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Full Length Research Paper

Bacteria from contaminated urban and hilly areas as a source of polyhydroxyalkanoates production

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Polyhydroxyalkanoates (PHA) production and extraction of different bacterial strains isolated from contaminated urban and hilly areas was conducted. The 30 bacterial isolates were Gram negative and belonged to *Pseudomonas, Citrobacter, Klebsiella, Escherichia* and *Enterobacter* genera. Bacterial level of resistance against antibiotics (Penicillin) and heavy metals (zinc, cadmium and copper) was determined. Bacterial isolates from contaminated urban areas were found to be more resistant. The screening for PHA production was done by the Sudan black staining. Among the urban area isolates, U17, U8 and U9 produced highest concentration of PHA (50.4, 40.6 and 37.9%) while in hilly area isolates H8, H6 and H9 showed highest production (45.8, 42.4 and 37.6%) by SDS digestion method. The percentage production was lowered when the extraction was done by sodium hypochlorite digestion method. Selected bacterial strains were optimized for PHA production at different growth conditions that is, pH, temperature and carbon sources. Bacterial isolates U8, U17 and H8 produced maximum amount of PHA 74, 69 and 59%, respectively, at pH 7, 37°C and using cooking oil as carbon source after 72 h. PHA polymerase *phaC1/C2* genes were successfully amplified from genomic DNA of three bacterial isolates showing 540 bp DNA fragment which confirmed the presence of *phaC1/C2* gene presence. It showed that the corresponding bacterial isolates would have been able to synthesize medium chain length PHA.

Key words: Polyhydroxyalkanoates, contaminated urban and hilly areas, bacteria.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are the environmentally friendly polyesters which can be accumulated to as much as 90% of cellular dry weight during unbalanced growth in the form of inclusion bodies in over 300 different microorganisms (Liu and Log, 2006). The production of PHA increases bacterial survival in nutrient depleted environments because PHAs can be degraded to provide energy and carbon source (Yang et al., 2006).

PhaC is essential for PHA synthase activity. Two forms of PHA synthase have been reported, an active form in PHA-accumulating cells and an inactive form in non-accumulating cells, where PhaC is more susceptible to degradation (McCool and Cannon, 2001). Polymers of various compositions are produced, depending on the substrate specificity of the PHA synthase and the carbon source on which the bacterium is grown, as well as the metabolic pathways involved in the utilization of the carbon source (Kolibachuk et al., 1999). More than 150 different hydroxyalkanoic acid derivatives have been identified as constituents of PHA (Yang et al., 2006). These PHA monomers can be synthesized in a variety of configurations to produce a wide range of material properties (Zhang et al., 2006).

Poly β-hydroxybutyrate (PHB) is the best known PHA and has been studied most often as a model product in the development of fermentation strategies. Although PHAs have been commercially developed and marketed, their widespread use has been hindered by the high cost of production. To circumvent this cost, it is needed to use cheap substrates, especially carbohydrates such as sugars or molasses (Klinke et al., 1999), crude carbon substrates from industrial food and agricultural wastes (Nikel et al., 2006).

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Abbreviations: PHA, Polyhydroxyalkanoates; **SDS,** sodium dodecyl sulfate; **PCR,** polymerase chain reaction

Objectives of research work include, comparison of the bacterial strains isolated from contaminated urban and hilly areas for different characteristics and their PHA production capabilities, confirmation of PHA production ability by amplifying the PHA polymerase gene (*phaC*) through polymerase chain reaction and to evaluate different bacterial strains to get maximum PHA production at their optimal conditions.

MATERIALS AND METHODS

Sample collection

Samples were collected from effluents of ice cream, pharmaceutical and tissue paper industry in sterilized bottles; soil samples from oil changing workshop, railway lube oil area, lubricant oil factory. Soil samples were also collected from hilly areas of Khanaspur and Ayubia (7000 ft high from sea level).

Growth conditions

Solid PHA detection media with 2% carbon source (glucose) at pH 6.8 - 7.0 was used for bacterial isolation. Bacterial isolates were identified by performing biochemical test. PHA producing isolates were detected macroscopically by observing the turbidity on PHA detection media and were further screened by Sudan black B staining after 72 h (Arnold et al., 1999). Three best PHA producer bacterial strains were selected for optimization. Optimization was done at pH 5, 7 and 9, at 25, 37 and 45°C, using glucose and cooking oil as carbon sources.

Antibiotics and heavy metal resistance

Resistance against antibiotic (Penicillin) and heavy metals (Copper, Cadmium and Zinc) was determined using plate dilution technique in nutrient agar (Sambrook and Russel, 2001).

