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Production and characterization of PHB from a novel isolate *Comamonas* sp. from a dairy effluent sample and its application in cell culture

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ABSTRACT

In the present study, media engineering was carried out to optimize various process parameters affecting fermentation for PHB production by *Comamonas* sp. The optimum conditions for PHB production were yeast extract concentration of 1%, pH 6.0 and inoculum concentration of 5.5%. Under optimized conditions the PHB yield was 0.523 g/g. The extracted polymer was characterized by FTIR and ^{1}H NMR. Nanomatrices and solid walled matrices were prepared using the extracted PHB. Cytotoxic studies revealed that both solid walled and nanomatrices prepared from the extracted PHB showed almost 100% survivals of H9c2 cells. This is the first report on application of extracted PHB from *Comamonas* sp. as an extracellular matrix for cell culture.

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1. Introduction

The extensive use of petroleum derived plastics leads to severe waste disposal problems in the environment. Poly-3-hydroxybutyrate (PHB) is a biodegradable polymer and is an eco-friendly alternative to conventional petroleum derived plastics. Since global petroleum reserves are finite, there is a need for search of alternative renewable source for plastics. In this context renewable materials from microorganisms can provide an alternative sustainable source for biopolymer production [1]. PHB is a biodegradable polyester and serves as a source for biodegradable plastics. They are produced by several bacteria as a carbon reserve material and can be accumulated around 90% of the dry cell mass under nutrient limitation especially under nitrogen and phosphorous limited conditions [2].

One of the main limitations for commercial production of PHB is the high production cost when compared with petroleum derived plastics. Several research and development activities are going on throughout the world for the development of a cost effective technology for the production of PHB. Several reports are available on PHB production using agro-industrial residues and waste stream from various industries as sole carbon source thereby reducing the production cost of PHB. Waste by-products such as crude glycerol, olive mill effluent, dairy effluent as well as waste cooking oil were reported for the production of PHB.

Most of the reports on PHB production were from species of *Bacillus*, *Pseudomonas* and *Alcaligenes*. There are several reports on PHB production from *Bacillus sphaericus* [3], *Bacillus firmus*, [4], *Alcaligens eutrophus* [5], *Corynebacterium glutamicum* [6] and *Pseudomonas* sp. [7]. There is only one report on PHB production by *Comamonas* sp. using biomass hydrolyzate as sole carbon source [8].

The objectives of the present study was to optimize various process parameters affecting submerged fermentation for poly-3-hydroxybutyrate (PHB) production by a dairy effluent isolate *Comamonas* sp. and its characterization as well as evaluation of nanomatrices and solid walled matrices for cell culture.

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Table 1
Plackett-Burman design for optimization of various process parameters affecting PHB production by *Comamonas* sp.

Run order	Glucose	pH	Incubation time	Inoculum conc.	Inoculum age	KH_2PO_4	$(NH_4)_2SO_4$	Yeast extract	PHB Yield (g/g)
1	5	5	72	5	18	0.5	0.5	0.1	0.155
2	1	5	72	5	18	3	3	0.1	0.040
3	5	5	24	1	18	3	0.5	0.5	0.098
4	1	7	24	5	24	3	3	0.1	0.109
5	5	7	24	1	18	0.5	3	0.1	0.072
6	1	5	24	1	18	0.5	0.5	0.1	0.099
7	5	7	24	5	24	0.5	0.5	0.1	0.011
8	5	7	24	1	24	3	0.5	0.5	0.091
9	1	5	24	5	18	3	0.5	0.5	0.121
10	1	7	72	1	18	0.5	0.5	0.5	0.120
11	5	5	72	1	24	3	3	0.5	0.069
12	1	5	24	1	24	0.5	3	0.1	0.126
13	5	7	72	1	18	3	3	0.1	0.107
14	5	5	72	5	24	3	0.5	0.1	0.085
15	1	5	72	1	24	0.5	3	0.5	0.133
16	1	7	72	1	24	3	0.5	0.1	0.065
17	5	7	72	5	18	0.5	3	0.5	0.124
18	5	5	24	5	24	0.5	3	0.5	0.093
19	1	7	72	5	24	0.5	0.5	0.5	0.110
20	1	7	24	5	18	3	3	0.5	0.228

2. Materials and methods

2.1. Microorganism and growth conditions

Comamonas sp. isolated from a Milma dairy effluent sample was used in the present study. The strain was maintained in nutrient agar slants. Inoculum were prepared in LB media and incubated at 30 °C for 24 h on a rotary shaker at 250 rpm. 1% (v/v) of seed cultures were transferred to 100 ml of fermentation media as and when required [8].

