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Screening of PHA (poly hydroxyalkanoate) producing bacteria from diverse sources

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Abstract

Synthetic plastics are non-degradable and cause waste disposal problems leading to environmental pollution. Bioplastics (polyhydroxyalkanoates) are considered good substitutes for petroleum derived synthetic plastics because of their similar physical and chemical properties. Main advantage of bioplastics is that they are of biological origin and can get degraded completely to CO_2 and water under natural environment by the enzymatic activities of microorganisms. Poly-β-hydroxyalkanoates (PHA) are polyesters of various hydroxyalkanoates, synthesized by numerous bacteria as an intracellular carbon and energy storage compound under limited nutrient conditions and with excess carbon. Poly- β- hydroxy butyrate (PHB) is the best known polyhydroxyalkanoate. Considering the industrial interest of PHA, this work has been undertaken for the screening of PHA producing bacteria from diverse sources. In the present study, an attempt was made to isolate efficient PHB producing bacteria from diverse environmental samples. Different industrial wastes and soil samples were screened for bacteria possessing the ability to accumulate poly hydroxyalkanoate (PHA) granules. About 23 bacterial isolates were found to be promising PHA accumulating bacteria. Screening for PHA producers was performed by using E 2 medium. Accumulation of PHB granules in the organisms was analyzed by Sudan black method.

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Introduction

In response to problems associated with plastic waste and its effect on the environment, there has been considerable interest in the development and production of biodegradable plastics. Polyhydroxy alkanoates (PHA) are polyesters that accumulate as inclusions in a wide variety of bacteria. Because of their inherent biodegradability, PHA are considered to be good candidates for biodegradable plastics, since they possess material properties similar to those of synthetic polymers currently in use and are completely biodegradable after disposal.

Poly-hydroxyalkanoates (PHAs) are inclusion bodies accumulated by some bacterial genera as reserve material, when culture medium is unbalanced due to limited oxygen, nitrogen, phosphorous, sulphur or magnesium and an excess of carbon source (Kim et al., 1994; Lee, 1996). Lafferty *et a*l. (1988) stated that the accumulation of PHA by microorganisms can be stimulated under unbalanced growth conditions, *i.e*., when nutrients such as nitrogen, phosphorus or sulfate become limiting, when oxygen concentration is low, or when the C: N ratio of the feed substrate is higher. PHB is accumulated by numerous microorganisms and is the best characterized PHA (Madison and Huisman, 1999). A number of bacteria such as *Azotobacter*, *Bacillus,* Archaebacteria, Methylobacteria, *Pseudomonas* have been found to synthesize PHA to varying levels. *Ralstonia eutropha* (formerly *Alcaligenes eitrophus*) has been the subject of much published research work because it can accumulate PHAs upto 80 per cent dry weight (Lee, 1996).

Plastics produced from PHAs have been reported to be truly biodegradable in both aerobic and anaerobic environment unlike many of the "so-called" biodegradable plastics made synthetically. PHAs are composed mainly of poly-betahydroxybutyric acid (PHB) and poly-beta hydroxyvaleric acid (PHV), although other forms are possible. β- hydroxy butyrate (PHB) is the best known polyhydroxyalkanoate. All bacteria capable of PHA synthesis accumulate PHA during the stationary phase of growth and these PHA granules facilitate cell survival during stressful conditions. Bioplastics have a wide range of agricultural, marine and medical applications (Arun et al.2006 and Kitamara et al. 2004).

This study includes screening of different bacterial isolates producing PHA from sewage and soil samples.

Materials and methods

Collection of samples

Soil samples, waste water samples (including industrial effluents, dairy waste, domestic sewage) and activated sludge samples were collected from various sources and used for the isolation of bacteria.

Enrichment of PHA producing microorganisms

The samples were inoculated in E2 broth medium (Lageveen et al., 1988) and incubated in a rotary shaker (150 rpm) at 30°C for 24-48 hrs. The growth obtained was then inoculated on E2 agar plates and incubated at 30°C for 48 hrs.

Rapid screening of isolates for PHA production by Plate assay method

All the isolates were qualitatively tested for PHA production using Sudan Black B dye (Juan *et al.,* 1998). Ethanolic solution of (0.05%) Sudan Black B was spread over the colonies and the plates kept undisturbed for 30 minutes. They are washed with ethanol (96%) to remove the excess stain from the colonies. The dark blue coloured colonies were taken as positive for PHA production.

Screening for PHA by Sudan black staining

After 48 hrs of incubation on E2 agar medium, the isolates were screened for PHA production by Sudan black staining (Burdon, 1942a; Lee, 1996). The isolated bacterial colonies were subsequently analyzed for Gram character. Identification of isolates was carried out by performing biochemical tests (Holt, 1994).

