co-3HV) was produced from synthetic waste using *H. palleronii.*
- *H. palleronii* showed growth up to 60 g/l volatile fatty acids concentration.
- Highest P(3HB-co-3HV) production was observed at 20 and 30 g/l VFA concentrations.
- P3HB and P(3HB-co-3HV) was characterized by NMR, GPC, TGA, and DSC analysis.
- Wastewater treatment along with biopolymer production reduces production cost.

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ABSTRACT

In the present study, synthetic wastewater (SW) was used for production of poly-3-hydroxybutyrate (P3HB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) P(3HB-co-3HV) using the bacteria *Hydrogenophaga palleronii*. SW at various volatile fatty acids concentrations (5–60 g/l) was evaluated for the growth and biopolymer production using *H. palleronii*. Substrate degradation was analyzed using total organic carbon (TOC) analyzer and high pressure liquid chromatography (HPLC). *H. palleronii* showed highest and lowest removal of TOC at 5 g/l (88 ± 4%) and 60 g/l (15 ± 6%) respectively. Among all the concentrations evaluated, bacteria showed highest biopolymer production with 20 g/l (63 ± 5%), followed by 30 g/l (58 ± 3%) and 40 g/l (56 ± 2%). Lowest biopolymer production was observed at 5 g/l concentration (21 ± 3%). Structure, molecular weight, and thermal properties of the produced biopolymer were analyzed. These results denoted that the strain *H. palleronii* can be used for degradation of high concentration of volatile fatty acids persistent in wastewaters and their subsequent conversion into useable biopolymers.

1. Introduction

The exploitation of plastics by the public has been increasing in the last decades. Though their existence has indisputably improved our daily lives, most of the plastics are produced by the use of petroleum derived compounds (Srikanth et al., 2012; Venkata Mohan et al., 2010). Also, plastics incline to persist in the environment due to their low biodegradable nature (Fradinho et al., 2014; Khanna and Srivastava, 2005). To overcome this, approachability has ascended in relation to the replacement of synthetic plastics by biodegradable bioplastics. Polyhydroxyalkanoates (PHAs) are naturally synthesized by many bacteria and intracellularly accumulated as granules, presents characteristics similar to synthetic plastics making it a promising material for biodegradable plastics production (Venkateswar Reddy et al., 2015; Fradinho et al., 2014; Laycock et al., 2013). Poly-3-hydroxybutyrate (P3HB) is one type of PHA, and polyester of 3-hydrobutyric acid that is accumulated by various bacteria (Kim et al., 2012).

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The perspective areas of PHA applications include the use of PHA as a filler for non-biodegradable plastics, disposable packages, agriculture systems for prolonged release of fertilizers and agrochemicals (Sudesh et al., 2000). Currently PHA based biopolymers are commercially available, industrially produced using pure culture under aseptic conditions and supplied with defined mediums (Fradinho et al., 2014). However, costs associated to these operational conditions increase PHA prices, economically limiting PHA application as a substitute for synthetic plastics (Fradinho et al., 2014; Reis et al., 2011). To compete with synthetic plastics, the production costs of PHA have to be reduced. The cost of media contributes most significantly to the overall production cost of PHA. Inexpensive substrates such as starch, glycerol from biodiesel production, molasses, plant oils and other cheap fatty acids draw the consideration of various researches (Ntaikou et al., 2009; Koller et al., 2005).

Objective of the present study is to convert volatile fatty acids (VFA) present in synthetic wastewater (SW) into useful biopolymers using *Hydrogenophaga palleronii*. In this study, examined the capacity of *H. palleronii* to produce PHA at various VFA concentrations. Effect of VFA concentration on bacterial growth and substrate degradation was also evaluated. Further, influence of VFA composition on structure and properties of PHA was evaluated. Considering the importance of utilizing waste streams as feedstock for PHA production, the VFA tested in this work are common products of waste and effluent fermentation (acetate, propionate, butyrate, lactate).

2. Methods

2.1. Bacteria

H. palleronii (also called as *Pseudomonas palleronii*, NBRC-102513) was collected from the Biological Resource Center, National Institute of Technology and Evaluation (NBRC), Japan. *H. palleronii* is a bacterium from the family of Comamonadaceae, has the ability to degrade different types of organic pollutants. Many authors reported about efficiency of this bacterium for degradation of various toxic compounds, but there are no reports about degradation of VFA present in wastewater using this bacterium.

