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Isolation, purification and characterization of PHB producing strains from dairy industry effluent

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ABSTRACT : Polyhydroxybutyrates are biodegradable thermopolyesters which are synthesised by many bacteria under stress conditions they are reported to produce biopolymers like PHB, which can be produced intracellularly as carbon and energy reserves. In this present study, twelve bacterial isolates isolated from dairy industry effluent collected from Aawin Industry, Trichy. However, only one bacterial isolate (DE 6) is selected based on high PHB production 0.068mg/ml. All the 12 bacterial isolates were characterised by morphological and biochemical test. Optimisation of culture conditions such as pH, temperature and incubation time was carried out for the isolate (DE 6). The maximum PHB yield was recorded under dry weight basis in minimal agar media as glucose as the sole carbon source at pH 6 and temperature 40^0C for 72 hours.

KEY WORDS : Polyhydroxybutyrate , Bioplastic, Biosynthesis

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INTRODUCTION

During the last decade environmental pollution and exhaustion of non-renewable sources have created much intrest in neutral materials like poly- β –hydroxybutyrate as a biodegradable (Howells, 1982). Food web contamination from potentially risky chemicals added to plastics during their manufacture (like bisphenol-A, phthalates and nonylphenols) is a parallel concern (Howells, 1982). Buildup of plastics in the marine environment is particularly worrisome. Conventional plastics do not biodegrade because it is gigantic, making them extra difficult to digest. Bioplastics considered as good substitutes for petroleum derived synthetic plastics because of their similar physical and chemical properties such as polypropylene (Doi *et al*., 1990; Steinbuchel and Hein, 2001 and Mercan *et al*., 2002). Bioplastics are simply plastics derived from renewable biomass sources, like plants and micro-organisms, whereas conventional plastics are synthesized from non-renewable fossil fuels, either petroleum or natural gas.

Polyhydroxyalkanoates (PHAs) are biodegradable monomers, naturally made by bacteria during fermentation of sugar, which can be combined to make high molecular weight polymers suitable for plastics. The biopolymer accumulated as storage material under stress condition (Barnard and Sander,1989 ; Sudesh *et al*., 2000; Chen *et al*., 2001; Kadouri *et al*., 2005; Berlanga *et al*., 2006; Lafferty *et al*., 1988 and Huisman *et al*.,1989). The most widely produced microbial bioplastics are polyhydroxyalkanoates (PHAs) and their derivatives (Madison and Huisman,1999; Witholt and Kessler, 2002

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and Kim and Lenz, 2001). Plastics produced from PHAs have been reported to be truly biodegradable in both aerobic and anaerobic environments (Page, 1995 and Cappuccino and Sherman, 1992).

MATERIAL AND METHODS

Effluent sample was collected in sterile bottle from effluent discharge unit at Dairy Industry, Tiruchirapalli. One ml of effluent sample is dispensed in 10 ml of sterile distilled water. This is mixed vigorously and 1ml from this is taken and added to another tube with 9 ml sterile distilled water to get a dilution of 10^{-1}. This serial dilution is repeated to get dilutions of 10^{-2}, 10^{-3},10^{-4},10^{-5},10^{-6}, 10^{-7}, 10^{-8} and 10^{-9}. For the isolation of organisms, 0.1 ml of each dilution was plated onto a nutrient rich medium by spread plate method for the propagation of microbial growth. The plates were incubated at 37^0C for 48 hours. The isolated colonies were selected and sub cultured on minimal agar medium for further studies (Cappuccino and Sherman,1992).

Screening of PHB producing isolates by Sudan Black B staining:

The isolated bacterial strains were screened for PHB production. Following the viable colony method of screening using Sudan Black B dye (Williamson and Wilkinson, 1958). The cultures were grown on minimal media supplemented with glucose (2%) as a sole carbon source, incubated at 40^0C for 48 hrs. after incubation, the plates were flooded with Sudan black B solution (0.3g in 95% ethanol) for the detection of microbial intracellular lipid granules and kept undisturbed for 20 minutes. The excess of Sudan black B was drained off. They were washed with 96 per cent ethanol to remove excess stain from the colonies. The dark blue coloured colonies were taken as positive for PHB production. All the positive isolates were assigned the code numbers based on their source of isolation.

