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ORIGINAL ARTICLE

Hypochlorite digestion method for efficient recovery of PHB from *Alcaligenes faecalis*

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Abstract We reported the optimum amount of PHB accumulated by *Alcaligenes faecalis* during its 24 h growth under nitrogen deficient conditions. After 24 h incubation decrease in the amount of PHB was recorded. Hypochlorite digestion of biomass of organism followed by extraction with a solvent system consisting of 1:1 mixture of ethanol and acetone resulted in efficient recovery of PHB vis-à-vis earlier methods. This solvent system gave a high recovery yield, i.e. 5.6 gL^{-1} vis-à-vis earlier reported yield, 1.34 gL^{-1} (by same method), 0.63 gL^{-1} (by chloroform extraction method) and 1.1 gL^{-1} (by dispersion method).

Keywords *Alcaligenes faecalis* · PHB · Hypochlorite · NPCM

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Introduction

Poly β-hydroxybutyrate (PHB) or polyhydroxyalkonoates (PHA) are class of polyesters produced and accumulated intracellularly by many gram-positive and gram-negative bacteria as a carbon and energy reserve granules. PHB and PHA have been regarded as an eco-friendly alternative to the synthetic polymers because they are biodegradable and therefore safe for the environment and possess properties similar to that of synthetic polymers currently in use [1–4]. PHB possesses only *R* side chains (lack *S* side chains) and are therefore biodegradable [1, 5, 6].

The potential of PHB as biodegradable thermoplastic has been long recognized but the less efficient recovery processes has hampered their commercialization for wide range of applications [7–9]. Time of extraction is equally important for the recovery of PHA, because during prolonged incubation under nutrient limiting conditions, PHB is utilized as a stored carbon and energy in presence of PHB depolymerase [10–12].

Method of extraction is equally important in getting high recovery yields; different methods used in the recovery cause severe degradation of PHB and therefore decrease in the recovery yield [2, 13–16]. The rate of degradation can be reduced several folds by digestion of non-PHB cell mass (NPCM) with sodium hypochlorite [15].

In present investigation we report the time dependent intracellular accumulation and utilization of PHB and digestion of NPCM for the efficient recovery of PHB from *Alcaligenes faecalis*.

Materials and Methods

Microbial culture

A. faecalis BCCM 2374 was obtained from the depository of School of Life Sciences, North Maharashtra University, Jalgaon. It was routinely maintained on nutrient agar at 4°C.

For the intracellular accumulation of PHB, *A. faecalis* (6×10^6 cells ml^{-1}) was grown carbon-rich growth medium containing gL^{-1}, glucose, 20; $(NH_4)_2SO_4$, 2.0; KH_2PO_4, 13.3; $MgSO_4 7H_2O$, 12.; citric acid, 1.7; trace element solution, 1.0 mlL^{-1} $FeSO_4 7H_2O$; 10, $ZnSO_4 7H_2O$, 2.25; $CuS0_4 5H_2O$, 1.57; $MnSO_4$-$5H_2O$, 0.5, $CaCl_2$-$2H_2O$, 2.0; $Na_2B_4O_7 10H_2O$, 0.23; $(NH_4)_6Mo_7O_{24}$, 0.1; pH, 7.2 [17] at 29°C at 120 rpm for 24 h. Broth was centrifuged (10000 rpm, 15 min) and the biomass was collected and grown in nitrogen deficient minimal medium consisting of gL^{-1} Na_2HPO_4, 3.8; KH_2PO_4, 2.65; NH_4Cl, 2; $MgSO_4$, 0.2; fructose, 2 and trace minerals [1.0 mlL^{-1}] pH, 7.0 ± 0.2 [18], for 48 h at 30°C at 120 rpm. The biomass from nitrogen deficient minimal medium was stained with Sudan black B and observed under bright field microscope [19].

For the extraction of PHB NPCM was digested with sodium hypochlorite at 37°C for 1 h, followed by extraction with hot chloroform and precipitated with ethanol and acetone (1:1). The precipitate was allowed to evaporate at 29°C to obtain PHB crystals [13, 16]. Extracted PHB crystals were redissolved in chloroform (5 mg in 5 ml) to estimate the PHB content of extract at 235 nm [20]. This method specifically measures PHB; PHB is converted into crotonic acid. Alternatively, the sodium hypochlorite digested biomass was subjected to hexane and propanol (1:1 v/v) for PHB extraction.