Extraction of PHA

Extraction of PHA was performed by two methods, sodium dodecyl sulfate (SDS) digestion method and Law and Slepecky method (1960). In SDS digestion method, SDS was added to the cell culture and the ratio of SDS to cell mass was 0.5 (Kim et al., 2003).

Isolation of *phaC* gene

Genomic DNA was isolated by Genomic DNA kit (Fermentas). DNA was amplified using 179R/L primers (1-179 ACAGATCAACAAGTTCTACATCTTCGAC and 1-179 RGGTGTTGTCGTTGTTCCAGTAGAGGATGTC) for *phaC* gene (Solaiman et al., 2000). PCR products were purified using PCR purification kit (Fermentas).

RESULTS

Isolation and Identification

Seventeen bacterial isolates were collected from contaminated urban areas and thirteen bacterial isolates were collected from contaminated hilly areas (Table 1). Based on Gram stain's results and biochemical reactions the PHA producing bacterial isolates belong to the genus *Pseudomonas, Citrobacter, Klebsiella, Escherichia* and *Enterobacter*.

Screening Of bacterial isolates for PHA production

The bacterial colonies were observed macroscopically for turbidity and further confirmation was done by Sudan black staining (Table 1). The colonies were found highly turbid for bacterial isolates U2, U4, U7, U9 and U14 of contaminated urban areas and H4 and H7 of contaminated hilly areas. On staining bacterial isolates, U4, U5, U8, U9, U11, U17, H6 and H8 showed granules in all cells. Bacterial isolates U12, H3, H4 and H9 showed no granules in their cells.

Optimization of growth conditions for PHA production

Bacterial isolates U8, U17 and H8 were selected for optimization of PHA production under different growth conditions by varying the temperature, pH and carbon source. The bacterial isolate U8, U17 and H8 showed highest percentage PHA production (74, 69 and 59% respectively) at 37°C with cooking oil as carbon source at neutral pH after 72 h of incubation (Figure 1).

Resistance against antibiotics and heavy metal ions

94% isolates in urban area and 69% of the hilly area isolates showed resistance for Penicillin. The strains U6, H9, H10, H11 and H12 exhibit sensitivity at 100 µg ml^{-1} level for the selected antibiotic. The 41% isolates of contaminated urban areas and 31% isolates of contaminated hilly areas showed maximum value of MIC (> 300 µg ml^{-1}) for penicillin (Table 1).

All the purified bacterial strains were found to be highly resistant for Cu and Cd up to the level of 8 mM ml^{-1} while 35% strains from urban contaminated area and 31% from hilly contaminated area exhibit sensitivity for Zn at the level of 1 mM ml^{-1}.

Extraction of PHA

Extraction of PHA from bacterial isolates was done by two methods after 72 h of incubation at 37°C (Table 1). By SDS digestion method, seven bacterial isolates (U8, U9, U11, U17, H6 and H9) produced maximum quantity of PHA (that is, 40.6, 37.9, 39.4, 50.4, 42.4 and 58.8%, respectively). By sodium hypochlorite digestion method, seven bacterial isolates (U7, U8, U10, U17, H8, H9 and H13) produced maximum PHA (that is, 23.3, 26.0, 22.6,

Table 1. Characterization of bacterial isolates purified from different contaminated urban (U) and hilly (H) sites.