Quantitative screening of PHB producing Isolates:

The selected strains were grown on minimal broth (pH) under standard culture conditions and incubated at 37^0C. During incubation, samples were retrieved after every 24 hrs for 4 days, to quantify the production of PHB (mg/ml) by chloroform extraction method.

Quantification of PHB production and selection of Isolate :

About 10 ml of bacterial cultures (24 – 96 hrs) grown in minimal broth was retrieved at an interval of 24 hrs and centrifuged at 10,000 rpm for 1o minutes. The supernatant was discarded and the pellet was suspended with 2.5 ml of 4 per cent sodium hypochlorite solution and 2.5ml of chloroform. The pellet suspension was incubated at 30^0C for 1hr. After incubation the suspension was centrifuged at 1500 rpm for 10 minutes. After centrifugation three phases were obtained. The upper phase consisted of hypochlorite solution which was removed and the middle phase (choloroform containing undisturbed cells) was separated by fliteration from the bottom phase (chloroform with PHB). The extracted chloroform phase was used to quantify the production of PHB by measuring the absorbance at 230 nm (Ishizaki and Tanaka, 1991).

Characterization of PHB producing isolates:

PHB producing strains were identified and characterized by morphological and biochemical characterization according to the Bergey's Manual of Determinative Bacteriology.

Morphological characterisation:

Morphological characters such as form, shape, size, colour and texture of the selected isolates were investigated according to Bergey's manual of Determinative Bacteriology (Ishizaki and Tanaka, 1991). by growing them on minimal agar medium.

Biochemical characterization:

Different biochemical tests were carried out includes IMVIC tests, catalase test, urease test and starch hydrolysis.

Effect of pH on bacterial growth for the production of PHB:

Minimal broth medium was prepared and the tubes were adjusted with pH 2,4,6,8 and 10, respectively. Inoculate each tube with the loopful of culture. Incubate the tubes in shaking incubator at 37^0C temperature for 24 hrs. After incubation observe the growth of culture in each tube. After incubation, the broth culture were subjected to PHB extraction by crotonic acid method. The extracted PHB was quantified by measuring the absorbance at 230 nm. The optimum pH was determined based on the amount of PHB produced.

Effect of temperature and incubation time on bacterial growth for the production of PHB:

In order to optimize the temperature for the production of PHB, the bacterial cultures were inoculated in minimal broth (pH 7) and incubated at temperature 20°C, 30°C, 40°C, 50°C and 60°C for 24, 48,72, 96 and 120 hours. All the isolates were incubated under standardised conditions for the optimization of temperature. After incubation, the broth culture were subjected to PHB extraction by crotonic acid method.

Molecular characterization of bacterial isolates:

The selected strains was identified by applied biosciences. The 16S rRNA gene was selectively with the 16S rRNA gene universal primer.

RESULTS AND DISCUSSION

The results and discussion commenced on screening of isolates from effluent sample, proceeded for standardised production of PHB by quantitative analysis. The discussion extended for the extracted PHB powder under physical optimised conditions and proceeded for partial purification.

Collection of effluent sample :

Twelve isolates were isolated from dairy effluent sample from Aawin industry, Trichy.

Isolation of PHB producing organisms from effluent samples:

All the tweleve isolates showed its growth on the minimal agar supplemented with 2 per cent glucose and the isolates were coded as DE1, DE2, DE3, DE4, DE5, DE6, DE7, DE8, DE9, DE10, DE11 and DE12. The PHB granules are synthesised by the micro-organisms under unbalanced growth conditions such as limited amount of O, N, P, S or trace elements such as Mg, Ca, Fe and high carbon and low nitrogen concentration (Lee, 1996). The isolates were further characterised by morphological properties, quantitative PHB production analysis, physiological and biochemical characteristics of isolates.

