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Mutagenesis of *Bacillus thuringiensis* IAM 12077 for increasing poly (-β-)hydroxybutyrate (PHB) production

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Abstract: A gram-positive bacterium that accumulated PHB was isolated from local garden soil in Bangalore. Based on its morphological and physiological properties, and nucleotide sequence (about 1.5 kb) of its 16S rDNA it was identified as *Bacillus thuringiensis* IAM 12077. In this study the PHB-producing capacity of putative mutant strains of *Bacillus thuringiensis* IAM 12077 was evaluated. A *Bacillus* sp. identified as a *Bacillus thuringiensis* IAM 12077 strain capable of producing 10%-15% dry cell weight (DCW) PHB when grown in nutrient broth for 48 h was subjected to random mutagenesis [physical (UV) and chemical (acridine orange)]. Among the UV-mutants screened, 19 putative mutants produced more PHB than the parental, while 2 strains produced less. Of these mutants, B8 exhibited promising PHB accumulation (24.68%; 1.54-fold) with more PHB production (1.3 g/l; 5.4-fold) than the parental strain. Chemical mutagenesis yielded putative mutants, of which 6 had a decrease, 3 had no change, and 2 had an increase (B3, C2) in PHB production. While the increase in accumulation (19.69%, 1.43-fold and 22.22%, 1.62-fold, respectively, for B3 and C2) was comparable to the increase shown by the UV mutant (B8), the yields did not concomitantly increase (0.43g/l, 1.13-fold and 0.6g/l, 1.57-fold, respectively).

Key words: *Bacillus thuringiensis* IAM 12077, Poly (-β-) hydroxybutyrate, random mutagenesis

Poli (-β-) hidroksibutirat üretiminin artışı için *Bacillus thuringiensis* IAM 12077'nin mutagenezi

Özet: PHB biriktiren gram pozitif bir bakteri Bangalore'nin yerel bahçe topraklarından izole edildi. Morfolojik ve fizyolojik özellikleri ve 16S rDNa nükleotit dizi analizine (yaklaşık 15 kb) göre *Bacillus thuringiensis* IAM 12077 olarak tanımlandı. Bu çalışmada *Bacillus thuringiensis* IAM 12077'nin varsayılan mutant suşlarının PHB üretim kapasiteleri çalışıldı. 48 saat nutrient brothda büyüdüğünde % 10-15 kuru hücre ağırlığında PHB üretme yeteneğine sahip *Bacillus thuringiensis* IAM 12077 olarak tanımlanan bir *Bacillus* sp. rasgele mutagenlere [fiziksel (UV) ve kimyasal (akridin oranj)] tabi tutuldu. Total UV mutant taramasında, iki suş PHB veriminde bir azalma gösterirken, 19 varsayılan mutant suş PHB üretiminde ebeynlerin üzerinde artış göstermiştir. Bu mutantların, (B8) ebeveyn suşların üzerinde PHB üretiminde bir artış ile (% 24,68; 1,54 kat) PHB birikimi ümit verici görülmektedir. Kimyasal mutajenlerde PHB üretiminde varasayılan mutantların yalnızca iki tanesinde (B3, C2) bir artış gözlenirken, 6 tanesinde bir azalma, 3 tanesinde önemsiz değişiklikler görülmüştür. Birikimdeki artış UV mutant B8 suşu ile görülen artış ile (B3 ve C2 için sırasıyla % 19,69; 1,43 kat ve % 22,22; 1,62 kat) karşılaştırılabilirken, verimlerde aynı anda bir artış (sırasıyla 0,43g/l; 1,13 kat ve 0,6g/l; 1,57 kat) bulunamamıştır.

Anahtar sözcükler: *Bacillus thuringiensis* IAM 12077, Poli (-β-) hidroksibutirat, rasgele mutajenler

Introduction

Global environmental concerns and solid waste management problems have generated considerable interest in the development of biodegradable plastics with the desired physical and chemical properties of conventional synthetic plastics. PHBs have found a wide range of applications as biodegradable and biocompatible polymers; however, the wide-spread substitution of conventional plastics has been limited by high production costs (1-4). Therefore, more efforts need be devoted to making this process economically feasible by increasing our understanding of the PHB accumulation process and improving productivity. In biotechnological terms, cheap substrates, mutations, and genetically modified high PHB-yielding bacteria (or plants) can be used for biopolymer production.

