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A statistical approach for optimization of polyhydroxybutyrate production by marine *Bacillus subtilis* MSBN17

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ABSTRACT

The important biological macromolecule polyhydroxybutyrate (PHB) producing *Bacillus subtilis* was isolated from the marine sponge *Callyspongia diffusa* and identified by means of 16S rRNA analysis. The central composite design (CCD) was used to optimize the PHB production using cheap raw materials such as pulp industry waste (PIW), tamarind kernel powder (TKP), palm jaggery (PJ) and green gram flour (GGF). The extracted polymer was characterized by ^{1}H NMR analysis. The PIW was fed at three different intervals and the maximum production of PHB (19.08 g/L) was attained after a period of 40 h of incubation of *B. subtilis*. Dissolved oxygen, sodium chloride and nitrogen source were found to be the critical control factors that affected the PHB polymer production. The present investigation demonstrates an inexpensive model of producing PHB green thermoplastics *in vitro* for biomedical applications.

1. Introduction

Polyhydroxyalkanotes (PHAs) is a natural polyesteric biological macromolecules that is synthesized and accumulated by prokaryotes in the form of intracellular granules in response to nutritional limitation or the presence of excess carbon source in the growth environment [1,2]. PHAs are completely biodegradable thermoplastics and have been the subject of great interest in bioprocess technology. PHAs are considered as good alternative compounds for petroleum-derived synthetic plastics and have immense applications in medicine, pharmacy, agriculture, food industry and also serve as the raw material for the production of enantiomerically pure chemicals as well as in paint industry [3,4]. Until the year 2009, there are more than 150 constituents of PHAs have been identified [5] and characterized. Polyhydroxybutyrate (PHB) is the one of the best characterized derivative of PHA. The cost of production is the major limiting factor that constrained the commercial application of PHB. Therefore, environment-friendly and cost-effective methods for large scale PHB production using novel microbial strains are inevitable for the commercial applications. One of the most important approaches to reduce cost is to use industrial wastes and the by-products as carbon sources [6]. This method is appreciative of the fact that it decreases costs that are involved in the disposal of waste materials and at the same time enables the production of value-added products.

The majority of PHB producing bacteria was isolated from soil and activated sludge [7]. Recently, new bioresources such as marine environments were explored regarding their potential to harbor new PHB producers [8–11]. Marine sponges are harbor a remarkable array of microorganisms and the marine sponge-associated endosymbiotic bacteria have been recognized as rich source of biological macromolecules that are of potential interest to various industrial sectors [12]. The dynamic change of surrounding sea water results the massive stress in marine sponges and it also directly affects the survival of bacterial endosymbionts associated in the marine sponges [12]. Inclusion of storage substances such as PHB is a common bacterial strategy that increases survival in changing surroundings [7]. The hypothesis of this study was that especially the marine sponges should provide conditions under which diverse PHB producing bacteria are enriched.

Abbreviations: ANOVA, analysis of variance; CCD, central composite design; DCW, dry cell weight; FT-IR, Fourier transform infrared spectroscopy; GC, gas chromatography; GGF, green gram flour; GPC, gel permeation chromatography; NMR, nuclear magnetic resonance; PHAs, polyhydroxyalkanoates; PHB, polyhydroxybutyrate; PIW, pulp industry waste; PJ, palm jaggery; RSM, response surface methodology; SAS, sterilized aged seawater; TKP, tamarind kernel powder; ZMA, Zobell marine agar.

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Gram-negative bacteria are commonly used in industries to produce PHB, whereas the gram-positive bacteria are not much exploited on commercial aspects despite the fact that *Bacillus* is the first gram-positive genus that has been identified to produce PHB [13,14]. However, when it comes to the question of the production, optimization of the various parameters seem to be an important factor to standardize and thus, enhance the production of PHB. Classical optimization such as one factor at a time method was followed previously which is unreliable and time consuming with key disadvantage of lack of interactive studies. The response surface methodology (RSM) is a powerful statistical tool for studying the interactive effects of different variables using statistical method and it has been successfully applied for optimization of various PHAs bioprocesses. The industrial PHB production requires a non-linear feed supply that can be provided by fed-batch cultivation [9]. Previously many reports are available for the synthesis of PHB by fed-batch process using industrial wastes as substrates such as sugar molasses and dairy industry waste [9,15].

The present study describes isolation and identification of gram-positive spore forming bacteria having high PHB productivity. The purified biopolymer was characterized by FTIR, GC–MS, GPC and NMR analysis by comparing with the standard PHB. Thereafter, effect of carbon, nitrogen and mineral sources were examined to evaluate their effect on PHB production. The special emphasis has been given to optimize the most significant variables such as substrates in order to increase the production of PHB by statistical optimization (RSM). This would be the first report elucidating the use of combination of pulp industry waste, tamarind kernel powder and palm jaggery as sources in the production of PHB. Also the optimized medium was studied in pilot scale bioreactor by fed-batch process.