2.2. Fermentative production of PHB

PHB production was carried out in 250 ml of Erlenmeyer flasks containing 50 ml culture media which had the following composition: $(NH_4)_2SO_4$, 2.0., KH_2PO_4-2.0., $MgSO_4.7H_2O$, 0.2., Na_2HPO_4, 0.2., yeast extract-0.2 and glucose-20 g/l. Media were inoculated with 1% inoculum and incubated at 30 °C for 72 h, centrifuged and the biomass pellets were lyophilized. Lyophilized pellets (0.5 g) was digested with concentrated H_2SO_4 (1 ml) at 90 °C for 30 min, cooled and then mixed with 4 ml of 0.014N H_2SO_4. Sample was diluted fifty times using 0.014N H_2SO_4 and filtered using 0.2 μm filter [3].

2.3. PHB assay

PHB assay was carried out by the method of Law and Slepecky [9]. Rezex (Phenomenex) ROA-organic acid column was used for HPLC analysis. Crotonic acid was used as standard. The HPLC conditions were oven temperature at 50 °C using 0.01N H_2SO_4 as mobile phase at flow rate 0.6 ml/min.

2.4. Media engineering for PHB production

2.4.1. Plackett–Burman design

A Plackett–Burman design was adopted to find out significant parameters affecting PHB production. The variables chosen were glucose, inoculum age, incubation time, pH, $(NH_4)_2SO_4$, inoculums concentration, K_2HPO_4 and yeast extract. The experiment consists of a total of 20 runs. Eight parameters were selected at two levels (Lower and higher levels) to select the significant parameters affecting PHB production by the selected strain. The experimental set up is shown in Table 1.

Table 2
Box–Behnken design for optimization of various process parameters affecting PHB production by *Comamonas* sp.

Run no:	Yeast extract	pH	Inoculum conc.	Biomass (g/l)	PHB Yield (g/g)
1	1	8	5.5	0.292	0.321
2	0.75	7	5.5	0.370	0.344
3	0.75	7	5.5	0.382	0.331
4	1	7	3	0.387	0.238
5	0.5	8	5.5	0.398	0.274
6	0.5	7	8	0.400	0.369
7	0.75	6	3	0.360	0.350
8	0.5	7	3	0.363	0.350
9	1	6	5.5	0.370	0.523
10	0.75	8	8	0.360	0.281
11	0.75	7	5.5	0.376	0.325
12	1	7	8	0.363	0.370
13	0.5	6	5.5	0.310	0.013
14	0.75	8	3	0.396	0.010
15	0.75	6	8	0.419	0.418

2.4.2. Box–Behnken design

The significant parameters affecting PHB production were selected from the Plackett–Burman design and optimized by adopting Box–Behnken design. The parameters selected were yeast extract, pH and inoculum concentration. The experiment consists of a total of 15 runs. Three parameters were selected at three levels (lower, middle and higher). The experimental set up is shown in Table 2.

2.5. Characterization of PHB

2.5.1. Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was carried out to detect different functional groups in PHB. The infrared spectroscopy detects the vibration characteristics of chemical and functional groups in the sample. The FTIR spectrum was recorded between 4000 and 400 cm^{-1} using a Shimadzu spectrometer (Japan). The extracted PHB as well as the standard PHB were dissolved in chloroform and layered on a NaCl crystal and after evaporation the polymer film was subjected to FTIR.

2.5.2. Nuclear magnetic resonance (NMR)

NMR spectroscopy is an effective method for observing the structure and dynamics of polymer chains both in solution and in solid state. The most important application of NMR spectroscopy is in the field of structure determination. NMR spectrum provides

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