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ORIGINAL ARTICLE

Understanding PHB Production profile in *Bacillus thuringiensis* IAM12077 and development of strategies for R-3 Hydroxybutyrate production

Vaishnavi Gowda And Srividya Shivakumar*
Department of Microbiology, School of Sciences, Jain University, Jayanagar, Bangalore 560011
Email: sk.srividya@jainuniversity.ac.in

ABSTRACT
Bacillus thuringiensis IAM12077 is a PHB producer with maximum accumulation of 0.5g/L after 48 h growth in glucose rich media with nitrogen deficiency. An attempt to understand the in-vivo PHB depolymerase of the system was made by altering resting cell conditions. Cells of 48h grown culture was harvested and resuspended in nutrient limiting conditions to trigger in-vivo depolymerisation. Highest depolymerisation of PHB yielding 0.56g/L 3HB was noticed under pH9, 60μM $MgCl_2$ and 8h incubation. An understanding of the in-vivo depolymerase system helps in formulating conditions to increase either PHB accumulation or in-vivo degradation to release enantiomerically pure 3-hydroxubutyrate (3HB).
***Key words**: In-vivo PHB depolymerisation, 3-Hydroxubutyrate, iPHAZ, in-vivo PHB depolymerase, enantiomer, R-Hydroxyalkonoates*

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INTRODUCTION

Enantiomeric purity is considered as an important parameter in pharmaceuticals, food supplements, flavours, fragrance and other chemicals. Dosage requirement and effectiveness of a drug varies when there is an unwanted enantiomer present. Such a racemic mixture can also have adverse side effects like the case reported by Thalidomide [1].Currently, synthesis of chiral drugs is either through asymmetric synthesis or through degradation of naturally occurring chiral pool [2]. Primary focus of biocatalysis and white biotechnology is the replacement of conventional chemical production processes with sustainable biotechnological approaches [3].

There are specific chemical molecules which can be used as chiral precursors of which (R)-hydroxybutyrate is most important because they contain two easily modifiable functional groups (hydroxyl and carbonyl group) and provide provisions to introduce a secondary chiral centre. Macrocyclic components of elaiophylin [4], peptide antibiotic visconsin [5], fungicides like vermiculin and norphyrenophorin [6] and pharmaceuticals like β-lactams and captopril [7, 8] are reported using R-HAs as building blocks.

One approach of production of enantiomerically pure R-HA is through the enzymatic hydrolysis of polyhydroxyalkonoates (PHA). PHA is viewed as the most suitable alternative to conventional plastic owing to their chemical and physical versatility [9]. Poly (3hydroxybutyrate) is the most widely studied PHA to be produced in an industrial scale.

At the cellular level, PHB is produced as an intracellular granule with accumulation to 90% of cellular dry weight during growth in unbalanced nutrient conditions by a wide range of gram positive and negative bacteria [10, 11]. Native intracellular PHB (nPHB) granules contain hydrophobic core enclosed in a layer of phospholipids and proteins. nPHB is in an amorphous state and when exposed to extracellular environment, denatures to semi-crystalline PHB (dPHB). Biodegradation of PHB is classified into two distinct categories of extracellular and intracellular degradation. Active mobilization of nPHB by the

producer is known as intracellular degradation. Utilization of dPHB by the extracellular enzymes secreted by a PHB degrading microorganism is extracellular degradation [12, 13]. Accumulated nPHB in a cell is enzymatically mobilized by intracellular PHB depolymerase (iPhaZ). System of intracellular PHB mobilization is not as clearly understood as extracellular PHB degradation system. First report of *in-vivo* PHB depolymerase system was in 1964 in *Rhodospirillum rubrum* [14]. Thereafter, numerous intracellular PHB depolymerases have been characterized. iPhaZ of *Paracoccus denitrificans*was previously reported to degrade protease treated nPHB granules to 3hydroxybutyrate (3HB) dimers and oligomers [15]. A novel iPhaZ was identified in *Bacillus thuringiensis* serovar '*israelensis*' which needed pre-treatment of nPHB with trypsin for nPHB degradation to release a considerable amount of 3HB monomers. Previously it has been demonstrated that iPhaZ cannot hydrolyse dPHB [16, 17, 18].
Bacillus thuringiensis IAM12077, a biphasic PHB producer accumulating up to 44% of cell dry weight on glucose was previously studied to optimize nutrient limiting conditions for highest yield of PHB [19].In the present study, an attempt to understand the dynamics of iPhaZ (intracellular depolymerase) activity towards triggering 3HB production in *Bacillus thirungiensis* IAM12077 was made.

