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POLY(3)HYDROXYBUTYRATE (PHB) PRODUCTION IN *BACILLUS THURINGIENSIS* IAM 12077 UNDER VARIED NUTRIENT LIMITING CONDITIONS AND MOLECULAR DETECTION OF CLASS IV *PHA* SYNTHASE GENE BY PCR.

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ABSTRACT

Limiting nutrient in the culture conditions is crucial for PHA production. In this study, *B. thuringiensis* IAM 12077 was grown in different culture conditions with limitation of potassium, nitrogen, sulphur and phosphorous. In all the limiting conditions used, a similar range of PHAs were produced with glucose as carbon source (10g/L). The PHA yields and accumulation obtained were found to be 1.61g/L, 65.9%; 1.86g/L, 64.1%; 1.66g/L, 65.9% and 1.7g/L, 68.9% dry cell weight (DCW) for potassium, nitrogen, sulphur and phosphorus limitations, respectively, showing that any of the above deficiency can trigger PHB production in this strain. Time course study in N_2 deficient medium revealed that though cell biomass remained almost steady from 0h after transfer to production medium till the end of fermentation (120h), PHB yield showed increase from 0.533 g/L at 0h to 4g/L by 48h and later gradually decreased, with PHB accumulation increase from 11% to 77% by 96h. PCR identified the presence of class IV *pha* synthase in *B.thuringiensis* IAM12077 suggesting the capability of this strain to accumulate different PHA monomers from structurally unrelated carbon sources which can be explored further.

KEY WORDS : *B.thuringiensis* IAM12077, nutrient limitation, PHB production, Class IV *pha* synthase, PCR

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INTRODUCTION

Polyhydroxyalkanoates (PHAs) are polyesters of 3-, 4-, 5- and 6-hydroxyalkanoic acids, produced by a variety of bacterial species under nutrient-limiting conditions with excess carbon. PHAs exhibit a high degree of polymerisation with molecular masses up to several million Daltons[1]. They are biodegradable, insoluble in water, non-toxic, biocompatible, piezoelectric, thermoplastic and/or elastomeric. These features make them suitable for applications in the packaging industry, medicine, pharmacy, agriculture, food industry, as raw material for enantiomerically pure chemicals and the production of paints[1]. About 150 different types of PHAs have been isolated from different bacterial species. The composition of the polymer synthesized is governed by two main factors, i.e. the bacterial strain used and the carbon source utilized to grow the bacteria. Currently, PHAs are produced on the industrial scale exclusively using Gram-negative bacteria (such as *Cupriavidus necator*, *Methylobacterium organophillum*, *Pseudomonas oleovorans* and recombinant *Escherichia coli*)[2]. Pyrogenic outer lipopolysaccharide (LPS) endotoxins copurify with PHAs produced. The presence of LPS induces a strong immunogenic reaction and is therefore undesirable for the biomedical application of the PHAs [2,3]. Gram-positive bacteria lack LPS and are hence better sources of PHAs for use in biomedical applications[4]. However, the presence of lipidated macroamphiphiles in PHAs isolated from this group of bacteria needs to be investigated[4]. The PHA synthases from the genus *Bacillus* have been assigned to a new class of PHA synthases because of their differences in subunit composition compared with the other three classes of PHA synthases[4]. Two proteins, PhaC, the normal PHA synthase enzyme, and PhaR have been proven to be essential for the PHA synthase activity by using *in vivo* and *in vitro* methods[5,6] . Detection of these classes of *pha* synthase in isolates would give an insight into the polymerization ability of the strain in a culture independent manner. The aim of the present study was to optimize PHB production by *B.thuringiensis* IAM12077 under different culture conditions with limitation of potassium, nitrogen, sulphur and phosphate. In addition, the molecular basis of PHA accumulation has been investigated.