Screening of PHB producing bacteria:

All the twelve isolates showed different Sudan Black absorption pattern were viewed from viable colony staining technique and reported in Table 1.

The isolates showed positive for the presence of lipophilic PHB granules observed as dark grey colour. Five isolates DE1, DE2, DE4, DE6 and DE 8 was showing maximum Sudan Black absorption pattern (+++) as shown in Table 1. Four isolates (DE3, DE9, DE10 and DE11) were showed moderate Sudan Black absorption pattern (++) . Growth pattern (+) showed by isolates DE5, DE7 and DE12 based on Sudan Black absorption. The efficient PHB producing strain was selected based on qualitative screening and the consolidated results are present in Table 1.

Quantitative screening of PHB positive isolates:

Five isolates were selected from plate screening method, based on the maximum Sudan Black absorption, indicating the accumulation of PHB.

Table 1: Growth pattern "+++" denotes maximum Sudan Black absorption, "++" denotes moderate Sudan Black absorption and "+"denotes minimum Sudan Black absorption

Sr. No.	Isolate name	Growth pattern on minimal agar plate
1.	DE 1	+++
2.	DE 2	+++
3.	DE 3	++
4.	DE 4	+++
5.	DE 5	+
6.	DE 6	+++
7.	DE 7	+
8.	DE 8	+++
9.	DE 9	++
10.	DE 10	++
11.	DE 11	++
12.	DE 12	+

Quantitaive screening for PHB production by chloroform extraction, under standard temperature and pH for (24 – 96 hrs) was performed. The selected isolates (DE1, DE2, DE4, DE 6 and DE 8) were compared with the standard strain *Bacillus cereus* MTCC 442 and the results were shown in Fig 1. The ioslates DE1 (0.152 µg/ml), B2 (0.149 µg/ml), DE 6 (0.165 µg/ml) showed maximum production of PHB at 48 hours of incubation. From the above analysis (DE1, DE2 and DE6 were selected for further analysis.

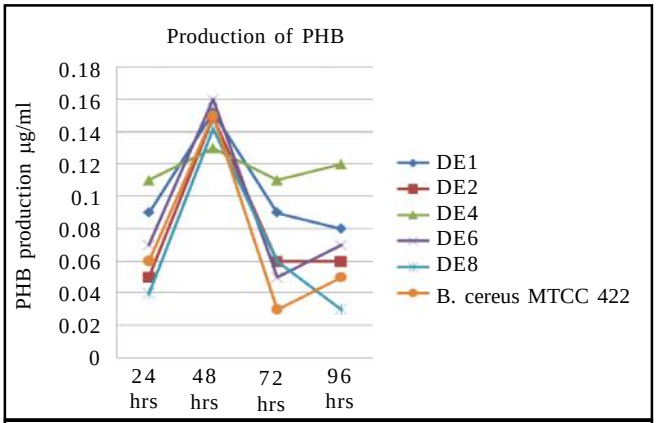

Fig. 1 : Optimisation of cultural conditions for the production of PHB

Morphological and biochemical characterisation of PHB positive isolate:

All the three selected isolates (DE1, DE2 and DE6) were further characterised by number of morphological and biochemical tests as shown in Table 2.

Based on the morphological and 19 different biochemical tests the isolates were DE1, DE2, DE6 were identified as *Acinetobacter* sp., *Acinetobacter* sp. and *Bacillus* sp., respectively.

Optimisation of physical parameters for maximum PHB production:

The physical parameters such as pH and temperature were optimised under standardised incubation time (48 hrs).

pH optimisation:

The optimisation of pH for the production of PHB at constant temperature and standardised incubation time was carried out and the results were presented in Fig 2.

