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Kinetics and model building for recovery of polyhydroxyalkanoate (PHA) from *Halomonas campisalis*

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ABSTRACT

Moderately haloalkaliphilic *Halomonas campisallis* MCM B-1027 isolated from the alkaline lake of Lonar, India was found to produce a biodegradable polymer, polyhydroxyalkanoate (PHA). Scale up production of PHA from *H. campisalis* MCM B-1027 was carried out in 120 L stainless steel (SS) fermenter using 85 L production medium with a yield of 51.56% PHA on dry cell weight basis. The recovery of PHA was studied by several methods such as solvent extraction of the polymer, chemical digestion of the non-PHA biomass and mechanical disruption of the cells. Highest rate of PHA recovery obtained was 63 mg/h with solvent extraction using rotary film evaporator. Further optimization of extraction of PHA from dry cells in chloroform using 10 L rotavapor was carried out. L9 orthogonal array was chosen to study the influence of time, rotation and temperature at three levels along with their interactive effects on extraction process. Maximum PHA recovery of 84.47% was obtained at 25 rpm, 60 °C, within 3 h. Time factor had highest 84.11% contribution in the extraction process. This study is the first report on model for batch extraction of PHA and its validation based on second-order mechanism developed to predict the saturation capacity of the solvent, initial rate of extraction at different temperatures and activation energy of the process.

1. Introduction

Polyhydroxyalkanoate (PHA) being intracellular product, it is recovered by disruption of cells, lysis, permeabilization, or extraction [1,2]. Effectiveness of PHA recovery varies from one host to another due to differences in cell-wall structure and PHA formation factors, and consequently, due to differences in cell disruption and the subsequent fractionation process [3]. PHA recovery can be achieved mainly by three ways; solvent extraction of the polymer, chemical or enzyme digestion of the non-PHA biomass, or mechanical disruption of cell wall and inner membrane.

In solvent extraction method chlorinated solvents are most commonly used for extraction and PHA is subsequently recovered by either evaporation or precipitation in a poor PHA solvent viz. hexane, octane, methanol, etc. [4]. PHA can be recovered without degradation, by using a solvent extraction method, which results in significant raw material (solvent) cost [5]. Low-boiling point chlorinated solvents such as dichloromethane and chloroform were found to give a high PHA quality and a high extraction yield without decomposition [6].

As an alternative to solvent extraction, aqueous enzymatic digestion methods have been developed by ICI Ltd. (US). However, this often requires additional digestion or solvent extraction steps for increasing the product purity. In view of the higher recovery costs described, a differential digestion method using sodium hypochlorite alone and in combination with chloroform has been reported [7–12]. Although this method is effective in the digestion of non-PHA cellular materials (NPCMs), it causes severe degradation of PHB, rendering it unsuitable for many applications. Yung-Hun et al. [13] evaluated different detergents under various conditions to extract PHA from *Ralstonia eutropha* and *Escherichia coli* cells. Using Taguchi approach Yasotha et al. [14,15] carried out optimization for recovery of medium chain length (mcl) PHA synthesized by *Pseudomonas putida* using enzymatic digestion treatment of alcalase.

PHA recovery based on mechanical dispersion of cell wall such as by cell homogenizer, bead milling and ultrasound, used in combination with chemical method, to enhance digestion of the non-PHA cellular material has been described [3]. Tamer et al. [16,17] demonstrated the comparison of high pressure homogenization, bead milling and chemical induced lysis or disruption of *Alcaligenes latus* for recovery of PHB. Various PHA recovery methods, advantages and disadvantages, and results expressed in terms of PHA purity and yield are stated by Kunasundari and Sudesh [18].

PHA is an attractive polymer because of its physical properties; it can replace many conventional petrochemical products in

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applications including molded goods, paper coatings, performance additives, packaging, medical and agricultural field, etc. [19–22]. A major thrust of past research was focused on the development of an alternative method for recovery of PHA and its effect on the characteristics of PHA recovered.

This study was carried out with research objectives: to screen the methods for recovery of PHA, to optimize the operating parameters, to study kinetics of PHA extraction, and development of a model for PHA recovery process and recovery of chloroform.

2. Materials and methods

2.1. Production of PHA in 120 L stainless steel (SS) fermenter

2.1.1. Microorganism and inoculum development

Halomonas campisalis MCM B-1027 isolated from the alkaline soda lake of Lonar, District Buldhana, Maharashtra, India, was used for production of PHA [23,24]. The seed inoculum of *H. campisalis* was prepared by growing the culture in 500 ml conical flask containing 200 ml nutrient broth (NB), pH 9.0, and supplemented with 3% (w/v) NaCl, at 150 rpm and 37° C for 18 h. 5 L erlenmeyer bottles (2 No.) containing 2 L NB as mentioned above were inoculated with 4% (v/v) culture. The broth was incubated at 37 °C, 125 rpm for 18 h. The optical density (OD) of the culture was adjusted to 1.70 ± 0.05 corresponding to10^9 cells/ml.

