BRAZILIAN JOURNAL OF MICROBIOLOGY xxx (2017) xxx–xxx

BRAZILIAN JOURNAL OF MICROBIOLOGY

http://www.bjmicrobiol.com.br/

Environmental Microbiology

Variation analysis of bacterial polyhydroxyalkanoates production using saturated and unsaturated hydrocarbons

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ARTICLE INFO

Article history:
Received 23 February 2016
Accepted 11 February 2017
Available online xxx
Associate Editor: Miguel J. Beltran-Garcia

Keywords:
Waste frying oil
Polyhydroxyalkanoates
Fluorescence microscopy
FTIR

ABSTRACT

Polyhydroxyalkanoates (PHA) are efficient, renewable and environment friendly polymeric esters. These polymers are synthesized by a variety of microbes under stress conditions. This study was carried out to check the suitability of waste frying oil in comparison to other oils for economical bioplastic production. Six bacterial strains were isolated and identified as *Bacillus cereus* (KF270349), *Klebsiella pneumoniae* (KF270350), *Bacillus subtilis* (KF270351), *Brevibacterium halotolerance* (KF270352), *Pseudomonas aeruginosa* (KF270353), and *Stenotrophomonas rhizoposid* (KF270354) by ribotyping. All strains were PHA producers so were selected for PHA synthesis using four different carbon sources, i.e., waste frying oil, canola oil, diesel and glucose. Extraction of PHA was carried out using sodium hypochlorite method and maximum amount was detected after 72 h in all cases. *P. aeruginosa* led to maximum PHA production after 72 h at 37 °C and 100 rpm using waste frying oil that was 53.2% PHA in comparison with glucose 37.8% and cooking oil 34.4%. *B. cereus* produced 40% PHA using glucose as carbon source which was high when compared against other strains. A significantly lesser amount of PHA was recorded with diesel as a carbon source for all strains. Sharp Infrared peaks around 1740–1750 cm^{-1} were present in Fourier Transform Infrared spectra that correspond to exact position for PHA. The use of waste oils and production of poly-3hydroxybutyrate-*co*-3hydroxyvalerate (3HB-*co*-3HV) by strains used in this study is a good aspect to consider for future prospects as this type of polymer has better properties as compared to PHBs.

Introduction

Polyhydroxyalkanoic acids (PHA) present inside prokaryotic cells are carbon and energy reserved granules stored during environmental stress conditions. About 300 different bacterial strains are identified to accumulate PHA.[1] All Gram positive, Gram negative, archea, halophilic, halotolerant, root nodule bacteria are able to produce PHA.[2,3]

Bacteria has the ability to produce PHA from a diverse range of carbon sources such as complex waste effluents to

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http://dx.doi.org/10.1016/j.bjm.2017.02.008

Please cite this article in press as: Tufail S, et al. Variation analysis of bacterial polyhydroxyalkanoates production using saturated and unsaturated hydrocarbons. *Braz J Microbiol.* (2017), http://dx.doi.org/10.1016/j.bjm.2017.02.008

plant oils, carbohydrates, short chain fatty acids, and alkanes. Wastes are discharged in large amounts from food processing industries and agricultural wastes that have the potential to be used as carbon sources for PHA production.[4]

Carbohydrates are a good source but the production cost remains high as compared to synthetic plastics, so the search must be continued. Carbon substrates including organic fatty acids have also been reported for PHA production.[5] Therefore to make PHA production more economical, one of the foremost steps is to isolate naturally occurring bacterial strains that utilize less expensive carbon sources such as molasses that are obtained either from sugar beets or sugarcane. Molasses normally sold for about 33–50% of the cost of glucose.

