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DETECTION OF POLYHYDROXYALKANOATE PRODUCING INDIGENOUS *PSEUDOMONAS* STRAINS BY GENOTYPIC AND PHENOTYPIC METHODS

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ABSTRACT

Polyhydroxyalkanoate is a natural biodegradable polymer that can be used as a substitute of petrochemical plastic. The present study has been conducted in order to assess the potential of native *Pseudomonas* species to produce environment friendly polyhydroxyalkanoates (PHAs) by using a combination of phenotypic and genotypic methods. A variety of environmental and clinical samples were screened for the detection of PHA producing *Pseudomonas* species. A total of 73 Pseudomonas strains were studied for the PHA production. The genotypic characterization of the isolates revealed the presence of *pha*C gene among 16 *P. aeruginosa* strains of which 13 were environmental and 03 were clinical isolates. Sudan Black B staining also revealed the accumulation of PHA granules among the *P. aeruginosa* strains which gave positive results in genotypic characterization.

Key words: *Pseudomonas aeruginosa*, Polyhydroxyalkanoate, PHAs gene, Sudan black B stain, Polymerase Chain Reaction (PCR).

INTRODUCTION

The exponential increase in human population has resulted in accumulation of bulk amount of non-degradable waste material globally at an alarming rate, particularly the petrochemical based plastic waste (Kumaravel *et al*., 2010). The accumulation of such non-biodegradable polymers affect the potential survival of many species (Kumaravel *et al*., 2010) and increases the demand for the development of biodegradable materials that are environment friendly (Shah *et al*., 2007).

The term biopolymer refers to those chemically unrelated products that are synthesized by microorganisms under various environmental conditions (Degeest *et al*., 2001). Seven classes of biopolymers are reported so far, namely, polynucleotides, polyamides, polysaccharides, polyisoprenes, lignin, polyphosphate and polyhydroxyalkanoates (Santhanam and Sasidharan, 2010). Bioplastics (Polyhydroxyalkanoates) are efficient substitutes for petroleum derived synthetic plastics since they have identical properties to synthetic polymers and can be completely degraded to water, carbon dioxide and methane by anaerobic microorganisms in various environments such as soil, sea, lake water and sewage and can be easily disposed of without causing harm to the environment (Santhanam and Sasidharan, 2010).

Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyalkanoates (HAs) synthesized by various microorganisms as intracellular carbon and energy reserve compounds and accumulated as granules in the cytoplasm of cells (Lee, 1996). Polyhydroxyalkanoates (PHAs) are considered as environment friendly polymers. These PHAs are accumulated inside the bacterial cell when the cell growth is restricted by the deficiency of nitrogen, oxygen or phosphorus and an excess amount of carbon source is present (Verlinden *et al*., 2006). About 300 different microorganisms can accumulate this polymer to as much as 90% of cellular dry weight in the form of inclusion bodies (Liu and Loge, 2001). PHAs are considered efficient in industrial packaging applications due to their biodegradability, water insolubility, non-toxicity, bio-compatibility, thermoplasticity, piezoelectronic and elastomeric features (Anderson and Dawes, 1990). PHAs have wide range of applications, including manufacture of bottles, packaging materials, films for agriculture, medical and pharmaceutical applications (Oliveira *et al*., 2004).

For the detection and identification of microorganisms capable of synthesizing and accumulating intracellular PHA granules, various methods are now being developed (Kung *et al*., 2007) including both phenotypic and genotypic techniques. Genotypic characterization employs various Polymerase Chain Reaction (PCR) protocols for the detection and amplification of PHA promoting genes (Sheu *et al*., 2000; Solaiman *et al*., 2000; Shamala *et al*., 2003; Solaiman and Ashby 2005). Phenotypic characterization involves various staining procedures such as Sudan Black staining (Schlegel *et al*., 1970), Nile blue A staining (Kranz *et al*., 1997), Nile red (Gorenflo *et al*., 1999; Spiekermann *et al*., 1999) and direct staining of bacterial colonies by fluorescence microscopy (Spiekermann *et al*., 1999).

A variety of Gram positive and Gram negative bacteria are well-known for PHA production. *Pseudomonas* species have widely been reported for their ability to synthesize and accumulate large amounts of PHA granules

intracellularly (Pham *et al*., 2004). The members of the genus represent a great degree of metabolic diversity and they can colonize a wide range of niches. Simple carbon sources such as, soap residue or cap liner-adhesives, bottled mineral water, antiseptics like quaternary ammonium compound, pool water or hot tubs, soil, water and infected wounds fulfill their nutritional requirement. *Pseudomonas aeruginosa* is ubiquitously found in soil and water, frequently on the surfaces of plants and often on the surfaces of animals (Lister *et al*., 2009). *Pseudomonas putida* GPo1 (formerly referred to as *Pseudomonas oleovorans* GPo 1) was the first organism reported to produce PHA (de Smet *et al*., 1983).