Results and discussion

For the past two decades, there has been a growing public and scientific interest in the development and use of biodegradable polymers as an ecologically useful alternative to plastics. Polyhydroxyalkanoates (PHA), synthesized by different genera of microorganisms has attracted attention as biodegradable plastics. They are accumulated intracellularly, as high as 90 per cent of cell dry weight under conditions of nutrient stress and act as a source of carbon and energy (Madison and Huisman, 1999).

In this work, attempts were made to isolate PHA accumulating bacteria from diverse sources and to select the efficient strains. Various samples collected from diverse areas were used for isolating PHA producers. The relative occurrence of PHA accumulating bacteria from these of samples was studied. A striking prevalence of PHA producing bacteria was observed in waste water samples (including industrial effluents, dairy waste, and domestic sewage) and activated sludge samples. Out of the 23 PHA producing isolates obtained from various samples, 14 were from sewage samples and 9 from soil samples (Table 1). PHA producers found in different soil samples were comparatively low. Similar results were reported by Sujatha et al (2005). They obtained higher PHA producers from tannery effluent and sewage sludge samples compared to garden and field soil samples. While isolating PHA accumulating bacteria from nature, it is necessary to screen rapidly a wide collection of bacteria in a short time. Stains specific to PHA are made use of in the detection of the granules. Viable colony staining technique was performed as a method for rapid screening of PHA accumulating bacteria. The PHA granule forming ability of the isolates was further studied by Sudan black staining method.

Table 1. Screening of PHA producing bacteria.

Sample Source	Designation of isolate	Gram Reaction	PHA Accumulation	
			Plate Assay	Sudan Staining
Khan river sewage	KS-1	Gram Positive bacilli	++++	++++
Khan river sewage	KS-2	Gram Positive bacilli	++++	++++
Khan river sewage	KS-3	Gram negative bacilli	+++	++++
Khan river sewage	KS-4	Gram negative bacilli	++	++
Khan river sewage	KS-5	Gram Positive cocci	++	++
Sewage treatment plant	STP-1	Gram Positive bacilli	++++	++++
Sewage treatment plant	STP-2	Gram Positive bacilli	++++	++++
Sewage treatment plant	STP-3	Gram Positive cocci	+	+
Sewage treatment plant	STP-4	Gram negative bacilli	+++	+++
Industrial effluent	IE-1	Gram negative bacilli	+++	+++
Industrial effluent	IE-2	Gram Positive bacilli	++++	++++
Industrial effluent	IE-4	Gram positive bacilli	++++	++++
Industrial effluent	IE-5	Gram negative bacilli	++	++
Industrial effluent	IE-6	Gram negative bacilli	+ + +	+++
Industrial soil	IS-1	Gram Positive bacilli	+++	++
Industrial soil	IS-2	Gram negative bacilli	+++	+++
Industrial soil	IS-3	Gram Positive bacilli	++	++
Industrial soil	IS-4	Gram positive bacilli	++	+++
Agricultural soil	AS-1	Gram negative bacilli	+++	+++
Agricultural soil	AS-2	Gram negative bacilli	+++	+++
Compost	C-1	Gram Positive bacilli	++++	++++
Compost	C-2	Gram positive bacilli	++++	++++

The bacterial isolates were stained by Sudan black staining method and observed microscopically. A total of 23 strains were found to accumulate PHA granules after observing microscopically by Sudan black staining method. All 23 isolates were also tested for PHA production following the viable colony screening method based on the intensity of staining. (Fig. 1) The black stained isolates were ranked in terms of + symbol. The poorly stained colonies were indicated with + symbol, medium stained colonies as ++ symbol, strongly stained colonies as +++ symbol while excellently stained as ++++ symbol. Out of the 23 isolates screened for PHA production 8 showed excellent staining by Sudan Black in Plate assay method.

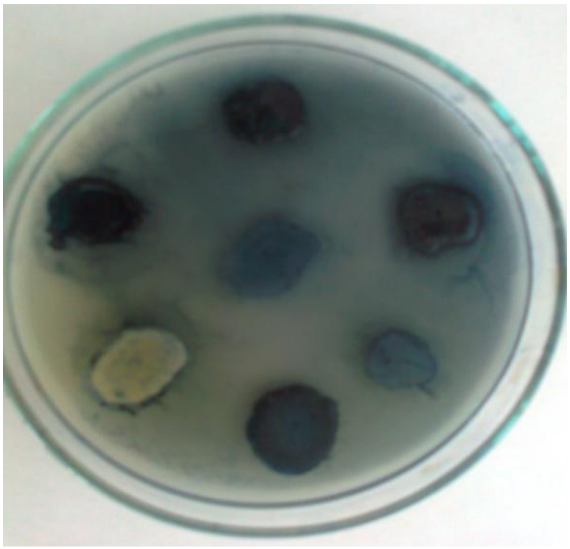

Fig. 1. Rapid screening of isolates for production by Plate assay method.

