MICROBIAL PRODUCTION OF POLYHYDROXYBUTYRATE AND ITS COPOLYMER BY AZOHYDROMONAS AUSTRALICA

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INDIAN INSTITUTE OF TECHNOLOGY DELHI

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MICROBIAL PRODUCTION OF POLYHYDROXYBUTYRATE AND ITS COPOLYMER BY AZOHYDROMONAS AUSTRALICA

by

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Department of Biochemical Engineering and Biotechnology

submitted
In fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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DEDICATED TO MY BELOVED PARENTS AND HUSBAND

CERTIFICATE

This is to certify that the thesis entitled **"Microbial production of polyhydroxybutyrate and its copolymer by *Azohydromonas australica*"** being submitted by **Ms. Geeta** to the Indian Institute of Technology Delhi, for the award of degree of "Doctor of Philosophy" in Biochemical Engineering and Biotechnology Department is a record of the original bonafide research work carried out by her, which has been prepared under our supervision and guidance in conformity with the rules and regulations of Indian Institute of Technology Delhi. The results contained in this thesis have not been submitted in part or full to any other University or Institute for the award of any degree or diploma.

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Date: ***Geeta***

Place:

Microbial production of polyhydroxybutyrate and its copolymer by *Azohydromonas australica*

ABSTRACT

Over the past years, usage of plastics such as polypropylene and polyethylene have increased significantly for different industrial applications because of their durability, strength, ease of moulding and resistance to chemicals. However, these petroleum derived polymers stay in the environment for a long time thereby contributing to the problem of solid waste management. This problem needs sustainable solution so that not only waste disposal problem is resolved but also disappearing petrochemical raw materials are replaced by renewable feed stocks. Polyhydroxyalkanoates (PHAs) polymers have been recognized as best substitutes for conventional plastics because they possess material properties similar to conventional plastics and are produced by fermentation of renewable raw materials e.g. sugars which provide sustainable biotechnological solution to the above problem. However, the high production cost has limited their use in a wide range of applications.

The industrial production of these biopolymers requires an improvement in concentration and productivity using inexpensive raw materials so as to make the production cost economically comparable with the conventional petrochemical-plastics. Thus the objective of the present study was to optimize PHAs production using *Azohydromonas australica*. *A. australica* (earlier known as *Alcaligenes latus*) is of particular interest for PHAs production as it produces biopolymer during growth phase and accumulates it up to 80% of cell dry weight. *A. australica* is also able to utilize an inexpensive carbon source, sucrose thereby allowing the use of different renewable resources rich in sucrose such as sugarcane juice and beet molasses etc.

Statistical optimization of nutrients medium recipe for high growth and PHB production by *A. australica* was attempted in the present investigation which resulted in a total biomass and PHB concentration of 3.66 g/L and 2.63 g/L respectively in shake flask cultivation. Experiments on culture growth inhibition by major limiting substrates (carbon and nitrogen) demonstrated a decrease in specific growth rate of culture beyond a particular sucrose (beyond 25 g/L) and nitrogen concentration (beyond 0.6 g/L). Batch cultivation in a 7 L bioreactor using statistically optimized medium exhibited an accumulation of high biomass concentration of 8.71 g/L containing 6.24 g/L PHB with an overall PHB content of 72% of dry cell weight.

Further enhancement in PHB concentration and productivity was obtained by repeated-batch cycle strategy which involved removal of 20% (v/v) of the culture broth and subsequent replacement with an equal volume of fresh medium. Three repeated batch cycles resulted in a total PHB concentration of 20.55 g/L and a PHB productivity of 0.29 g/L.h at the end of cultivation (69 h).

Batch cultivation was then carried out in a custom made 8 L stainless-steel bioreactor using NAD(P)H probe for *on-line* NAD(P)H fluorescence measurement in order to develop a method for *on-line in-situ* monitoring of biomass concentration during PHB fermentation process. A linear correlation between biomass concentration and net fluorescence was obtained during initial and late log phase of the fermentation which can serve as an *on-line* indicator of biomass concentration and; a sharp decrease in fluorescence signal towards the end of cultivation is an important indicator of culture starvation due to substrate depletion inside the bioreactor which can be an appropriate signal in guiding fresh nutrients feeding strategies for high PHB accumulation.

The average batch kinetic data and substrates inhibition data was then used for the development of mathematical model for PHB fermentation process. Statistical validity of the model to describe the batch experimental kinetics was demonstrated by 'F' test with a confidence level of 99 %. The model was extrapolated to fed-batch cultivation by taking the mass balance around the bioreactor and various nutrients feeding strategies were designed using the developed model which were then experimentally implemented. These involved fed-batch cultivation under constant feed rate, decreasing feed rate, pseudo-steady state of sucrose and nitrogen limitation. Among all these cultivation strategies, the highest PHB concentration and productivity of 29.64 g/L and 0.6 g/L.h respectively was achieved in fed-batch cultivation under limiting nitrogen concentration.

In order to improve the thermo-processability of PHB and decrease its brittleness, efforts were made to produce PHB copolymer by incorporating other monomer units such as 3-hydroxyvalerate into PHB polymer. Prior to bioreactor cultivation, preliminary shake flask experiments were conducted using different organic acids (valeric acid, propionic acid, sodium propionate etc.) at appropriate concentration levels (2 g/L, 4 g/L and 6 g/L) to identify the best organic acid for poly (3HB-co-3HV) production. Valeric acid (4 g/L) was found to be the most suitable organic acid for incorporation of valerate unit into PHB polymer for poly (3HB-co-3HV) production.

Batch cultivation for poly (3HB-co-3HV) production was thereafter carried out in a 7 L bioreactor using intermittent valeric acid addition. It resulted in a total biomass and poly (3HB-co-3HV) concentration of 7.27 g/L and 5.94 g/L respectively. Thereafter fed-batch fermentation under nitrogen limiting concentration was carried out which involved pulse feeding of valeric acid. This fed-batch strategy yielded a reasonably high poly (3HB-co-3HV) concentration of 21.59 g/L and a productivity of 0.43 g/L.h in 50 h.

An innovative integrated fed batch-continuous reactor operating strategy was then developed which involved programmed feeding of fresh nutrients into the bioreactor. A poly (3HB-co-3HV) concentration of 24.65 g/L translating into an overall PHAs productivity of 2.18 g/L.h was obtained. This represented a 4.9-fold increment in productivity as compared to fed-batch cultivation under nitrogen limitation for copolymer production.

CONTENTS

CHAPTER 4. RESULTS AND DISCUSSION