MATERIALS AND METHODS

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

PHB accumulating *Bacillus thuringiensis* IAM 12077 isolated in the previous study was used[7]. The strain showed capability to utilize a variety of mono and disaccharides, pentoses and hexoses for PHB production[7]. It also produced appreciable levels of PHB from lactose and whey[8] and several agroindustrial residues[9]. 24 h Nutrient broth grown culture of *Bacillus thuringiensis* IAM 12077 was centrifuged at 8000xg for 10-15 min and the culture pellet was transferred to N_2 deficient medium (pH 7.0) containing (g/L) 10 starch, 0.2 $MgSO_4$, 0.1 NaCl, 0.5 KH_2PO_4, 2.5 peptone, and 2.5 yeast extract[10] . Production studies were carried out in 250ml flasks containing 50 ml culture medium and incubated at 37°C on a rotatory shaker at 120 rev/ min for 48h. The PHB production in biphasic growth condition was performed with glucose (10g/L).[11] Further, PHB production after 48h was determined in the second phase of growth[10].

Extraction and determination of PHB

After 48 h incubation at 37°C, 5 ml of the culture was taken and centrifuged at 10,000 x g for 15 min. The supernatant was discarded and the pellet was treated with 5 ml of sodium hypochlorite and incubated at 30°C for 2 h. After incubation, the mixture was centrifuged at 10,000 rpm for 15 min and then washed with distilled water, acetone, methanol and diethyl

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ether respectively for washing and extraction. Finally the residue was extracted with boiling chloroform and filtered through Whatman No. 1 filter paper. The chloroform extract was evaporated to dryness[12]. Determination of PHB was performed routinely by dry weight estimation. For dry weight estimation, the pellet after extraction was dried to constant weight[11].

Media optimization (PHA production under various nutrient limiting conditions

a. Nitrogen

A semi-defined PHA production medium, Kannan and Rehacek medium[13] , was used with modification for the production of PHA under nitrogen-limiting conditions. The pH of the medium was adjusted to 6.8 before autoclaving. The medium contained (g/L) glucose 20; yeast extract, 2.5; KCl, 3; $(NH_4)_2SO_4$, 5.0 and 100 ml of defatted soybean dialysate (prepared from 10 g of defatted soybean meal in 1000 ml of distilled water for 24 h at 4ºC). The inoculum was prepared in 250-ml Erlenmeyer flasks containing 30-ml sterile nutrient broth. Flasks were incubated at 30ºC for 24 h on a rotary shaker at 250 rev /min. A total of 10 ml of these seed cultures was transferred into 50 ml sterile Kannan and Rehacek medium in one 250mL flasks, at 30ºC and a speed of 250 rev /min. The shaken flask cultures were harvested and assayed for PHA production at various time intervals.

b. Potassium

A previously reported potassium-deficient production medium[14] was used for PHA production under potassium-deficient conditions. The pH of the medium was adjusted to 6.8 before autoclaving. The medium contained (g /L) glucose 20; peptone 10; casein 5; NaCl 13. The inoculum was prepared as above and the inoculated production medium was used for 300-ml shaken flask cultures as described above.

c. Sulphur

Kannan and Rehacek medium[13] containing 8g/L ammonium chloride instead of the ammonium sulphate was used for PHA production under sulphur-deficient condition. The pH of the medium was adjusted to 6.8 before autoclaving. The inoculum was prepared as above and the inoculated production medium was incubated and treated as described above.

d. Phosphate

A previously reported phosphate-deficient production medium[15] (Rubia *et al.,* 1986) was used for PHA production under phosphate-deficient conditions. The pH of the medium was adjusted to 6.8 before autoclaving. The medium contained (g/ L) glucose, 20; potassium nitrate, 0.5; $MgSO_4.7H_2O$, 0.2; calcium chloride, 0.1; sodium chloride, 0.1. The medium also contained (mg /L) K_2HPO_4, 8.0 and KH_2PO_4, 2.8. The inoculum was prepared as above and the inoculated production medium was used for 50 mL shaken flask cultures as described by Valappil *et al*., 2007). [16]

Comparison of PHB production by B.thuringiensis IAM12077 on two different Nitrogen deficient media

