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Optimization and fed-batch production of PHB utilizing dairy waste and sea water as nutrient sources by *Bacillus megaterium* SRKP-3

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ABSTRACT

A gram positive bacterium (designated strain SRKP-3) that potentially accumulated polyhydroxyalkanoates (PHAs) was isolated from brackish water. From its morphological and physiological properties and nucleotide sequence of its 16S rRNA, it was suggested that strain SRKP-3 was similar to *Bacillus megaterium*. A four-factor central composite rotary design (CCRD) was employed to optimize the medium and to find out the interactive effects of four variables, viz. concentrations of dairy waste, rice bran, sea water and pH on PHB production. Using response surface methodology (RSM), a second-order polynomial equation was obtained by multiple regression analysis and a yield of 6.37 g/L of PHB dry weight was achieved from the optimized medium at pH 9. The same medium was utilized for fermentor studies by fed-batch culture. The dairy waste is fed at three different time intervals at 0th, 12th and 24th hour to keep the carbon source as excess and PHB production was checked for every 3 h. Maximum production of PHB (11.32 g/L) occurred at 36th hour. Dissolved oxygen was found to be major limiting nutrient that affected the PHB synthesis.

1. Introduction

Polyhydroxyalkanoates (PHAs) and their derivatives are the most widely synthesized microbial bioplastics (Kim and Lenz, 2001; Witholt and Kessler, 2002). These bioplastics were produced by a large number of bacteria in response to unfavorable growth conditions (deprivation of any of limiting nutrient) and also the carbon source should be in excess (Anderson and Dawes, 1990; Khanna and Srivastava, 2005). Until now, approximately 150 constituents of PHAs have been identified (Joa~o et al., 2009). Among various constituents, poly (3-hydroxybutyric acid) (PHB), an intracellular microbial thermoplastic, is the principal polyhydroxyalkanoate (PHA), is widely produced by many bacteria including *Bacillus megaterium* (Kulpreecha et al., 2009). Nevertheless, the most attractive feature of PHAs is their biodegradability to CO_2 and H_2O. Although PHAs have been recognized as a good candidate to replace conventional petrochemical plastics its high cost of production is the main factor which has restricted the broader application of PHAs as a commodity plastic. Improvement in PHAs production strategies i.e. by feeding appropriate carbon and nitrogen sources at suitable concentrations (Kulpreecha et al., 2009) can lead to the reduction in the cost of the final product implying wider use of PHAs in daily life (Li et al., 2007). According to Choi and Sang (1997), 40–48% of the total production costs are ascribed to the raw materials where the carbon source could account for 70–80% of the total expense. A production process based on waste carbon sources is the requirement of the day, instead of noble ones (Wolf et al., 2005).

Dairy industry waste is one among the chief pollutant which has high BOD and COD content (Orhon et al., 1993). The main end-product of dairy industry, cheese whey generated from the pressing of cheese, is mainly regarded as a pollutant due to its high biological oxygen demand and its disposal is being managed at considerable cost. Also separation of whey from dairy waste is a costlier process and often out of the range of economical feasibility for small and medium sized plants. Crude dairy waste is now rapidly becoming a 'waste product' with an associated disposal cost. In recent years, there has been an increasing trend towards more efficient utilization of agro-industrial residues (Pandey et al., 2000), for the production of various fermentation related products. Rice bran, one among the agricultural by-products, was previously used in the production of PHB (Gao et al., 2008), which can reduce the total cost associated with the production of PHB.

Process optimization of fermentations has long been used to enhance the yield and productivities of many bioprocesses (Khanna and Srivastava, 2005) and it has proved to be successful in substantially improving the product yield and productivity of many

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bioprocesses. Conventional one factor at a time method was followed previously which is painstaking, time consuming with the key disadvantage of lack of interaction studies. Response Surface Methodology (RSM) provides a systematic and efficient research strategy for studying the interaction effects of various parameters using statistical methods (Deepak et al., 2008). It has been extensively applied in microbial fields in the recent years.

The complexity in PHB (PHAs) production requires a non-linear feed supply, which can be provided by fed-batch cultivation (Patnaik, 2008). Previously, many reports are available for the synthesis of PHB by fed-batch cultivation. Kulpreecha et al. (2009), has reported the synthesis of PHB using molasses, urea and minerals. Therefore, in the present study, we describe the isolation and identification of gram positive bacterium having higher PHB productivity. The purified PHB was characterized by FT-IR. Thereafter, effect of carbon, mineral and nitrogen sources were examined to analyze their effect on PHB production. By taking the best concentration of substrates and conditions, statistical media optimization was carried out by Response Surface Methodology. To our knowledge this is the first report elucidating the use of combination of dairy waste, rice bran and sea water as sources in the production of PHB. Also, the optimized medium was studied in fermentor by fed-batch culture.