A number of *Bacillus* species have been reported to accumulate 9%-44.5% dry cell weight (DCW) PHA (5-9). The amylase and protease-producing ability of *Bacillus* isolates makes them attractive for cost-effective PHB production from alternative carbon sources, like agro-waste and food waste rich in starch and protein. We isolated a *Bacillus* sp. identified as a *Bacillus thuringiensis* IAM 12077 strain capable of producing 0.2 g/l of PHB when grown in nutrient broth for 48 h, which is comparable to that produced by *B. megaterium*, and higher than that produced by *B. squirmus* and *B. subtilis* (6); however, to date, no study has reported the attempted mutagenesis of *B. thuringiensis* strains for increased PHB production. Hence, the present study aimed to enhance PHB accumulation in the *B. thuringiensis* strain via random mutagenesis.

Materials and methods

Isolation of PHB-producing *Bacillus* sp.

Different soil samples were collected from Bangalore and its environs. Screening to obtain spore-forming bacteria was performed by heating samples at 80 °C for 10 min and then plating them on nutrient agar (NA). The resulting bacterial colonies were tested for PHB accumulation by staining with Sudan black (0.3% in 96% ethanol). Bluish-black colonies indicating PHB production were characterized. Different PHB-positive *Bacillus* spp. identified as gram-positive spore-forming rods were chosen for further study.

Characterization of the isolated bacteria

The morphological and physiological properties of the isolates were investigated according to *Bergey's Manual of Determinative Bacteriology* (10). The sequencing of 16S rDNA and taxonomic studies of strain IAM 12077 were performed at Chromous Biotech Pvt., Ltd., Bangalore, India. A partial 16S rDNA fragment (~1.5kb) was amplified using high-fidelity PCR. The PCR product was sequenced bi-directionally using the forward, reverse, and internal primers. The sequence data were aligned and analyzed in order to identify the bacterium and the nearest related strains.

Production of PHB in nutrient broth

The strain was grown in nutrient broth culture medium containing 2.5% peptone, 1.0% yeast extract, and 0.5% beef extract. Cultures (50 ml in 250-ml conical flasks) were inoculated with a 5% (v/v) inoculum and incubated at 37 °C with vigorous orbital shaking at 120-150 rpm. To make a solid medium, 1.5% agar was added to the broth.

Mutant isolation

UV light (254 nm) and acridine orange (75 μg/ml) were used as physical and chemical mutagens, respectively. In mutant isolates, concentrations with a 99.99% lethality ratio were used. The possible mutants were isolated on nutrient agar and the colonies were stained with Sudan black (0.3%). Bluish-black colonies indicative of PHB production were selected for PHB analysis. PHB production of the mutants was determined by growing the cells in nutrient broth for 48 h (6).

Extraction and determination of PHB

After 48 h of incubation at 37 °C, 5 ml of the culture was removed and centrifuged at 8000 rpm 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 ether. Finally, the residue was extracted with boiling chloroform and then was filtered through Whatman No. 1 filter paper. The chloroform extract

was evaporated to dryness (11). Determination of PHB was performed by dry weight estimation. For dry weight estimation, following extraction the pellet was dried to constant weight. The mean and standard deviation were calculated from at least 3 independent experiments run in duplicate.

Cell dry weight

After centrifugation of the culture medium, the supernatant was discarded and the cell pellet was washed with distilled water. The washed pellet was re-suspended in 1 ml of distilled water, transferred to pre-weighed boats, and dried to constant weight at 60 °C. The dry weight of the cells was determined by drying the washed cells to constant dry weight.

Statistical analysis

Mean and standard deviations were calculated from at least 2 independent experiments run in duplicate.

Results

Of the 66 *Bacillus* spp. screened from different soil samples, the isolate that yielded 0.2 g/l PHB and 10%-15% DCW of PHB when grown in nutrient broth for 48 h was selected for further study. The morphological and taxonomical features of the isolate were examined (Table 1). Based on its biochemical characteristics, the isolate was characterized and identified as the endospore-forming, gram-positive *Bacillus thuringiensis*. Analysis of the partial nucleotide sequence of the 16S rDNA (~1.5 kb) identified the isolate as *Bacillus thuringiensis* IAM 12077, with a homology close to that of *Bacillus cereus* IAM 12605 (Figure 1).

In the present study production of PHB by *Bacillus thuringiensis* IAM 12077 was detected when in nutrient broth for 24-48 h. Growth of the strain increased up to 96 h (0.25 g/l-1.9 g/l), but PHB yield increased only up to 48 h (0.066 g/l-0.2 g/l) after which time production declined.

In the the present study mutagens (physical and chemical) were applied to PHB-producing *B. thuringiensis* IAM 12077 (11%-13%) as a strategy for obtaining a strain with higher PHB production. Chemical mutagenesis yielded putative mutants, of which 6 strains (A4, B2, C1, D3, D1, and E1) showed a decrease in PHB production, 3 (A2, C3, and E2) showed no change, and 2 strains showed an increase (B3 and C2) (Figure 2a). While the increase in PBH accumulation (19.69%, 1.43-fold and 22.22%, 1.62-fold, respectively, for B3 and C2) was comparable to

Table 1. Physio-biochemical characteristics of the isolate, *Bacillus thuringiensis* IAM 12077.