MATERIALS AND METHODS

Microorganism used in the study and PHB production under biphasic growth conditions

PHA accumulating *Bacillus thuringienesis* IAM12077 [20]was used in the study. Overnight grown culture of *Bacillus thuringienesis* IAM12077 in nutrient broth was centrifuged at 8000 x g for 10-15 minutes and the culture pellet was transferred to N deficient medium (pH 7) containing (g/L); 20 glucose, 0.2 $MgSO_4$, 0.1 NaCl, 0.5 KH2PO4 , 2.5 Yeast Extract, 0.5 Citric Acid. 250 mL flasks containing 50 mL culture was harvested after 48 h growth on a rotary shaker at 120 rpm at 37^0C.

Extraction and Determination of PHA

Five mL of the 48 h culture was centrifuged at 8000 x g for 15 minutes. Supernatant was discarded and to the pellet 5 mL of sodium hypochlorite was added and the mixture was incubated at 30^0C for 2 hours. Post incubation, the pellet treated with sodium hypocholorite was centrifuged at 10,000 x g for 15 minutes and washed with distilled water, acetone and methanol for washing and extraction, respectively. PHA pellet was then gravimetrically quantified by drying to a constant weight [21].

Time optimization for PHB production

The PHB production in biphasic growth condition was determined at various time intervals with 10 (g/L) glucose as carbon source. PHB production as a function of time was determined in the second phase of growth with glucose.

Effect of pH, time and $MgCl_2$ on *in vivo* PHB depolymerization

B.thurngiensis IAM12077 cells were grown in PHB production mediafor 48 h at 30 °C under shaking conditions. After centrifugation (Remi, 4 °C, 4065 x g, 15 min) cell pellet was suspended in 20 ml of three different pH [0.2M citrate buffer (pH 4.0) distilled water (pH 7.0), and Glycine-NaOH (pH 9.0)] and incubated at 37°C under static condition. Aliquots were withdrawn after vortexing at regular time intervals from4-24 h and stored at –10°C. Withdrawn samples were then centrifuged at 1017 x g for 10 min [22]. Pellet was used for the estimation of biomass; the supernatant was used for the estimation of 3-HB. The identification of the PHB depolymerisation product was carried out.
The effect of $MgCl_2$ on PHB depolymerisation in *B.thuringiensis* IM12077 was studied using the pellet of 1 litre of 24 h grown culture suspended in 20 ml of optimized pHcontaining a specified concentration of $MgCl_2$ (20-60 mM).

Estimation of 3HB

Amount of 3HB released was determined using β-Hydroxybutyrate Colorimetric Assay Kit # 700190 from Cayman Chemicals, USA.

Statistical Analysis

The mean and standard deviation was calculated from at least two independent experiments in duplicate. The analysis of variance was performed using SPSS version 20 statistical package and mean comparisons were carried out using Duncan's multiple range test.

RESULTS AND DISCUSSION

Bacteria which synthesize PHA are divided into two groups. The first group accumulates PHA during stationary phase and requires limitation of N, P, Mg or oxygen,and an excess of carbon source. The most important microorganism for industrial production *Ralstonia eutropha,* belongs to this group. The second group, accumulating PHA during the growth phase, includes *Alcaligenus latus*, a mutant strain of *Azotobacter vinelandii*, *A. bijerinckii* [23] or recombinant strains of *E.coli* bearing the PHA operon of *R.eutropha*. Our strain, *B.thuringiensis* IAM12077, belongs to the 1st group because PHA accumulates

during the stationary phase. It has been reported to produce 44% PHB in biphasic growth condition indicating that while the 1st phase is used for the development of biomass, the 2nd phase is preferentially utilized for PHB production by the cells [21].

Using the optimized conditions of growth, in glucose unbuffered media, nutrient limitation gave highest PHB yield of 0.5 g/L averaging cell accumulation of 35 to 44 % (Table 1). Maximum PHB accumulation was observed at 48 hours in the second phase of growth (Figure 1) with comparative high DCW. PHB yield remained constant till 72 hours. A slight drop in accumulation (0.48-0.45g/L) was observed in 96 hours. Statistically there was no significant difference in PHB yield from 24-96 hours, depicting the stable state of PHB in the cell. For further studies, polymer extracted after 48 hours of growth in the 2nd phase was used. Residual pH of the system checked at the time of extraction had decreased to pH 5 from pH 7.