Name of bacterial isolates	Sudan black	MIC (µg ml^{-1}) penicillin	PHA % by SDS digestion method	PHA % by Law and Slepecky Method	Minimum Inhibitory concentration (MIC)(mM ml^{-1}) $CuSO_4$	$CdCl_2$	$ZnCl_2$	Name of bacterial isolates	Sudan black	PHA % by SDS digestion method	PHA % by Law and Slepecky method	MIC (µg ml^{-1}) penicillin	Minimum inhibitory concentration (MIC) (mM ml^{-1}) $CuSO_4$	$CdCl_2$	$ZnCl_2$
U1	+	100	29.4	18.5	3	S	7	U16	+	17.4	21.1	> 300	4	5	7
U2	+	50	20.9	16.7	4	S	7	U17	+++	50.4	23.3	> 300	6	1	7
U3	+	50	18.7	14.7	3	S	7	H1	+	16.4	13.7	> 300	4	4	6
U4	+++	> 300	28.5	10.5	4	1	4	H2	++	33.1	6	100	3	1	3
U5	+++	100	27.5	12.6	7	4	7	H3	-	25.7	6.9	300	4	1	6
U6	++	S	23.5	18.6	7	S	7	H4	-	33.6	17.4	100	7	S	3
U7	++	50	18.2	23.3	8	3	5	H5	+	28.4	8.3	> 300	7	4	6
U8	+++	> 300	40.6	26	5	3	8	H6	+++	42.4	17.5	200	7	S	2
U9	+++	> 300	37.9	17.2	3	S	6	H7	++	33.7	21.3	200	4	5	3
U10	++	300	21.1	22.6	3	3	1	H8	+++	45.8	22.7	> 300	3	3	5
U11	+++	200	39.4	12.9	4	1	7	H9	-	37.6	24.3	S	5	4	7
U12	-	100	13.1	16.4	6	4	1	H10	++	33.8	18.3	S	3	S	2
U13	+	200	34.2	3	3	3	5	H11	++	28.7	20.8	S	2	1	5
U14	++	200	32.5	13.9	3	2	5	H12	++	34.1	12.9	S	3	S	1
U15	++	> 300	22.6	15.4	7	S	5	H13	+	31.1	22.5	200	2	3	4

23.3, 22.7, 24.3 and 22.5%).

Gene amplification

Amplification of gene fragment of PHA synthase (*phaC*) was done using primers (1-179L and 1-179R), which were based on 550 bp conserved sequence flanked by *phaC1* and *phaC2* gene fragments in *Pseudomonas* species. Six PHA producing isolates (U6, U8, U9, U17, H6 and H8) were subjected to PCR amplification of *phaC1/C2* gene fragment. After PCR, three isolates (U9, U17 and U6) gave positive results by showing ~540 bp bands on 0.7% agarose gel (Figure 2).

DISCUSSION

Growing concern about environmental pollution has renewed interest in the development of PHAs, which are totally biodegraded by microorganisms present in most environments. Also, these polymers can be produced from different renewable carbon sources. Contaminated environments contain essential nutrients and are enriched in conditions for PHA production. So these environments have a large number of PHA producing bacterial strains. It is difficult to perform analysis of PHA at large scale therefore no systematic study can be done on bacterial strains accumulating PHA growing in different contaminated environments (Nikel et al., 2006). In this study samples were collected from contaminated sites as these soils help bacteria to accumulate PHA within their cells. It has been suggested for diverse ecological systems that the accumulation, degradation and utilization of PHAs by several bacteria under stress is a mechanism that favors their establishment, proliferation, survival and competition, especially in competitive environments where carbon and energy sources are limiting, such as those encountered in the soil and rhizosphere. Understanding the role that PHAs play as internal storage polymers is of fundamental importance in microbial ecology (Kadouri