Test	**Observation**
Gram's stain	Gram-positive
Spore staining	Central, oval, bulging
Cell shape	Rods
Cell size	> 3 μm
Colony character	White, raised, irregular
Motility	+
Catalase	+
Oxidase	-
Indole	-
Methyl Red	-
Voges-Proskauer	-
Citrate utilization	+
Casein hydrolysis	+
Aesculin hydrolysis	+
Starch hydrolysis	+
Urea hydrolysis	-
Growth at 50 °C	+
Growth in 10% NaCl	-
Anaerobic growth	+
TSI	Acid slant/alkaline butt, gas, no H_2S
Sugar Utilization	**Result (acid/gas)**
Glucose	+/-
Galactose	+/-
Arabinose	+/-
Mannitol	-/-
Maltose	+/-
Mannose	+/-
Raffinose	-/-
Rhamnose	-/-
Sucrose	+/-
Salicin	+/-
Lactose	+/-
Fructose	+/-
Xylose	+/-

+ positive; - negative.

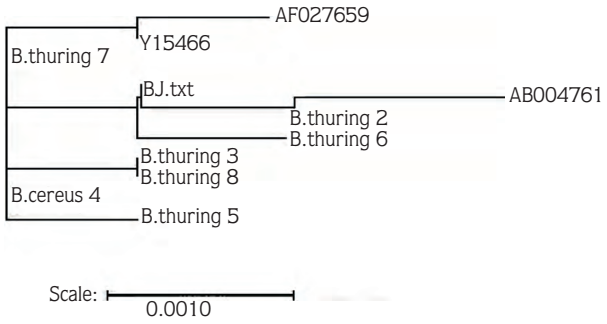

Figure 1. Phylogenetic tree representing close homologs of *Bacillus thuringiensis* IAM 12077. The tree was constructed by neighbor-joining on a p distance matrix.

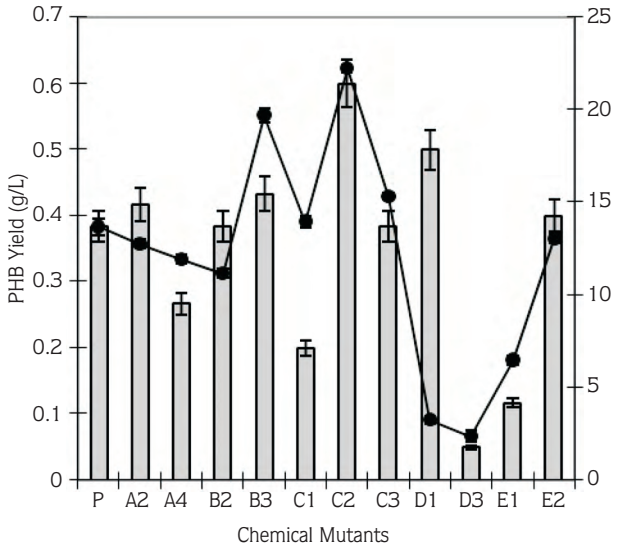

Figure 2a. The effect of chemical mutagenesis on PHB yield (-●-) and accumulation (■) in *Bacillus thuringiensis* IAM 12077.

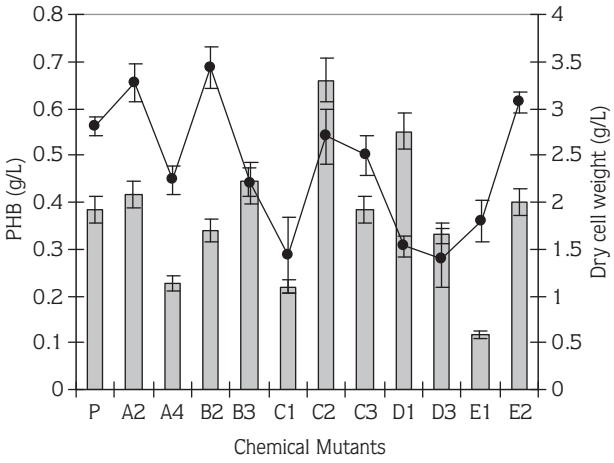

Figure 2b. PHB yield (■) and DCW (-●-) of chemical mutants of *Bacillus thuringiensis* IAM 12077 strains.

the increase shown by the UV mutant B8, their yields did not concomitantly increase (0.43 g/l, 1.13-fold and 0.6 g/l, 1.57-fold, respectively). This can be attributed to the decrease in cell density in the mutant strains (2.2 g/l and 2.7 g/l, respectively), as compared to the parental (2.8 g/l) (Figure 2b). As cell density in the mutant strains did not increase, the increase in PHB production can be credited to the mutant strains' increased capacity for PHB accumulation.

