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Formulation and optimization of a novel media comprising rubber seed oil for PHA production

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ABSTRACT

Formulation and optimization of a media comprising rubber seed oil[1] (RSO) as a low cost substrate for polyhydroxyalkanoates (PHA) biosynthesis in the bacterial strain *Bacillus cereus* was implemented. The formulation study using general full factorial design concluded that 1.5% (v/v) RSO in distilled water is sufficient to obtain PHA content as high as 604.77 mg/g cell dry weight and a total yield of 2.16 g/L within a growth period of 72 h at 150 rpm. Response surface methodology based design was used for the optimization of production parameters. Accordingly, PHA content of 641.14 mg/g cell dry weight and a total PHA yield 2.56 g/L were obtained in 69.2 h with RSO concentration of 1.567% (v/v) and an agitation of 130 rpm. Characterization of the polymer was performed by Fourier transform infrared spectroscopy, X-ray diffraction crystallography, scanning electron microscopy, differential scanning calorimetry and thermogravimetric analysis. Scale up studies and industrialization of this media for PHA production will result in value addition to rubber seeds that are currently commercially insignificant.

1. Introduction

Petroleum based conventional plastics have been a major cause for environmental pollution for the past few decades, thereby necessitating the development of a degradable alternative for the same. Despite the pollution related problems, the material is of high demand owing to its durability and mechanical properties (Kunasundari and Sudesh, 2011). Dependency on the depleting fossil fuels for production of plastics is a second reason for the need to develop a renewable alternative (Hottle et al., 2013). Polyhydroxyalkanoates (PHA) are a widely studied and completely biodegradable alternative for these non-biodegradable plastics. They are polymers synthesized in bacteria as storage forms of carbon under conditions of non-carbon nutrient deficiency. Once extracted, these polymers make an excellent alternative for conventional plastics owing to the similarities in physical properties. The existence of PHA has been reported as early as 1926 (Kynadi and Tharamel, 2014) and a number of microbial strains that synthesize PHAs of different kinds have been reported since then. There are different types of PHAs produced based on the monomer present. The most common type of PHA produced is poly(3-hydroxybutyrate) (Zinn et al., 2001). Some of the other types of PHA that have been reported are polyhydroxyvalerate, polyhydroxyhexanoate, polyhydroxydecanoate etc. (Braunegg et al., 1998). These biopolymers are stored in bacteria as immiscible intracellular granules of lipid nature (Sudesh et al., 2002). The type of PHA synthesized, the size and number of PHA granules accumulated etc. depend on factors like the microbe used and the nature of substrate provided (Ha and Cho, 2002). The most explicitly studied bacterial species for PHA production is *Cupriavidus necator* (Chee et al., 2010).

Commercialization of PHA faces a major hitch in the form of high production cost. This can be attributed to the following reasons; high cost of substrate and expensive purification procedure. This study deals with some cost reduction strategies that can be employed for PHA production. About 45% of the production cost for PHA is in the form of substrate. A number of agricultural substrates have been studied for PHA production among which vegetable oils are of good relevance and are easily available in the market (Koller et al., 2009). Jatophra oil, rape seed oil, palm oil, coconut oil etc. have been studied as good plant oil substrates for PHA production (Alias and Tan, 2005; Ng et al., 2011; Obruca et al., 2010; Solaiman et al., 1999). Rubber seed oil (RSO) has not been reported elsewhere other that in our previous study as an efficient substrate for PHA accumulation. This oil has negligible commercial use, therefore can be procured at a very low cost and has been observed to be a better substrate in comparison to a few other vegetable oils. Therefore, formulation of media and optimization of production parameters have been attempted using RSO. The media

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[1] RSO: Rubber seed oil, PHA: Polyhydroxyalkanoates, DCE: Dry coconut cake extract, CDW: Cell dry weight

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formulation study also attempts to replace commercially available salts used in the conventional media with a cost effective alternative. High cost of purification of PHA from Gram negative bacteria like *C. necator* can be attributed to co-purification of lipopolysaccharide endotoxins along with the polymer (Chen and Wu, 2005; Valappil et al., 2007). This will necessitate additional high cost purification steps. Therefore this study utilizes wild *Bacillus cereus*, a Gram positive strain isolated from oil contaminated field. *Bacillus* species has been previously reported to accumulate PHA (Aarthi and Ramana, 2011; Singh et al., 2009). The advantage of isolating the strain from oil contaminated field is that it can efficiently metabolize oils.

2. Materials and methods

2.1. Raw materials, organism and PHA production media

Rubber seed oil, the substrate used in this study, was obtained by cold pressing rubber seeds collected from the rubber plantations in Kerala. Dry coconut cake, the growth supplement, was obtained from the local market. *Bacillus cereus* was isolated from oil contaminated soil of Calicut district, Kerala. The isolate was identified by 16S rRNA sequencing and the sequence has been deposited in GenBank database under the name *Bacillus cereus* strain STV1180 with the accession number KU761536.