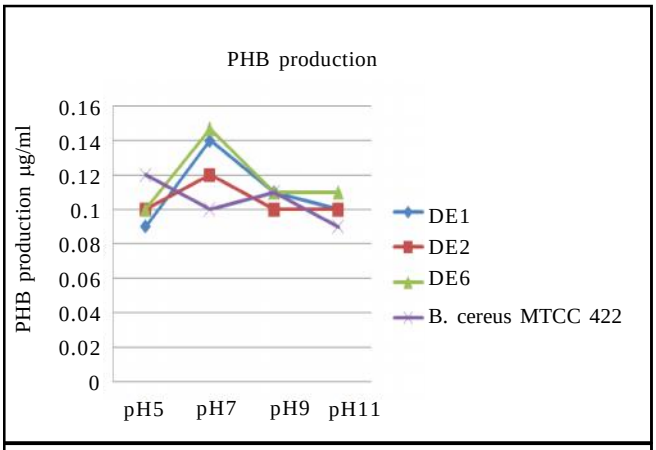

Fig. 2 : Optimisation of pH for PHB production

Temperature optimisation:

The optimisation of temperature for the production of PHB at constant temperature and standardised incubation time was carried out and the results were presented in Fig 3 .

Table 2 : Morphological and biochemical characterisation of PHB positive isolates

Isolate	Indole	Methyl red	Voges proskauer	Citrate	TSI				Catalase	Oxidase	Urease	Starch hydrolysis	Carbohydrate	Nitrate	Casein hydrolysis	Coagulase	Gelatin	ONPG	Esculin	Motility	Sporulation	L-Phenyl alanine	Gram staining	Cocci/ rods
					Slant	But	H2S	Gas																
DE 1	-	-	-	-	AK	Ak	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Rods
DE 2	-	-	-	+	Ak	Ak	-	-	+	-	+	-	-	-	-	-	+/-	-	-	-	-	-	-	Coccobacilli
DE 6	-	-	-	+	Ac	Ak	-	-	+	-	-	+	+	+	+	-	+	-	+	+	+	+	+	Rods
B.cereus																		-	+	+	+			Rods
MTCC	+	-	+	-	Ak	Ac	-	+/-	+	+	+	+	+	-	+	+	+					-	+	
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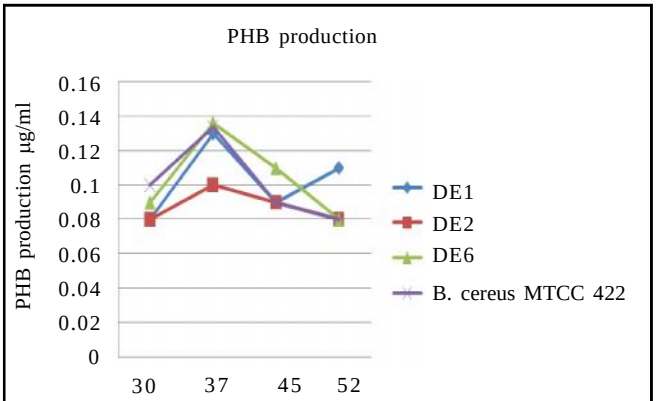

Fig. 3 : Optimisation of temperature (^{0}C) for PHB production

Molecular characterisation of high PHB positive isolate :

PCR amplification of genomic DNA with universal primers specific for 16S rRNA amplification. The PCR product was bidirectionally sequenced using 16S specific primers Sequence data was aligned and analysed for finding the cosest omologs for the sample. The overall results and morphological and biochemical characterisation the high PHB producer (DE6) was selected for molecular identification. Based on nucleotides homology and phylogenetic analysis the strain DE6 was detected to be *Bacillus cereus* (Accession number - KU512626).

Conclusion:

The main aim of this present study was to isolate the PHB producing bacteria from dairy industry effluent sample. Now-a-days researchers are focusing on biopolymer producing micro-organisms for developing biodegradable plastics. The medium used for the PHB isolates was simple medium and less cost effective and the PHB yield from these isolates was high compared with the earlier reports. Among 5 isolates DE6 showing high PHB production. The PHB produced from this strain will further be characterized by analytical techniques like FT IR and GC MS analysis.

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