Juan et al (1998) employed viable colony screening method for the rapid detection and isolation of PHA producing Rhizobium *meliloti* strains by using 0.02% alcoholic solution of Sudan Black B. Colonies unable to incorporate the Sudan Black B appeared white, while PHA producers appeared bluish black. Hartman (1940) was the first to suggest the use of Sudan Black B, as a bacterial fat stain. Subsequently, Burdon et al (1942a) confirmed the greater value of this dye and modified the procedure for demonstrating intracellular fatty material in bacteria by preparing microscopic slides of bacteria stained with alcoholic Sudan Black B solution and counterstained with safranin.

The cultural characteristics of the PHA producing bacterial colonies obtained on E2 were studied (Figure 2). Gram staining showed that among 23 isolates, 12 were Gram positive bacilli, 2 were Gram positive cocci and 9 were Gram negative bacilli. Biochemical analysis (Holt, 1994) revealed that the PHA producing strains belonged to the genera- *Bacillus* (12 isolates), *Pseudomonas* (5 isolates), *Azotobacter* (4 isolates) and *Staphylococcus* (2 isolates).

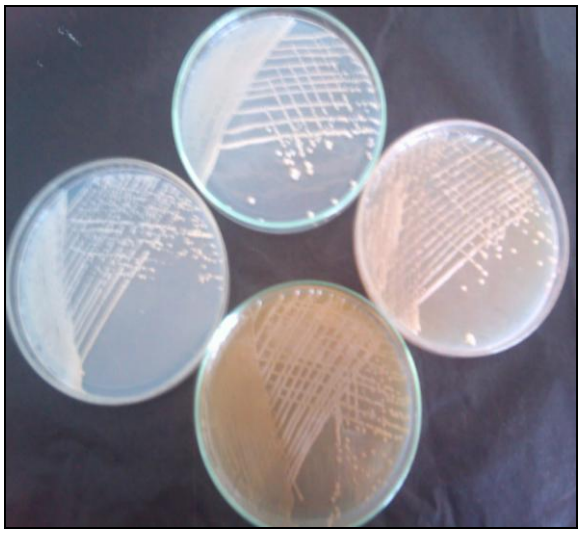

Fig. 2. Colony morphology of PHA producing bacteria on E2 medium.

Polyhydroxyalkanoic acids (PHA) represent a complex class of storage polyesters that are synthesized by a wide range of different Gram-positive and Gram-negative bacteria. In our study, most isolates were *Bacillus* species and showed large intracellular accumulation of PHA. Many workers have reported production of PHA by various species of *Bacillus*. Yuksekdag et al. (2004) have reported PHA production by *Bacillus subtilis* and *B. megaterium*. Production and characterization of PHA produced by *Bacillus megaterium* NCIM 2475 was also reported by Otari et al (2009). Full et al (2006) have studied on production of PHA by *Bacillus* species from industrial wastes.

Yilmaz et al. (2005) have determined production of PHA by *Bacillus* spp.

PHA production by *Psuedomonas* species has also been studied. Lageveen et al. (1988) have reported the formation of Poly-(R)-3-Hydroxyalkanoates by *Pseudomonas oleovorans*. Microbial production of polyhydroxy alkanotes (PHA) from *Pseudomonas oleovorans* using different carbon sources was reported by Santhanam and Sasidharan (2010). Ayub *et al.* (2004) isolated *Pseudomonas* sp. 14-3, a strain from Antarctic environments that accumulated large quantities of polyhydroxybutyrate (PHB) when grown on octanoate.

Followed by genus *Bacillus* and *Psuedomonas*, *Azotobacter* and *Staphylococcus* were the other bacterial genera obtained in our work. Parshad et al. (2001), Page (1992), have studied on PHA produced by *Azotobacter* spp. Roy et al (2009) have reported poly beta hydroxybutyric acid production by *Staphylococcus* species isolated from activated sludge.

Conclusion

The search for promising strains of PHA producers is a continuous process and development of efficient polyhydroxyalkanoate producing bacteria is the need of the hour. On the basis of data obtained in the present work it can be concluded that *Bacillus* species isolated can be employed in the production of PHA.

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