2. Materials and methods

2.1. Isolation and screening of PHB polymer producers

Marine sponge *Callyspongia diffusa* was collected from the southeast coast (10°026.51° N 79°13′45.39° E) of India by SCUBA diving at a depth of 10–15 m. Sponges that were completely intact were used for microbiological analysis. The specimens were kept idle for about 2 h in sterilized aged seawater (SAS) to remove the loosely associated microorganisms from the inner and outer sponge surfaces. This method is primarily done to avoid cross contamination of bacterial species. Nearly 1 cm^3 of sponge tissue was excised from the internal mesohyl (endoskeleton) area using a pair of sterile scissors for the purpose of isolating sponge-associated bacteria. The excised portion was thoroughly washed three times with SAS to remove any bacteria within the current canals and tissue was homogenized with phosphate buffered saline using a tissue homogenizer. The homogenized sample was plated on various isolation media including sponge agar 1, sponge agar 2 [16], Zobell marine agar (ZMA), and Emerson agar (EA) (Himedia, Mumbai). Amphotericin B (30 μg/μl) was added to inhibit the growth of fungi and the plates were incubated at 28 °C for 7 days in the dark. Morphologically distinct colonies were selected and purified by repeated sub culturing for at least three times on ZMA and stored at 4 °C. PHB producing strains were screened by the viable colony staining method on ZMA using Nile blue A (1 μg/ml) as the staining agent. The colonies were directly examined for fluorescence by exposing them to UV light to detect the accumulation of PHB. Amongst the various species isolated, the efficient PHB producer MSBN17 was selected by Sudan black B staining method [17].

2.2. Identification of PHB polymer producer

The strain MSBN17 was morphologically and biochemically identified based on the Bergeys's manual of determinative bacteriology [18]. The DNA was isolated from MSBN17 and purified [19]. From the genomic DNA, nearly full-length 16S rRNA sequences were amplified by using primers 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′). A 25-μl reaction volume PCR was performed by using 10 ng of genomic DNA, 1× reaction buffer (10 mM Tris–HCl at pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, and 0.1%Triton X-100), 0.4 mM (each) deoxynucleoside triphosphates (Invitrogen), 0.5 U of DNA Polymerase (New England Labs, UK), and 1 mM of both the forward and reverse primers. A PCR temperature profile of 95 °C for 3 min was used initially; then 30 cycles of 95 °C for 45 s, 55 °C for 45 s, 72 °C for 1.45 min and a final extension step at 72 °C for 10 min was used. The various PCR products were cloned using the TOPO TA Cloning kit [19] based on the manufacturer's instructions (Invitrogen) for sequencing. The 16S rRNA gene sequence obtained from the MSBN17 was compared with the other bacterial sequences using NCBI mega BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for their pair-wise identities. Multiple alignments of these sequences were carried out by Clustal W 1.83 version of EBI (www.ebi.ac.uk/cgi-bin/clustalw/) with a transition weight of 0.5. Phylogenetic trees were constructed using MEGA 5.0 version (www.megasoftware.net) by means of the neighbor joining (NJ), minimum evolution (ME), and unweighted pair group method along with the arithmetic mean (UPGMA) algorithms. Nucleotide composition of each aligned sequence was predicted by the BioEdit software package.

2.3. Production and purification of PHB polymer

A loop full of the sponge isolate MSBN17 was inoculated into 50 ml of Luria Bertani (LB) broth (1% casein enzymic hydrolysate; 0.5% yeast extract; 1% sodium chloride; at a pH of 7.5) in a 250 ml Erlenmeyer flask, which was subsequently incubated at 30 °C for 24 h, aerobically. The 5 ml of broth culture was inoculated into a 500 ml shake flask containing 200 ml of LB broth and incubated at 30 °C for 36 h on a rotary shaker at 250 rpm to make the starter inoculum. The seed culture (5%, v/v) having a cell density of 10^6 cells/ml was inoculated into 500 ml of modified production medium containing pulp industry waste (PIW) (20%, v/v), tamarind kernel powder (TKP) (2%, w/v), palm jaggery (PJ) (1.5%, w/v), green gram flour (GGF) (0.4%, w/v), and NaCl (2.5%, v/v) and incubated at in a Erlenmeyer flask (250 rpm) at 30 °C for 48 h. The growth was measured in terms of the OD values at A_{600}. Nearly 50 ml of the culture broth was removed aseptically for the analysis of dry cell weight (DCW), PHB accumulation, substrate utilization, and pH change during the process of fermentation. Later, cells were harvested by centrifugation at 10,000 × *g* for 10 min. The harvested pellet was washed with ethanol and distilled water (each wash at 8000 × *g* for 10 min), and then lyophilized. PHB was extracted by modified protocol using 4–6% sodium hypochlorite solution (Rankem) [20]. The solution containing lyophilized cells was kept for 15–20 min at 37 °C. The PHB granules were collected by centrifugation at 12,000 × *g* for 12 min and the pellet was washed with water, methanol and acetone respectively (each wash at 8000 × *g* for 10 min). The polymer was dissolved in chloroform and precipitated using ice-cold methanol. The precipitated polymer was dried in rotary evaporator and weighed.

2.4. Characterization of PHB polymer

The purified PHB was thoroughly mixed with potassium bromide (KBr) and then dried. The dried sample was subjected to Fourier-transformed infrared (FT-IR) spectrophotometer analysis