Similar observation was reported by Valappil *et al*. (2007) in *Bacillus cereus* SPV wherein the initial pH of the culture decreased from 7 to 4.5 [24]. The authors also observed that the on reaching the minimum pH, there was increased consumption of glucose leading to rapid accumulation of PHB during stationary phase. Concentration of PHB remained constant from the period of highest accumulation till 64 hours. Stability of the produced polymer concentration implies the lack of polymer utilization to support growth or sporulation. The drop in pH is attributed to the secretion of acids associated with PHB production [23, 25, 26]

Depolymerisation of PHA to their respective hydroxycarboxylic acid has been the method of choice to obtain enantiomerically pure (R)-(-)-hydroxycarboxylic acid, which finds its use as chiral monomer intermediate for synthesis of some pharmaceuticals & pesticide. Lee [27] has shown production of different (R)-(-)-hydroxycarboxylic acids from corresponding PHAs by their *in vivo* depolymerization in *Alcaligenes latus*, *Ralstonia eutropha*, *Pseudomonas* sp. The depolymerisation efficiency was found to be varying and was dependent on pH and temperature. By establishing an efficient *in vivo* PHB depolymerization system, 3HB can be extracted as a by-product of growth replacing the current practice of degradation post production and extraction of PHB.

In an attempt to understand the optimal assay requirements of iPHAZ of *B. thuringienesis* IAM12077, influencing factors like resting time, pH and concentration of Mg^{2+} were evaluated. Different time intervals (h) (4, 16, 20, and 24) were tested to estimate the amount of 3HB released from resting cells of *Bacillus thuringiensis* IAM12077. At the same time, for each time interval, three pH levels (4, 7 and 9) were set up to explore the effect of pH on *in- vivo* PHB depolymerase. Mg^{2+} ions are known to enhance the activity of PHB depolymerase [22]. The *in vivo* depolymerase exhibited broad pH optima and showed similar levels of 3HB release in all the three pH tested (Figure 2). However, the highest 3HB yielding combination of resting cell incubation in pH 9.0 was further tested to determine the effect of Mg^{2+} ions (0µg, 20 µg, 40 µg, 60 µg). Highest yield of 3 HB (0.56g/L) from resting cells was obtained under the following conditions of pH 9, 8 h resting and concentration of 60 µM $MgCl_2$(Figure 3).

The study by Uchino *et al.* (2008) [28] reported low 3HB production at acidic pH of 4, increasing the pH to 10 produced significantly higher levels of 3HB. A similar yield of 3HB in the wild type of *Methylobacterium* sp ZP24 was reported by Nath *et al.* (2005) [22] of 0.6g/L at pH 4 with 40mM of $MgCl_2$indicating strain specific profiles of iPHAZ. 3HB dehydrogenase further breaks down 3HB to acetoacetate. A mutant deficient in the enzyme will show increased accumulation of 3HB due to subsequent arrest of PHB mobilization within the cell. Nath *et al*., (2005) also made an attempt to further increase the iPHAZ activity by inducing random mutation using chemical mutagens to obtain 3HB-dehydrogenase mutants.

Bacillus thuringiensis possesses an inherent error free SOS repair mechanism which makes application of a similar strategy to increase 3HB yield through mutated iPHAZ, ineffective[29].This suggests that for the current organism, mutation strategies must be site directed to achieve the desired results.

Analyzing the PHB production profile of *Bacillus thuringiensis* IAM12077 helped in the identification of the specific time during which PHB productivity was maximum. Harvesting the polymer at the said time and later subjecting it to enzymatic degradation can offer an economical production strategy of 3HB.