2. Methods

2.1. Isolation of PHAs producing microorganism

The brackish water samples were collected from Gulf of Mannar, Tuticorin (It lies between 8° 47′ to 9° 15′ N latitude and 78° 12′ to 79° 14′ E longitude) in sterile falcon tubes and transferred to lab under aseptic conditions in ice. PHAs producing strains in brackish water were isolated using Nile red dye staining method (Spiekermann et al., 1999). Maximum producer of PHAs was screened based upon the intensity of fluorescence under UV-light and PHAs production.

2.2. Characterization of the isolated bacteria

The morphological and physiological properties of the isolate (SRKP-3) were investigated according to Bergy's manual of determinative bacteriology (Holt et al., 1994).

2.3. 16S rRNA gene sequence analysis

The genomic DNA was extracted from 1 ml of isolated culture using the AccuPrep genomic DNA Extraction kit (Bioneer, Korea). From the genomic DNA, nearly full-length 16S rRNA sequences were amplified by PCR using primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′). The PCR mixture consisted of 5 μL of 10′ PCR buffer (final concentrations: 100 mM KCl, 20 mM Tris–HCl pH 8.0), 2.5 mM of $MgCl_2$, 2.5 mM of each dNTP, 1 μL of each primer, 1 μL of the template DNA, and 5.0 units of *Taq* polymerase (TaKaRa, Japan) for a total volume of 50 μL. The thermal cycling program used was as follows: initial denaturation at 95 °C for 5 min; 35 cycles consisting of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and a final extension step consisting of 72 °C for 7 min. Amplified PCR products were analyzed by agarose gel electrophoresis, purified with High Pure PCR Product Purification Kit (Roche, Germany) and sequenced by Cosmotech, Co. Ltd., Korea. Sequence alignment and analysis of similarity of the 16S rRNA genes were performed with CLUSTAL W (Thompson et al., 1997) and DNA baser RC2 (v2.9.97) programs. A similarity search was done in the Ribosomal Database Project (RDP).

2.4. Media and growth conditions

A loop of isolated strain SRKP-3 was inoculated into 20 ml of Luria–Bertani (LB) medium (1% tryptone; 0.5% yeast extract; 1% NaCl, pH 7.0) in a 100 ml Erlenmeyer flask, which was subsequently aerobically cultured at 37 °C for 24 h. Then, 2 ml of the cultured broth was inoculated to a 1 L shake-flask containing 200 ml of LB medium. The flask was maintained at 37 °C for 24 h. Growth was monitored spectrophotometrically by measuring culture's absorbance at 600 nm and dry weight periodically. The cells were harvested by centrifugation, washed twice with phosphate-buffer and air dried. Weight of the dried mass was considered as the dry weight of the sample.

2.5. Production of PHAs

Five Percentage overnight culture of the isolated strain SRKP-3 was inoculated into the modified production medium composing Dairy waste – 25% v/v, rice bran 3% (w/v) and sea water 30% (v/v), in 500 ml Erlenmeyer flasks and incubated at 37 °C for 36 h. After incubation the samples were centrifuged at 4000*g* for 5 min. The pellet was washed twice with distilled water and lyophilized. PHAs was extracted from dried cells by the solvent extraction method and quantified.

2.6. Purification of synthesized PHAs

PHAs were isolated from cell material by extraction with 25% sodium hypochlorite and chloroform at 37 °C for 3 h in screw-cap tubes for small quantities. The chloroform solutions were centrifuged to remove any cellular debris and concentrated by rotary evaporation. PHAs polymer was precipitated from the chloroform solution with chilled methanol (1:3) added drop wise. The precipitated polymer was separated by centrifugation at 3500*g* and the methanol–chloroform mixture was decanted. Then the polymer was once again dissolved in chloroform and precipitated in methanol in order to obtain highly purified polymer. Finally, the obtained pellet was dried in vacuum at room temperature and weighed.

2.7. Fourier transform-infrared spectroscopy

Purified PHAs (2 mg) was thoroughly mixed with KBr and dried. The dried sample was subjected to FT-IR spectrum using a Fourier Transform IR spectrophotometer, Shimadzu (Japan). The spectrum obtained was compared with that of the commercially available PHB (Sigma, USA).

2.8. Effect of substrates on the production of PHAs

Effect of various substrates (Dairy waste, rice bran and sea water) on the production of PHAs was determined. The quantity of substrates was taken from low number to high number. The range of substrate concentration are, For dairy waste (150 ml/L to 550 ml/L), rice bran (20 g/L to 60 g/L), sea water (150 ml/L to 550 ml/L) and the concentration of the substrates contributes for maximum production of PHAs was taken for CCRD.

2.9. Effect of time and pH on PHAs production

To find the effect of time on the growth and production of PHAs, the strain SRKP-3 was inoculated into the production medium (dairy waste (25% v/v), rice bran (3%) and sea water (25% v/v)) and samples were collected for every 12 h. The range of initial pH of the medium under consideration was 6–11. For determination