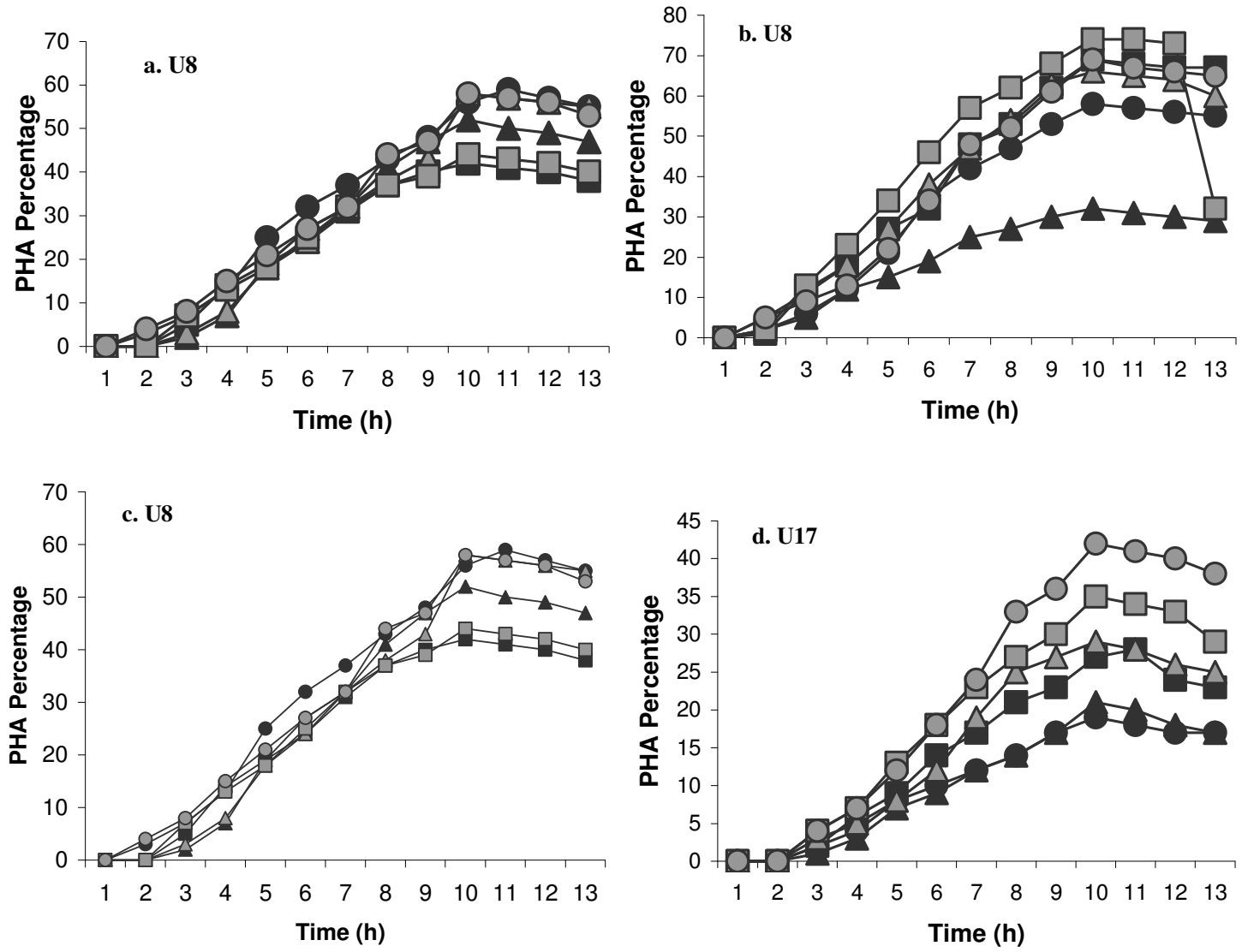


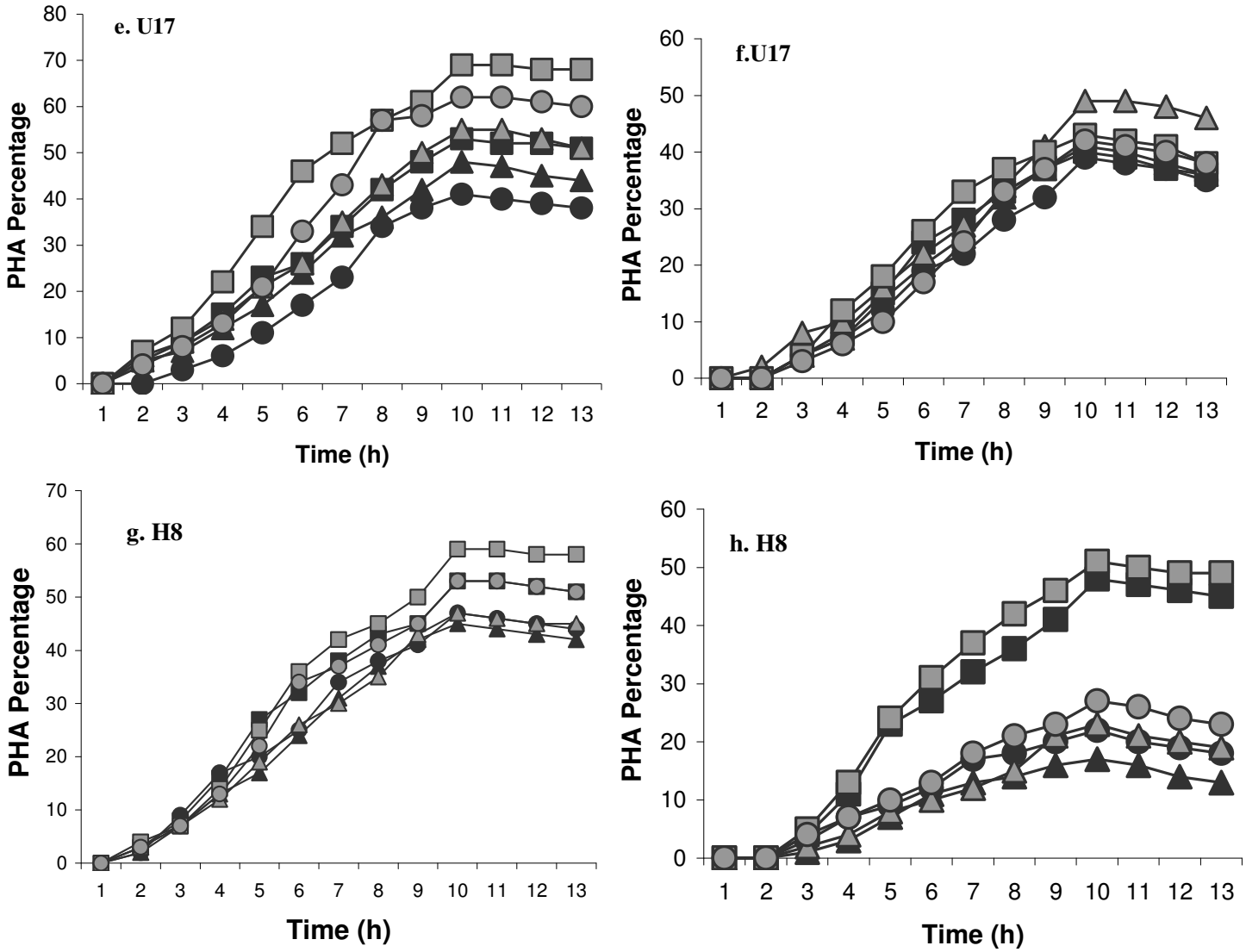
Figure 1. Comparative analysis of percentage PHA production by selected bacterial strains (U8, U17 and H8) under different growth conditions. (a, d, g = pH 5; b, e, h = pH 7 and c, f, I = pH 9). **1a - d.**

