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Process optimization for poly-β-hydroxybutyrate production in a nitrogen fixing cyanobacterium, *Nostoc muscorum* using response surface methodology

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Abstract

A five-level-four-factor central composite rotary design was employed to find out the interactive effects of four variables, viz. concentrations of acetate, glucose and K_2HPO_4, and dark incubation period on poly-β-hydroxybutyrate (PHB) production in a N_2-fixing cyanobacterium, *Nostoc muscorum*. Acetate, glucose and dark incubation period exhibited positive impacts on PHB yield. Using response surface methodology (RSM), a second order polynomial equation was obtained by multiple regression analysis. A yield of 45.6% of dry cell weight (dcw) was achieved at reduced level of nutrients, i.e. 0.17% acetate, 0.16% glucose and 5 mg l^{-1} K_2HPO_4 at a dark incubation period of 95 h as compared to 41.6% PHB yield in 0.4% acetate, 0.4% glucose and 40 mg l^{-1} K_2HPO_4 at a dark incubation period of 168 h under single factor optimization strategy.

Keywords: CCRD; Chemoheterotrophy; *Nostoc muscorum*; Poly-β-hydroxybutyrate; RSM

1. Introduction

In today's modern era of science and technology plastics have become one of the most widely used materials all over the world. Their applications are nearly universal: components in automobiles, home appliances, computer equipments, packages and even medical applications are areas, where plastics clearly have become indispensable. How much ever we may applaud about the quality of plastics and its uses in day-to-day life, they have long been vilified because they are environmentally unfriendly, i.e. they are not biologically degradable.

The search for biodegradable plastics has led to a number of partially and completely biodegradable products. Amongst all, microbially-formed polyhydroxyalkanoates (PHAs) offer much potential for significant contributions as "bioplastics". Poly-β-hydroxybutyrate is the most widespread and thoroughly characterized PHA found in bacteria. It is accumulated as a carbon and/ or energy storage material in various microorganisms usually under limiting nutritional conditions such as N and P stresses in presence of excess carbon (Steinbüchel, 1991; Byrom, 1994; Liebergesell et al., 1994; Yu, 2001). Until now, PHB is been produced by heterotrophic bacteria with the help of fermentation technology. Cyanobacteria, however, are indigenously the sole prokaryotes that accumulate PHA by oxygenic photosynthesis. More than 100 cyanobacterial strains screened so far, about 70% of them were found to contain PHB at concentrations ranging from 0.04% to 6% of dry cell weight (dcw) under photoautotrophic growth conditions. Most recently however, Nishioka et al. (2001) and Sharma and Mallick (2005a) demonstrated accumulation ranging from 43-55% (dcw), respectively in *Synechococcus* sp. MA19 and *Nostoc muscorum* under phosphate-limited and chemoheterotrophic growth conditions.

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Earlier studies revealed that PHB yield in cyanobacteria was a function of various encompassing cultural and nutritional conditions. Growth phase, light-dark cycles, temperature, pH (Stal, 1992; Sharma and Mallick, 2005b; Panda et al., 2006), nutrient limitations, viz. nitrogen (Stal, 1992; Lama et al., 1996; Wu et al., 2001) and phosphorus (Nishioka et al., 2001; Panda et al., 2006), mixotrophy (Wu et al., 2001; Sudesh et al., 2002; Panda et al., 2006), chemoheterotrophy (Miyake et al., 1996; Sharma and Mallick, 2005a) and gas exchange limitation (Sharma and Mallick, 2005a) have emerged as critical variables for enhanced PHB accumulation in cyanobacterial cell. Process optimization for PHB yield in *Rhizobium meliloti* was studied by taking variables such as complex sucrose, urea, inoculum size and K_2HPO_4 by Lakshman et al. (2004). Khanna and Srivastava (2005) optimized the concentration of KH_2PO_4, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$ and fructose for PHB production in *Wautersia eutropha* using central composite design. It is worth mentioning here that no report on process optimization for PHB yield in cyanobacteria using any statistical tool is yet available. Present study, therefore, envisaged to optimize some nutritional factors, viz. concentrations of acetate, glucose and K_2HPO_4, and dark incubation period for enhanced PHB production in *N. muscorum* by response surface methodology.

2. Methods

2.1. Organism and experimental conditions

N. muscorum Agardh was grown axenically in NO_3-free BG-11 medium (Rippka et al., 1979) at 25 ± 1 °C, pH 8.0, under 14 h light (75 μmol photon $m^{-2}s^{-1}$ PAR): 10 h dark cycles. Phosphate deficiency was obtained by replacing K_2HPO_4 of the medium with equi-molar concentration of KCl. For chemoheterotrophic growth condition, cultures supplemented with glucose and acetate at different concentrations were incubated under complete darkness following Sharma and Mallick (2005a).

2.2. Growth and dry weight measurement

Growth was measured in terms of chlorophyll *a* following Mackinney (1941). Cell dry weight was determined gravimetrically following Rai et al. (1991).

2.3. Extraction of poly-hydroxyalkanoates (PHAs)

Extraction of PHAs was done following Yellore and Desia (1998) with certain modifications. A known amount of cyanobacterial cells was suspended in methanol at 4 °C (overnight) for removal of pigments. The pellet so obtained after discarding the supernatant was dried at 60 °C. The polymer was extracted in hot chloroform followed by precipitation with cold diethyl ether. The sample was then centrifuged at 11,000 × *g* for 20 min to get the pellet. The pellet was washed with acetone and was dissolved again in hot chloroform.

2.4. Assay of poly-β-hydroxybutyrate (PHB)

PHB was quantified following the propanolysis method of Riis and Mai (1988) with the help of gas chromatography (Clarus 500, Perkin Elmer) in split mode (1:50, v/v), equipped with Elite-1 dimethylpolysiloxane capillary column (30 m × 0.25 mm × 0.25 μm) and flame ionization detector. Benzoic acid was used as the internal standard.

2.5. Experimental design

A five-level-four-factor central composite rotary design (CCRD) obtained by using the software Design-Expert 6.0.10®, Stat-Ease Inc. Minneapolis, USA was employed to find out the interactive effects of four variables, viz. concentrations of acetate, K_2HPO_4, and glucose, and duration of dark incubation on PHB production. Stationary phase cultures of *N. muscorum* were transferred to BG-11 medium with varying concentrations of K_2HPO_4, glucose and acetate as given in Table 1. Duration of dark incubation was imposed as per the experimental design. Central composite design at the given range of the above parameters in terms of coded and actual terms is presented in Table 2.

2.6. Statistical analysis

The experimental data obtained from the design were analyzed by the response surface regression procedure using the following second-order polynomial equation:

$$Y_i = \beta_0 + \sum_i \beta_i x_i + \sum_{ii} \beta_{ii} x_i^2 + \sum_{ij} \beta_{ij} x_i x_j$$

where Y_i was the predicted response, $x_i x_j$ were independent variables, β_0 was the offset term, β_i was the *i*th linear coefficient, β_{ii} was the *i*th quadratic coefficient and β_{ij} was *ij*th interaction coefficient. However, in this study, the independent variables were coded as A, B, C and D. Thus the second order polynomial equation could be presented as follows:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD$$

Table 1
Levels of factors used for optimization of nutritional factors for PHB production

Variable	Name	Level				
		−2 (−α)	−1	0	1	2 (+α)
A	Acetate (% w/v)	0.05	0.10	0.15	0.20	0.25
B	K_2HPO_4 (mg l^{-1})	−10	0	10	20	30
C	Glucose (% w/v)	0.05	0.10	0.15	0.20	0.25
D	Period of dark incubation (h)	42	84	126	168	210