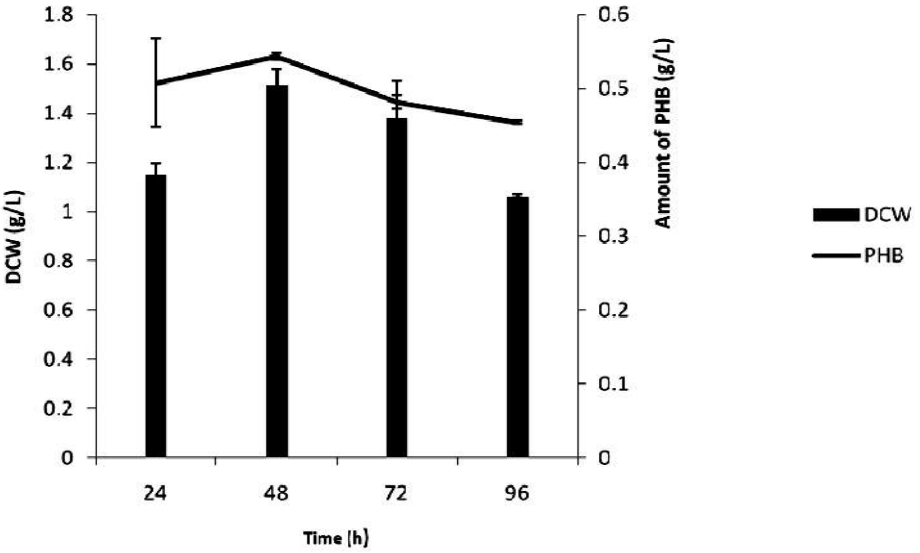

Figure 1: Time course of PHB production by *B.thuringiensis* IAM 12077

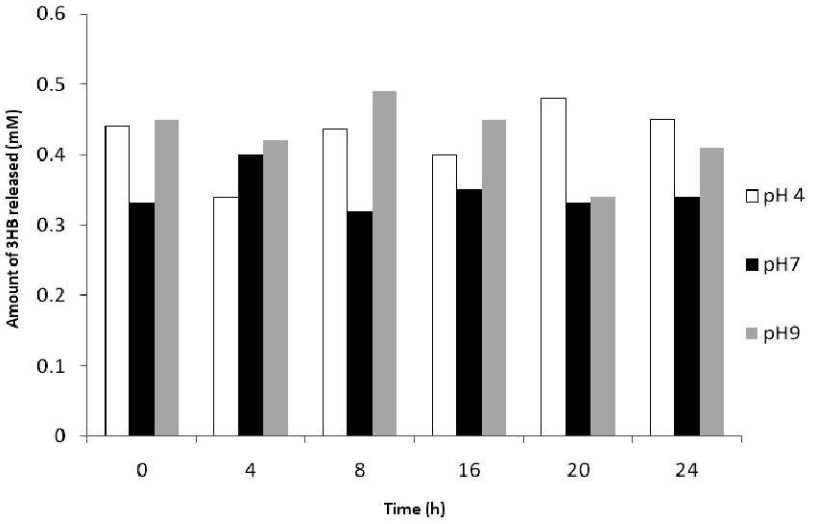

Figure 2: Effect of time and pH on *in vivo* PHB depolymerization

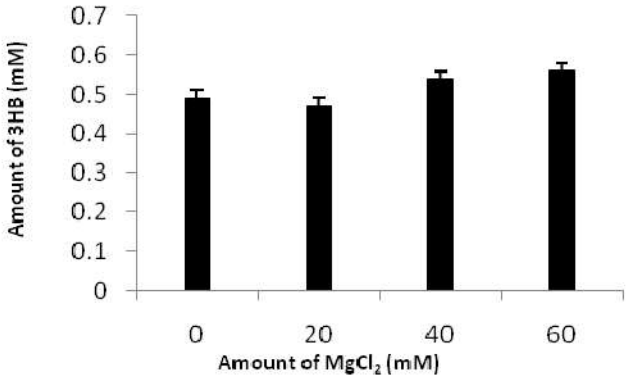

Figure 3: Effect of $MgCl_2$ on *in vivo* PHB depolymerization

Table 1: Production kinetics of PHB in *Bacillus thuringiensis* IAM12077

Time (h)	24	48	72	96
PHB(g/L)	0.508	0.544	0.482	0.454
DCW(g/L)	1.148	1.514	1.378	1.06
QpgPHB/h	0.0211	0.011	0.006	0.004
QxgDCW/h	0.048	0.032	0.019	0.011
Yp/s gPHB/[S]	0.025	0.027	0.024	0.023
Yx/s gDCW/[S]	0.057	0.076	0.069	0.053
Pp/x Yp/x/Yx/s	0.44	0.36	0.35	0.43
% Production	44.2	35.9	34.97	42.8
QpgPHB/DCW/hr	0.0018	0.0074	0.0049	0.0045

CONCLUSION

A two step process of PHB extraction followed by degradation can be combined if the production parameters were varied to support *in-vivo* PHB degradation. In order to formulate such strategies, the *in-vivo* degradation profile must first be understood.

An understanding of the *in vivo* effect of pH in the accumulation and degradation PHB offers novel production strategies for increased production of either the polymer or its monomeric units.

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