Conventional PHA production medium used in the study comprises 0.46 g/L K_2HPO_4, 0.2 g/L KH_2PO_4, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.2 g/L $(NH_4)_2SO_4$ and a carbon source of choice, most often glucose (Sangkharak and Prasertsan, 2008). All the experiments in this study have been conducted in 150 mL Erlenmeyer flasks with a culture volume of 50 mL. The inoculum used throughout the study was 5% (v/v) 18 h old *B. cereus* culture. All studies were conducted at room temperature as a cost reduction strategy.

2.2. Biomass production of B. cereus in DCE

Growth of *B. cereus* in dry coconut cake extract (DCE) was attempted to study the feasibility of employing the extract as a growth supplement. DCE was prepared by boiling 25 gm of dry coconut cake in 500 mL of water for 30 min. The solution was filtered, made up to 500 mL and stored at 4 °C. Biomass yield on utilization of varying dilutions of the DCE as growth media for 24 h was investigated and the results were compared to that obtained using conventional PHA production medium and nutrient broth (peptone 0.5% (w/v), yeast extract 0.3% (w/v) and sodium chloride 0.5% (w/v)).

2.3. GFF design for formulation of RSO media

A general full factorial (GFF) experimental design using Minitab software was employed for a novel media formulation. The input variables investigated were substrate concentration, presence of salts and the substitution of salts using DCE. The probability of this substitution was studied using a design that involved the presence and absence of salts as well as various concentrations of DCE starting from zero. The levels of different variables are as given in Table 1. The response variables analyzed were PHA content per gram of biomass and PHA yield per liter of medium. Agitation and production time were set at 150 rpm and 72 h. PHA was extracted by a modified non-halogenated solvent method using ethanol and water (Mohammadi et al., 2012). The extracted polymer was estimated by crotonic acid assay (Law and Slepecky, 1961). Analysis of variance (ANOVA) for the model was performed by F-test. Interactions and main effects plots were used to determine the optimal media composition.

Table 1
Factors analyzed and their levels in GFF design for media formulation.

Factor	Levels	Values
RSO concentration (% (v/v))	4	0.5, 1.0, 1.5, 2.0
DCE concentration (% (v/v))	5	0, 5, 10, 25, 50
Salts	2	presence, absence

Table 2
Parameters analyzed and their levels for RSM optimization of production parameters.

Parameter	Values				
	−1.68	−1	0	+1	+1.68
RSO (% (v/v))	0.91137	1.15	1.500	1.85	2.08863
Agitation (RPM)	19.093	80	120	180	220.908
Time (h)	9.546	30.00	60	90.00	110.454

2.4. RSM optimization of production parameters

A central composite design (CCD) with three independent variables was employed for response surface methodology (RSM) based optimization of the production parameters using the software Minitab. The input variables chosen were RSO concentration, agitation and production time. According to CCD, each parameter was examined at 5 levels as shown in Table 2. The design comprised 20 experiments with six axial points and six replicates at the center point. Response variables chosen were the same as that of the media formulation study explained in Section 2.3. PHA extraction and estimation were also as mentioned in Section 2.3.

Regression analysis of the CCD model was performed to express PHA productivity in the form of a quadratic polynomial while the authenticity and significance of the model was checked by ANOVA. Model was optimized using the response optimizer function to obtain maximum outcome. The predicted optimum condition was validated.

2.5. Substrate analysis to determine fatty acid utilization

Rubber seed oil was analysed for fatty acid composition by gas chromatography–mass spectrometry (GC–MS) before and after fermentation. Substrate oil was steam sterilised prior to analysis, to bring about any heat induced change in composition that might occur during sterilization of fermentation medium. Residual oil from the fermentation medium was separated by centrifugation at 10000 rpm with the addition of hexane. Hexane layer was pipetted out and the residual oil was extracted by using rotary evaporator at 70 °C. The sample was further oven dried at 105 °C for 15 min to remove any residual solvent and then used for analysis. Association of Analytical Communities (AOAC) official method 969.33 was used for fatty acid methyl ester (FAME) preparation from the test samples using boron triflouride catalyst (Cunniff, 1999) followed by GC–MS with flame ionization detector using JEOL GCMATE II GCMS.

2.6. Characterization of PHA

Fourier transform infrared (FT-IR) spectroscopy study was performed to determine the functional groups present in the polymer. Attenuated total reflection (ATR) mode of the JASCO FT-IR 4700 spectrophotometer was used in an exploration window of 4000–400 cm^{-1} for the analysis. Morphology of the outer surface as well as fractured surface of the polymer was analyzed using scanning electron microscopy (SEM). The polymer films were coarsely ground using mortar and pestle; sputtered with a thin layer of gold and analyzed on a Hitachi Variable Pressure Field Emission Scanning Electron Microscope SU 6600. The micrographs were analyzed using ImageJ 1.48 V software to measure pore size. The crystalline structure of PHA was confirmed using X-ray diffraction (XRD) studies with Ni-filtered Cu Kα (40 kV, 40 mA) radiation source employed by powder