The ubiquitous occurrence of *Pseudomonas* species and their ability to grow under diverse nutritional and environmental conditions makes them a strong candidate for the large scale production of PHA granules. The aim of the study was phenotypic and genotypic detection of PHA production by indigenous *Pseudomonas* strains from various environmental sources.

MATERIALS AND METHODS

Bacterial isolates

Indigenous strains of *Pseudomonas* were isolated from various environmental and clinical specimens. The bacterial isolates were subjected to Gram staining and identified using RapId strips (*Remel*).

Genotypic characterization of PHA-producing *Pseudomonas*

A total of 73 *Pseudomonas* strains were screened for their ability to accumulate poly- hydroxyalkanoates (PHA) by detecting the presence of *pha*C genes as described by Solaiman *et al*. (2000).

Polymerase chain reaction for *pha*C gene

A single colony from pure culture was suspended aseptically in 500µl Endonuclease free water and heated to 95˚C for 10 minutes. All the isolates were screened for *pha*C1 and *pha*C2 using I-179L and I-179R primers (I-179L: 5'-ACAGATCAACAAGTTCTACATCTTCGAC-3'; I-179R: 5'-GGTGTTGTCGTTGTTCCAGTAGAGGATGTC-3'). The PCR condition was as follows: initial denaturation at 94˚Cfor 5 minutes, followed by 30 cycles of denaturation at 94˚C for 1 minute, annealing at 50˚C for 2 minutes and extension at 72˚C for 2 minutes. A final extension was performed at 72˚C for 5 minutes. The amplified products were electrophoresed in 0.8% agarose containing ethidium bromide and visualized under ultra violet transilluminator.

Phenotypic characterization of PHA-producing Pseudomonas

The seventy three Pseudomonas strains were also screened for the accumulation of intracellular PHA granules by Sudan Black B dye.

Cultivation on specialized medium

The organisms were grown on modified E2 medium supplemented with glucose as carbon source as described earlier (Berekaa, 2012) to promote PHA accumulation.

Staining with Sudan Black B dye

A thin smear of bacterial cells was heat fixed and stained with a 3% Sudan Black B (w/v in 70% ethanol) solution for 10 minutes. The slide was then decolorized in xylene and counter-stained with Safranin (5% w/v in distilled water) for 10 seconds, washed with distilled water and blotted dry. The cells were observed microscopically under oil immersion lens (Wei *et al*., 2011).

RESULTS AND DISCUSSION

The exponential increase in human population has resulted in accumulation of bulk amount of non-degradable waste material globally (Kumaravel *et al*., 2010). Each year, millions of tonnes of plastic wastes are burried in landfill sites around the world, posing a threat to the environment. This growing problem requires development of biodegrable products. The most efficient environment friendly, biodegradable polymer is Polyhydroxyalkanoate (PHA) which resembles various synthetic thermoplastics and elastomers. The PHAs originate biologically and are degraded completely to carbon dioxide and water by microorganisms present in a wide range of habitat, such as soil, water and sewage (Anderson and Dawes, 1990). A variety of microorganisms like *Azotobacter, Bacillus*,

Pseudomonas, Archaea, can produce this polymer, however, *Pseudomonas* species have been studied intensively for the production of PHA (Kung *et al*., 2007, Phanse *et al*., 2011).

Table 1. Distribution of PHA-producing Pseudomonas strains.

Samples	Frequency	*Ps. aeruginosa*			*Pseudomonas* spp.		
		Isolates	*phaC*	Inclusion granules	Isolates	*phaC*	Inclusion granules
Envrionmental (n=40)							
Food	13	13	3	3	0	0	0
Water	8	6	5	5	2	0	0
Lens care solutions	15	14	4	4	1	0	0
Soil	4	1	1	1	2	0	0
Clinical (n=35)							
Blood	8	5	1	1	0	0	0
Pus	5	3	0	0	0	0	0
Urine	6	6	1	1	0	0	0
Others	16	16	1	1	4	0	0
Total	**75**	**64**	**16**	**16**	**9**	**0**	**0**