24 h Nutrient broth grown culture of *Bacillus thuringiensis* IAM 12077 was centrifuged at 8000rpm for 10-15 min and the culture pellet was transferred to N_2 deficient medium (pH 7.0) containing (g/L) 10 glucose, 0.2 $MgSO_4$, 0.1 NaCl, 0.5 KH_2PO_4, 2.5 peptone, and 2.5 yeast extract[10]. The inoculum was prepared in 250-ml Erlenmeyer flasks containing 30-ml sterile nutrient broth. Flasks were incubated at 30ºC for 24 h on a rotary shaker at 250 rev /min. A total of 10 ml of these seed cultures was transferred into 50 ml sterile medium in one 250mL flasks, at 30ºC and a speed of 250 rev /min[11]. The shaken flask cultures were harvested and assayed for PHA production at various time intervals. PHB production under N_2 deficient Kannan & Rehacek media (1970)[12] and the above mentioned production media by Mercan et al. were compared.

PCR detection of Class IV pha synthase

The nucleotide sequence of B. megaterium PHA gene cluster (Gene bank No 4160467)

was used as primer[17]. Primers were synthesized by Biotech Desk Pvt. Ltd., Tarnaka, Secunderabad. DNA isolation was carried out using Chromous Biotech–Genomic DNA Minispin: Bacteria kit (#RKN15) procured from Chromous Biotech Pvt. Ltd., Sahakar nagar, Bangalore. The sequences of the primers and the expected amplicon size is given below (Table 1).

Table1
Sequences of the Primers used and the expected amplicon size.

Primer Name	Sequence	Corresponding region of the *B.megaterium* gene	Expected amplicon size (bp)
BIF	5'-AACTCCTGGGCTTGAAGACA-3'	5900–5920	
BIR	TCGCAATATGATCACGGCTA	6509–6489	590

The optimized PCR reaction mixture was used to amplify the genomic DNA of bacteria used as templates. The amplification was carried out using genomic DNA (1 µl) as template and B1F: B1R set of primers reaction mixture contained: 1X PCR amplification buffer [10mM Tris-HCl (pH 9), 1.5mM $MgCl_2$, 50mM KCl and 0.1%gelatin], 200µM each deoxynucleotide triphosphate, 1U Taq DNA polymerase, 2.5 µM each primer, in 50 µl reaction mixture. The thermal cycler program run on a Corbett Thermocycler consisted of one cycle at 94°C for 0.3min, 50- 60°C for 0.45 min (standardized), 72°C for 1.15min followed by incubation at 72°C for 10 min and a final incubation at 4°C. PCR products were analyzed by gel electrophoresis in 1.2% agarose gels containing ethidium bromide (5µg/ml). The amplified DNA fragments were visualized by U.V illumination and the images were captured using a Nikon digital camera.

Statistical analysis

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

RESULTS AND DICUSSION

PHB production undergrowth limiting conditions

PHB production by *B.thuringiensis* IAM12077 under different culture conditions with limitation of potassium, nitrogen, sulphur and phosphate was investigated. Under nitrogen limiting conditions, cell biomass reached 2.93 g/L, PHB yield obtained was 1.866g/L amounting to accumulation of 64.16% DCW. Under sulphur deficient conditions, cell biomass reached 2.53g/L supporting a PHB yield and accumulation of 1.66g/L and 65.9% DCW. Similarly phosphorous and potassium limitation also yielded cell biomass of 2.46g/L and 2.73g/L with PHB yields of 1.7g/L and 1.66g/L with accumulation values of 68.93 and 60.93%, respectively. Thus in all the limiting conditions used, a similar range of PHAs was produced, with glucose as carbon source (10g/L), respectively (Fig.1), suggesting that any of the above nutrient deficiency can trigger PHB production in this strain.

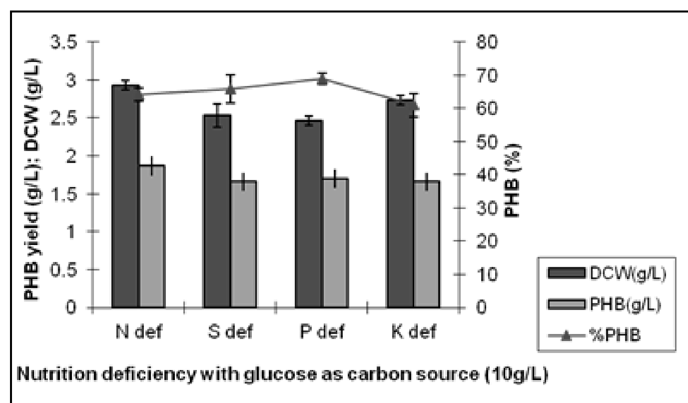

Figure 1
Media optimization with nutrition deficiency using glucose (10g/L) as carbon source under biphasic conditions.