Among the UV mutants that were screened, 19 putative mutants showed an increase in PHB production, as compared to the parental, while 2 strains showed a decrease. Of the mutants screened, the most promising mutant (B8) showed PHB accumulation (24.68%, 1.54-fold) with an increase in PHB production (1.3 g/l, 5.4-fold), as compared to the parental strain (Figure 3a). The 5.4-fold increase in PHB production of the mutant could be attributed to the 3.5-fold increase in its cell density, as compared to the parental (1.5-5.26 g/l) (Figure 3b).

Discussion

The use of PHB as a substitute for non-biodegradable petroleum-based plastics is substantially more expensive than using fossil-based counterparts and offers no performance advantage other than biodegradability. Furthermore, the

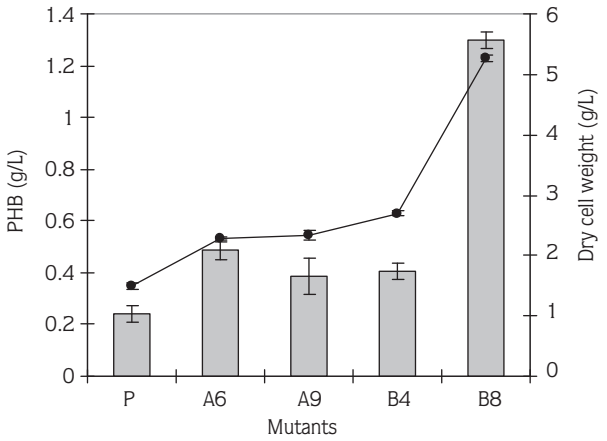

Figure 3a. The effect of UV mutagenesis on PHB yield (-●-) and accumulation (■) in *Bacillus thuringiensis* IAM 12077.

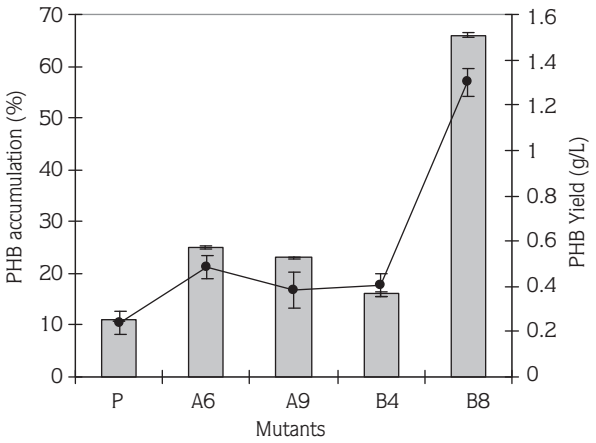

Figure 3b. PHB yield (■) and DCW (-●-) of UV-mutagenized *Bacillus thuringiensis* IAM 12077 strains.

continuous depletion of petroleum sources has emphasized the need for biodegradable microbial plastics. Today, most research efforts in this field concentrate on the isolation of PHB-producing microorganisms from different sources and improvement in the PHB production ability of microorganisms via less expensive substrates and genetic modification for high-yield biopolymer production.

Mutations, both chemical and physical, have been used to improve industrial strains. Hikmet et al. reported an increase in the PHB yield of *B. megaterium* Y6, *B. subtilis* K8, and *B. firmus* G2 via mutation (6). Dave et al. reported DCW of 70% PHB in optimum culture conditions for *Bacillus* sp. IPCB-403, while Findlay and White (1983) reported the presence of PHB in *B. megaterium* chromatographically (12,13). Chen et al. also studied D(-)-3-hydroxyalkanoate in 11 different *Bacillus* spp. and observed that PHB accounted for 50% of DCW in the bacteria (14). Other researchers have reported PHB production changes in *Bacillus* mutant strains (6). Some of the *Alcaligenes eutrophus* strains used for commercial PHB production have DCW of approximately 96% PHB (1,6). Researchers have concentrated on increasing PHB yields to this level in other bacteria using mutations. Our study yielded a putative UV mutant strain (B8) of *Bacillus thuringiensis* IAM 12077, with increased cell density (3.5-fold), a concomitant increase in PHB production (from 0.24 g/l to 1.3 g/l), and a 1.5-fold increase in accumulation (from 16% to 24.5%), as compared to the parental strain. Further molecular characterization of such mutant strains—at the protein and gene level—may shed light upon the strategies that could be used for targeted strain improvement. Growth and media optimization research with mutants will aid the development of potential industrial strains for PHB production.

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