2.1.2. Conditions for fermentation

Production of PHA was carried out in 120 L SS fermenter (Biochem Engineering Pvt. Ltd. Pune) with 85 L working volume. Production medium (PM) consisted of 1% maltose, 0.1% yeast extract, 0.5% NaCl, 0.038% $MgSO_4{\cdot}7H_2O$, 0.0128% $CaCl_2{\cdot}2H_2O$, 0.075% KCl, 0.02% NaBr. PM was sterilized *in situ* for 30 min. Maltose and $CaCl_2{\cdot}2H_2O$ were autoclaved separately and transferred aseptically to the fermenter. The initial pH of the production medium was adjusted to 9.5. During process, PM was inoculated with 4% (v/v) inoculum, and temperature maintained at 30 °C for 24 h. Aeration was 0.21 vvm till 12 h of fermentation followed by dissolved oxygen (DO) (DO Sensor model-D400-B070-PT-D9, Broadly James Corp., USA) level in the range 1–5% at 60 rpm. Samples were analyzed for cell growth (600 nm) and maltose by DNSA method [25].

2.1.3. Cell harvesting using continuous centrifuge and drying

At the end of fermentation the broth was centrifuged by continuous centrifuge (Model-AS 16 Y open type, Pennwalt India Ltd.) at 15,000 rpm. The cell pellet was treated with 3-fold (w/v) methanol for 15 min. The recovered cells were dried at 55 °C for 3 h.

2.2. Quantitation and characterization of PHA

2.2.1. PHA quantitation

PHA was quantitatively measured with minor modification in gravimetric method described by Ramsay et al. [26]. Three volumes of methanol were added to PHA solution, precipitate of PHA was recovered by filtration and dried in oven at 55 °C for 1 h.

2.2.2. Molecular weight determination and CHNS elemental analysis

Molecular weight and molecular weight distribution of the PHA formed at 40, 50, and 60 °C were determined by Gel Permeation Chromatography (GPC) (Thermoquest). Chloroform was used as a mobile phase and polystyrene as a standard. The molecular weight analysis of standard PHB and standard PHB-co-PHV 9:1were carried out.

The purity of polymer was confirmed using gas chromatography (GC) [23].

Elemental analysis of PHA was performed using elemental analyzer (Vario EL III, Elementar, Germany). Sulfanilic acid was used for calibration of instrument. PHB and PHB-co-PHV (7:3) (Fluka Chemika, Switzerland) were used as standards.

2.3. Screening of different methods for recovery of PHA from the dry cells

Recovery of PHA was carried using different methods according to the flow chart (Fig. 1).

2.3.1. Digestion of non-PHA mass

Sodium dodecyl sulfate (SDS): The cell mass of the culture was treated with 0.1% (w/v) SDS. The mixture was kept in a shaker incubator at 150 rpm, 37 °C for 30 min. The contents were centrifuged (Kubota, Japan) at 3200 rpm for 5 min. The pellet obtained was retreated as above. The white polymer obtained was dried at 65 °C for 30 min [27].

SDS and sodium hypochlorite: Wet cell mass was heated at 100 °C for 1 min and rapidly cooled to 55 °C for 30 min followed by freezing at 4 °C for 30 min. The cells were stored at −20 °C for 48 h. The freezed cell mass was suspended in 1% (w/v) SDS and incubated at 55 °C in a rotary shaker at 150 rpm for 30 min. The contents were centrifuged (Kubota, Japan) at 6200 rpm for 10 min. The pellet obtained was suspended in 4% (v/v) sodium hypochlorite. The solution was incubated at 30 °C for 30 min and centrifuged at 6200 rpm for 10 min. The polymer obtained was dried at 55 °C for 12 h [28].

Dispersion of sodium hypochlorite and chloroform: Lyophilized cell mass (0.3 g) was treated with a mixture of 50 ml chloroform and 50 ml 4% (v/v) sodium hypochlorite [29]. The mixture was incubated in a rotary shaker at 150 rpm at 35 °C for 90 min. The dispersion was centrifuged at 4200 rpm for 10 min. PHA was recovered from chloroform phase by evaporation of solvent. The film of PHA obtained was weighed and expressed as % PHA on dry cell weight (DCW) basis.

The optimization studies on recovery of PHA using sodium hypochlorite and chloroform were carried out for 30–150 min (30, 60, 90, 120 and 150 min) with 0.3% (w/v) cell mass. The PHA recovered at different treatment time was collected, dissolved in chloroform and the films were casted. The quality of films was checked in terms of transparency, and physical appearance.

2.3.2. Disruption of wet cell mass followed by Soxhlet extraction using chloroform

According to the method described by Mahapatra et al. [30], wet cell mass of culture was washed with cold acetone, and centrifuged at 10 °C. The cell mass was mechanically disrupted by simple grinding. The whole mixture was washed with distilled water and centrifuged. The lysed cell pellet was again washed with acetone and subjected to Soxhlet extraction with chloroform as a solvent. The extraction was carried out for 16 h. PHA film formed was weighed and expressed as % PHA on DCW.

2.3.3. Recovery of PHA using chloroform

Ambient temperature: The lyophilized cell mass (0.3 g) was washed with hot acetone for 10 min followed by mixing with 50 ml of chloroform. The mixture was incubated at ambient temperature for 48 h. The clear polymer solution was obtained after filtration. The PHA film formed was expressed as % PHA on DCW basis [10]. The optimization studies were done with respect to the time of treatment from 2 to 40 h (2, 4, 8, 16, 24, 32 and 40 h) for 0.3% (w/v) cell mass.

Refluxing: Preliminary experiments were carried out with the different amount of cell mass which varied from 0.1 to 0.8% (w/v) in chloroform volume 50 ml by keeping treatment time constant