Intracellular mobilization of PHB was studied by growing *A. faecalis* (6×10^6 cells ml^{-1}) in nitrogen-deficient medium at 29°C at 120 rpm for 48 h. Samples withdrawn after 6 h interval up to 48 h were subjected for measuring the amount of residual intracellular PHB [20] and growth (optical density at 620 nm).

Results and discussion

Occurrence of turbid colonies on nutrient agar plate and appearance of blue-black droplets of PHB against pink cytoplasm during Sudan Black B staining indicated the presence of PHB. Kim et al. [17] and Smibert and Krieg [20] have also reported that PHB accumulating cells produce more turbid colonies on nutrient agar and appear pink when stained with Sudan Black B.

A. faecalis accumulates optimum amount of PHB under nitrogen-limiting conditions in minimal medium during 24 h growth [21]. The amount of intracellular PHB during 24 h growth was 5.6 gL^{-1}. When correlated with growth curve it was found that maximum PHB accumulated at the end of active (exponential) phase of growth. After 24 h growth, towards, stationary phase/decline phase, continuous decline in the amount of PHB was observed by reaching 48 h growth it remained only 0.7 gL^{-1} (Fig. 1). This continuous decline in the amount of PHB from 5.6 gL^{-1} to 0.7 gL^{-1} indicated the intracellular mobilization of PHB by the organism. PHB producing microorganisms are reported to utilize the intracellular PHB as reserve carbon and energy when the nutrients are exhausted from medium [11]. *A. faecalis* also possesses PHB depolymerase, which help in intracellular mobilization of PHB [10–12].

Sodium hypochlorite digestion of NPCM resulted in the lysis of cells without affecting the PHB. Extraction of PHB with a mixture of hexane and propanol resulted in poor recovery yield (1.92 gL^{-1}). The solvent system consisting of 1:1 mixture of ethanol and acetone proved to be specific and efficient recovery method capable of specifically lysing the NPCM without affecting PHB. Rawte and Mavikurve [16] have also reported the usefulness of acetone and ethanol in the extraction of PHA. However, with ethanol and acetone (1:1 v/v) high recovery yield i.e. 5.6 gL^{-1} was obtained *vis-à-vis* earlier reported yields, 1.34 gL^{-1} (by same method) [16], 0.6 gL^{-1} (by chloroform extraction method) [13] and 1.1 gL^{-1} (by dispersion method) [14]. Table 1 gives the comparative account of recovery yield of PHB with various methods.

Conclusion

A. faecalis accumulate optimum amount of PHB under nitrogen deficient conditions during 24 h incubation and has

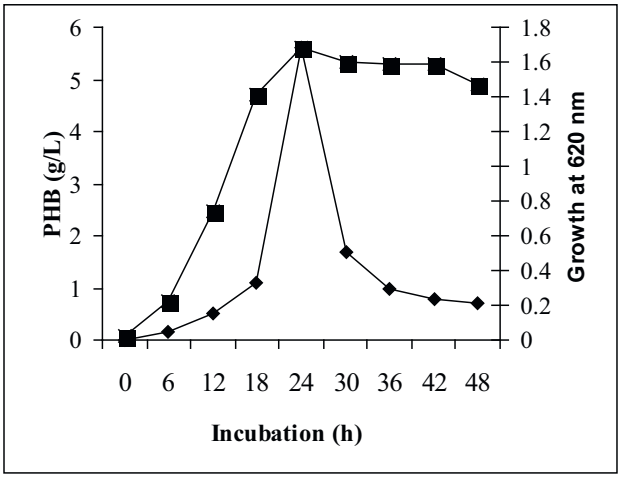

Fig. 1 Growth curve and intracellular accumulation and mobilization of PHB in *A. faecalis*

Table 1 Efficiency of PHB recovery methods

Recovery methods of PHB	PHB recovery yield (gL^{-1})	Reference
Hypochlorite digestion of non-PHB cell mass	5.6	This method
Hypochlorite digestion	1.34	[10]
Chloroform extraction method	0.63	[8]
Dispersion method	1.1	[9]
Hexane propanol method	1.92	

the capacity to internally mobilize this PHB as a carbon and energy reserve under nutrient limiting conditions. 1:1 mixture of ethanol and acetone give efficient and high recovery yield of PHB over the earlier reported methods.

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