Valappil *et al.,* (2008)[18] has reported varied production of PHA by *B. cereus* SPV in different culture conditions with limitation of potassium, nitrogen, sulphur and phosphate. The nitrogen-deficient medium used in their study was by far the best medium in terms of both the cellular growth (2 g/ L) and PHA accumulation (38% DCW) which is in agreement with our observation. The authors further observed production of P (3HB-3HV) copolymer under the potassium limiting condition, using glucose as the carbon source which needs to be explored in our study.

PHB production by B.thuringiensis IAM12077 in alternate N_2 deficient medium
Comparative Poly β-hydroxybutyrate (PHB) production by *B.thuringiensis* IAM 12077 was investigated in biphasic growth conditions using glucose in two different media- Mercan et al. (2002)[10] and Kannan & Rehacek (1970)[13]. Though in both cases nitrogen deficiency triggered PHB production, higher level of PHB accumulation was achieved by Kannan & Rehacek medium (1.09 folds) though PHB yield (1.76 folds) was higher in N_2 d efficient medium of Mercan et al. (2002). The higher yield of PHB in the Mercan medium is due to higher biomass achieved in this medium as compared to Kannan & Rehacek medium.

Table 2
Comparison of PHB production by B.thuringiensis IAm12077 in two different N_2 deficient media

N_2 deficient Media	Dry cell weight (DCW) (g/L)	PHB yield (g/L)	PHB accumulation (%) DCW
Mercan et al. (2002)	5.7	3.3	58.5
Kannan & Rehacek Media (1970)	2.93	1.866	64.16

Comparison of growth and PHB production kinetics of B.thuringiensis IAM 12077 in two different N_2 deficient media

The time course of aerobic growth and PHB production by *B.thuringiensis* IAM12077 under optimized conditions was measured at different time intervals after transfer into the nitrogen deficient (N_2) medium (Fig. 2). Cell biomass remained almost steady from 0h after transfer to production medium till the end of fermentation (120h). However, PHB yield showed increase from 0.533 g/L at 0h to 4g/L by 48h and later gradually decreased, with PHB accumulation increase from 11% to 77% by 96h. The PHB yield and PHB accumulation increased from 1.2g/L to 4g/L (3.33 fold) and 43.33 % to 77.9 % (1.79 fold), respectively. The production profile of the strain shows that after transfer to N_2 deficient medium, PHB production can be achieved within 24h after which the PHB level is steadily maintained for almost 120h of growth, showing high productivity by the strain. The slight decrease in PHB production after 30h could be due to the fact that the microorganism could synthesize PHB until the sporulation stage and after that the remaining bacterial cells consume the PHB. Yilmaz *et al.,* (2005)[19] reported that some *B. sphaericus* strains were able to synthesize PHB up to 32.55% (w/v). Yuksekdag (2004)[20] reported *B.megaterium* and *B.subtilis*, both to have produced maximum PHB at the 45th hour of incubation followed by a similar decline. Our strain achieves maximum level of PHB within 24h under similar growth conditions, making it a more industrially viable strain with respect to faster productivity. Bacteria able to synthesize PHA can be divided into two groups. The first group, accumulating PHA during the stationary phase, requires limitation of N, P, Mg and oxygen, for example, and an excess of carbon sources. 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* [21] 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 64.1% 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[11].