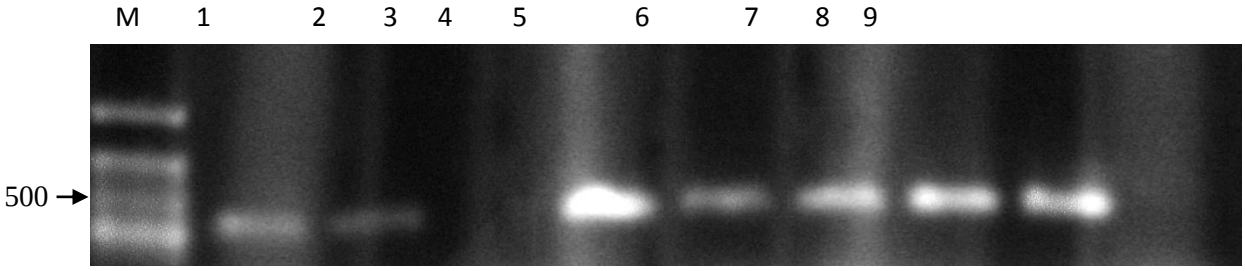

Figure 1. Agarose gel electrophoresis of PCR products for *phaC* genes from *Pseudomonas aeruginosa* strains (M=100bp DNA ladder ; lanes 1,2,4-8= positive; lanes 3 and 9=negative)

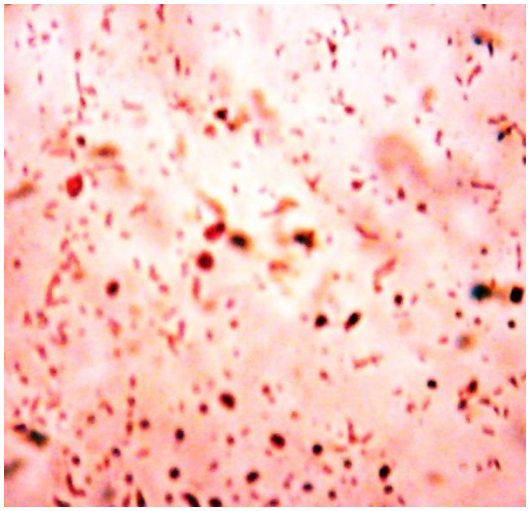

Fig. 2. Sudan Black staining of PHA producing *Pseudomonas aeruginosa* showing dark intracellular granules.

The present study was conducted in order to assess the potentials of Pseudomonas species to produce environment friendly polyhydroxyalkanoates (PHAs). In this study, 70 samples from diverse sources were screened and 73 *Pseudomonas* strains were isolated (Table 1). Sixty four isolates were identified as *Ps. aeruginosa*, while the remaining 9 isolates were identified as *Ps. alcaligenes* (7), *Ps. flavescens* (1) and *Ps. mendocina* (1). Of these 73 isolates, 16 strains of *Ps. aeruginosa* were positive for *pha*C gene as detected by PCR (Figure 1). DNA fragment of ~540bp was successfully amplified in these strains. The ability to produce PHA was also confirmed by performing Sudan Black B staining to observe the accumulation of PHA granules intracellularly (Figure 2). All the strains harboring the *pha*C gene also showed the presence of PHA granules when cultures growing on modified E2 medium were stained with Sudan Black B stain. In the present study, PHA production was detected not only among the environmental isolates of *Ps. aeruginosa* (13/34; 38%) but also by 10% (3/30) of the clinical isolates. However, none of the other *Pseudomonas* species, isolated in the current study, was able to produce PHA (Table 1). Similar pattern was also investigated in other studies where out of 1027 environmental isolates of Pseudomonas and other Gram negative strains, 14% possessed the *pha*C gene (Kung, *et al.*, 2007). Furthermore, in another study, 30 (85.71%) out of 35 Pseudomonas strains isolated from soil were found positive for the gene encoding Polyhydroxybutyrate (Sujhata *et al*., 2005). However, no reports of PHA producing clinical isolates were found in literature.

The present study confirms that the molecular detection of *pha*C gene can be used as a rapid tool for the detection of PHA producing bacteria, as a 100% correlation was observed between the genotypic and phenotypic characterization. The use of PCR method only demonstrates the presence of PHA synthase gene but fails to provide any information regarding the growth conditions favoring the production of large amounts of PHA. Therefore, use of a combination of detection methods is suggested to overcome the drawbacks associated with individual detection methods and help in the selection of a suitable candidate organism for mass scale production of the polymer (Kung *et al*., 2007, Chien *et al*., 2007).

The first step in the production of biodegradable plastics is the isolation of a suitable PHA-producing strain. While the genotypic identification of isolates proves to be a rapid tool, the phenotypic characterization is also important for the commercial PHA production. These indigenous *Ps. aeruginosa* isolates obtained in this study provide a valuable collection of PHA producing strains which can be used in further studies for strain improvement and large scale production of PHA.

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