2.2. Culture media

For the growth of *H. palleronii* nutrient broth, and SW were used as the media. Composition of the SW was slightly different from the originally proposed by Kourmentza et al. (2015). The SW contained 1.0 g $(NH_4)_2SO_4$, 1.0 g K_2HPO_4, 0.2 g NaH_2PO_4, 0.2 g $MgSO_4$. $7H_2O$, 0.05 g NaCl, 0.05 g $CaCl_2$, 8.3 mg $FeCl_3$. $6H_2O$, 1.4 mg $MnCl_2.4H_2O$, 1.17 mg Na_2MoO_4. $2H_2O$, and 1 mg $ZnCl_2$ per one liter of deionized water. One ml of a trace elements solution was added to one liter of SW. The trace elements solution contains 0.786 g $CuSO_4$. $5H_2O$, 5.0 g $FeSO_4$. $7H_2O$, 12.609 g $NaMoO_4$. $2H_2O$, 4.05 g $NiCl_2$. $6H_2O$, 4.398 g $ZnSO_4$. $7H_2O$, 2.453 g $CoCl_2$. $6H_2O$, 0.75 g KI, 3.0 g H_3BO_3, 5.0 g $MnCl_2$. $4H_2O$ and 5.0 g EDTA in one liter of distilled water (Kourmentza et al., 2015). The carbon source composition of SW contains acetate and propionate, among the two carbon sources acetate was used as the main carbon source (Table 1). Different carbon concentrations (5, 10, 15, 20, 30, 40, 50 and 60 g/l) were used in SW in order to identify the optimum concentration for bacterial growth (Table 1). Real wastewaters sometimes contains the higher concentration of VFA and will inhibit the bacterial growth. Hence in the present study we used higher concentrations (up to 60 g/l) of VFA to know the survival capacity of these bacteria. The pH of the medium was adjusted to 7 and autoclaved before adding to the flasks.

2.3. Growth curve analysis

A loop of *H. palleronii* strain was initially inoculated into 50 ml of nutrient broth in 500 ml flasks, and kept in shaking incubator under dark condition at 30 °C for overnight at 180 rpm. For growth experiments 4 ml (4% v/v) of the overnight grown culture was inoculated into different shake flasks containing 100 ml of SW with different carbon concentrations *i.e.*, 5–60 g/l. The experiments were conducted for 192 h. Samples were collected at different time intervals, and growth was monitored spectrometrically by measuring the absorbance at 600 nm using UV-spectrometer (UV-1800, Shimadzu, Japan).

2.4. PHA production

Cultures grown with SW at different carbon concentrations were collected at 120 h and centrifuged. The resulting pellet was suspended in SW. The composition of SW was same as mentioned in Section 2.2, but low nitrogen and phosphorous concentrations (0.1 g/l) were used in order to create stress for accumulation of PHA granules. Also experiments were conducted by using only acetate (at 20 g/l) as carbon source in SW in order to know the difference in biopolymer composition. All the conditions were maintained as like in growth phase. Culture was collected and the PHA was extracted and analyzed as described in Section 2.5.3.

2.5. Analysis

2.5.1. Total organic carbon (TOC) measurement

Total dissolved organic carbon from clarified samples at 0 h and 120 h was analyzed in a Shimadzu TOC automatic analyzer to know the removal of carbon concentration in SW. Sodium acetate and propionate standards (10–500 mg/l) are used to produce the TC and IC calibration curves.

2.5.2. Analysis of VFA utilization by bacteria

The concentrations of acetate and propionate at different time intervals were analyzed on HPLC (Shimadzu) with an RI detector and Shim-pack SCR-102 (H) column (Shimadzu, Kyoto, Japan). Samples collected for HPLC analysis were acidified with phosphoric acid (10%, wv^{-1}) to stop the biological reaction and centrifuged at 8000*g* for 10 min. The resulting supernatant was filtered and analyzed directly by HPLC. Filtered and degassed 5 mmol/l perchloric acid was used as mobile phase at a flow rate of 1.0 ml/min. The column was maintained at a temperature of 40 °C in a thermostat chamber. Acetate and propionate concentrations were calculated from the area of the curve obtained for 1 mM of the standards. All results were presented as average and standard deviation of the data from three independent experiments.

2.5.3. Extraction and estimation of PHA

Extraction and estimation of PHA was performed following the procedure reported with slight modification (Law and Slepecky, 1960; Venkata Mohan and Venkateswar Reddy, 2013). The biomass pellet was separated from the substrate by centrifugation (6000*g* for 10 min at 10 °C) and the resulting pellet was washed with acetone and ethanol separately to remove unwanted materials. The pellet was suspended in 4% sodium hypochlorite and incubated at room temperature for 3 h. The resulting mixture was centrifuged (6000*g* for 10 min at 10 °C) and the supernatant was discarded. The pellet with lysed cells after washing simultaneously with acetone and ethanol was dissolved in hot chloroform and was passed through filter paper (Whatman, cat no-1440–070, 7 cm Diameter, 8 Micron pore size) to separate the polymer from cell debris. PHA were separated from the chloroform by filtration, and concentrated using Rotary evaporator (Eyela N-1000) followed by drying

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