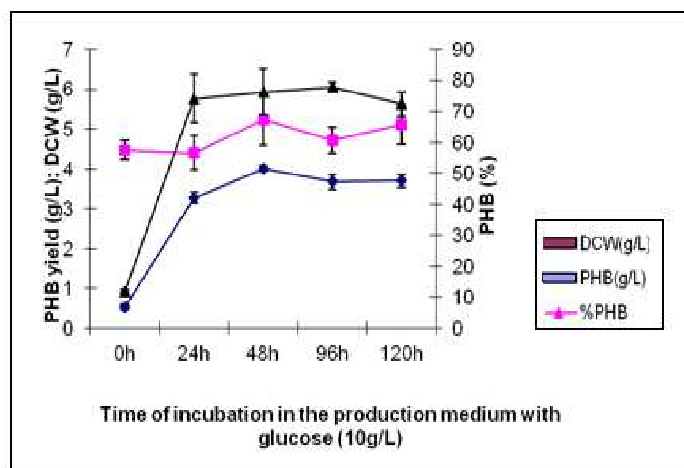

Figure 2
Time optimization for PHB production using media containing Glucose (10g/L) under biphasic growth conditions.

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Detection of class IV *pha* synthase gene from *B.thuringiensis* IAM 12077

Chen *et al.,* (1991) [22] reported that many species of *Bacillus* produce PHA and since then the gene for PHA synthesis has been cloned from *B. megaterium*. It was of interest to see whether primers based on that *pha* synthase sequence could enable the identifications of other PHA-producing *Bacillus* of a type similar to that present in *B. megaterium*. Polymerase chain reaction was carried out with *B.thuringiensis* with the B1F : B1R set of primers. The isolate reacted positively and the amplicon size observed was identical to that obtained (about 600 bp) with DNA from *B. megaterium* (Fig.3; Table 1). Similarly, amplification occurred with DNA from *B. laterospsorus* and *B. sphaericus* when the set of *B. megaterium* primers were used[17]. PHA-producing bacteria are classified into three groups based on *pha* synthases present in them. Recently it has been shown that *B. megaterium* possesses enzymes that are distinctively different from all known *pha* synthases in sequence and arrangement and hence may form a separate class by itself [5]. PCR protocol has been developed for bacteria isolated from the environment by colony PCR[23] and for specific detection of genes coding medium chain length PHA in *Pseudomonas* spp.[24] . Shamala *et al.,* (2003) have studied 10 isolates of *Bacillus* spp. capable of producing PHA similar to *B. megaterium* type and identified them using the PCR[16]. Overall the results confirm that the PCR protocol is applicable for rapid detection of PHA producers among *Bacillus* spp. Furthermore, *B. megaterium* type of PHA producer may be detected even in the absence of growth of bacteria in limiting media for the production and accumulation of PHA.

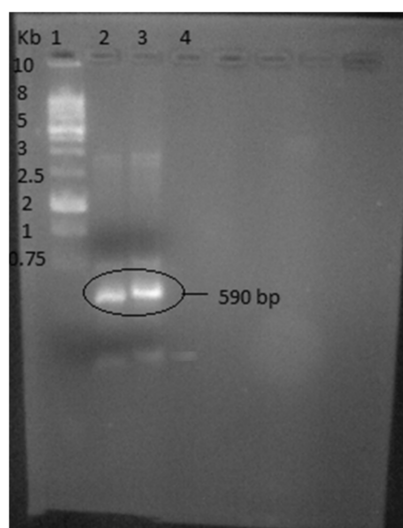

Figure 3

Amplified PCR product, where M-marker DNA (Lambda digest); lanes 2 & 3 represent the sample DNA loaded in 5µl, 10µl, repectively and lane 4- negative control (E.coli genomic DNA).

CONCLUSION

These results show, for the first time, that *B. thuringiensis* IAM12077 is capable of producing PHAs under different culture conditions of potassium, nitrogen, sulphur and phosphate limitations. Detection of class IV *pha* synthase gene in this strain shows the possibility to explore this strain for synthesis of a variety of PHAs feeds of related and unrelated

substrates. *Bacillus cereus, Bacillus subtilis, Bacillus megaterium* and *Bacillus thuringiensis* can utilize a wide variety of agroindustrial materials for PHB production. This can be exploited for the production of PHB at commercial level. Utilisation of Agro-industrial materials in production of biodegradable plastic (PHB) will not only ensure the low production cost but also solve the problem of management of waste material to a certain level[25].

CONFLICT OF INTEREST

Conflict of interest declared none.

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