In order to reduce the production cost researchers are trying to produce PHA from plant oils. These oils have been projected to be more efficient carbon sources for industrial PHA production than sugars.[6] Industrial-scale processes for the production of PHA from plant oils are currently being developed.[7] Different plant oils such as palm kernel oil, palm olein, crude palm oil, palm acid oil, canola oil, soya bean oil, corn oil and olive oil have been reported.[8,9] *Comamonas testosteroni* has been studied for its ability to synthesize medium chain length mcl-PHA from vegetable oils such as castor seed oil, coconut oil, mustard oil, cotton seed oil, groundnut oil, olive oil and sesame oil. Kahar and coworkers reported that the recombinant strains of *Ralstonia eutropha* are able to use plant oil such as soybean oil,[10] although the use of plant oils for plastic production may create a sense of competition for food sources. Various agro-industrial wastes like wheat bran, potato starch, sesame oil cake, groundnut oil cake, cassava powder, jackfruit seed powder and corn flour were tested for PHA production by *Bacillus sphaericus* which showed that jackfruit seed powder led to the production of maximum 19% PHBs. Many other agricultural and industrial wastes have also been reported as carbon sources including cassava bagasse hydrolyzate, babassu, soy cake, cane molasses and whey for PHB production.[11] Some scientists have tried waste plant oils as substrate. It has been shown that *Cupriavidus necator* produced 1.2 g/L PHA from waste frying oil that is in accordance with previous results obtained with glucose.[12]

The aim of this research work was to check the PHA production ability of purified strains using cheap carbon sources. We analyzed production ability of lipase producing bacterial strains using four different carbon sources including glucose, waste frying oil, diesel and cooking oil. Study of growth kinetics of bacterial strains and product accumulation kinetics using different oils parallel with glucose as standard carbon source was carried out. Then chemical analysis of the produced PHA using Fourier transform infrared spectroscopy (FTIR) was performed to identity monomer type of PHA our bacterial strains are able to produce by using these carbon sources.

Materials and methods

Bacterial strains

Strains were obtained from Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore and were named accordingly using the abbreviation for strain (STN) STN-6 to STN-11. All the strains were streaked on L-agar plates and incubated at 37 °C for 48 h to get isolated colonies. PHA detection agar (PDA)[13] containing 5 g/mL Nile blue was used for the direct screening of PHA producers. Medium (PDA) was supplemented with 20 g/L glucose. Plates were streaked and incubated at 37 °C for 24–48 h.[14]

Optimization of bacterial strains for pH

In order to find optimal pH for bacteria to grow and accumulate PHA granules, strains were grown on PDA medium having pH range 3–9. Optical density (O.D.) was recorded after every 24 h till 72 h at 600 nm and graphs were plotted.

Analysis of lipase enzyme activity of bacterial isolates

Purified strains were tested for their ability to utilize oils and the activity of lipase enzyme was checked by growing them on tributyrine agar.[15]

Sudan black B staining

Sudan black B (0.3%) in (ethyl alcohol) was used for detection of PHA inclusion bodies in cells. Heat fixed smears of strains were prepared and stained with Sudan black B for 15 min.[14] Slides were examined under Olympus CX31 microscope (Olympus, Inc, USA) using 100× objective lens with oil immersion.

Fluorescent staining

For fluorescent microscopy, 10 μL of 72 h old bacterial culture and 50 μL of acridine orange (0.1%) were taken in an eppendorf tube and incubated at 30 °C for 30 min. Pellet was collected by centrifugation at 4000 × *g* for 5 min.[16] On clean microscopic slide smear was prepared and observed under 100× objective lens of Leica BZ-01 fluorescence microscope (Leica Microsystems, Germany).

Growth kinetic studies and growth conditions

Shake flask fermentation process was carried out using 250 mL Erlenmeyer flasks containing 100 mL of PDA medium with glucose, waste frying oil (WFO), cooking oil and diesel as carbon substrates at 2% concentration and sterilized by autoclaving at 121 °C and 15 lb/in.^{2} for 20 min. The oil molecules were separated using sonicator[12] and then shaking in incubator so that bacteria were able to utilize it. Experimental design includes four flasks for each carbon source at 2% concentration and was inoculated with 24 h old bacterial culture from seed culture medium. One flask was kept as control and all were incubated at 37 °C and 100 rpm in a shaker. The aliquots (2 mL) of incubated medium were taken out at regular intervals of 8 h till 72 h from both the control and inoculated medium. Bacterial growth was determined by recording optical density at 600 nm. Along with it, 15 mL sample was collected every 24 h till 72 h and biomass was obtained by centrifugation at 4000 × *g* for 20 min. Pellet was lyophilized and biomass weight was calculated.

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