et al., 2002). Penicillin resistant strains are more prevalent in contaminated urban areas due to exposure of these areas to the antibiotics. Antibiotics are, presently, of common use and so they expose the environment in return. Hilly contaminated areas are not as much exposed so there is prevalence of sensitive bacterial strains even now. As with antibiotic resistance determinants, toxic heavy metal resistance determinants are pre-existent to recent human activities that create polluted environments (Silver, 1996).

Due to increased pollution of heavy metals in air, water and soil, all organisms face stress from contaminants, which increases the resistance in microorganisms up to these increased concentrations (Silver, 1996). These results predicted that copper resistance is almost equally prevalent in contaminated urban and hilly areas, but zinc resistant strains are only present in contaminated urban areas and cadmium resistant strains are equal to none in both the environments. The resistance in the bacterial strains developed depends upon the level of exposure of these areas by the corresponding heavy metal.

On staining different bacterial isolates showed granules in all cells, due to attachment of Sudan black (lipophilic dye) with PHA (Schegel et al., 1970). Sudan black analysis showed that bacterial strains of contaminated urban areas are more competent to produce PHA as compared to bacterial strains of contaminated hilly areas.

After extraction of PHA by both of SDS digestion and sodium hypochlorite digestion methods, it could be safely concluded that bacterial isolates of contaminated urban



Figures 1e - f.

areas are more prone to produce PHA as compared to those of contaminated hilly areas. The reason behind is that the environment in the contaminated urban areas is more supportive to PHA production as these areas fulfills the basic requirement of PHA production of surplus of carbon and absence of nitrogen. Growth of the PHA producing bacteria was greatly affected by different growth conditions (Huijberts et al., 1992).

Sugars such as glucose, sucrose and oils are the most common main carbon sources for PHA production because they can be obtained at a relatively low price (Tsuge, 2002). Results showed that bacterial strains had ability to utilize medium chain length carbon sources and gave better results using it. Irrespective of growth temperature, U8 isolate was fond of utilizing cooking oil and its PHA production capability become high when incubation temperature was also suitable.

Genetics of PHA production was studied by amplifying the gene fragment of PHA synthase. Six PHA producing strains (U6, U8, U9, U17, H6 and H8) were subjected to PCR amplification of *phaC1/C2* gene fragment. On PCR three strains (U9, U17 and U6) gave positive results by showing ~550 bp bands on agarose gel. The other three isolates were also PHA producers but not amplified on PCR, maybe due the non-complementation of their PHA synthase to the designed primers or isolate may not contain the PHA synthase of the *Pseudomonas* type (Shamala et al., 2003) or they posses the short chain length *phbC* gene which could not be amplified by the designed primers. Sheu et al. (2000) used PCR based strategy to confirm the PHA producers from different *Pseudomonas* after screening by Sudan black staining. *Pseudomonas oleovorans* contains two PHA polymerases, which are encoded by *phaC1* and *phaC2* of the *pha* gene

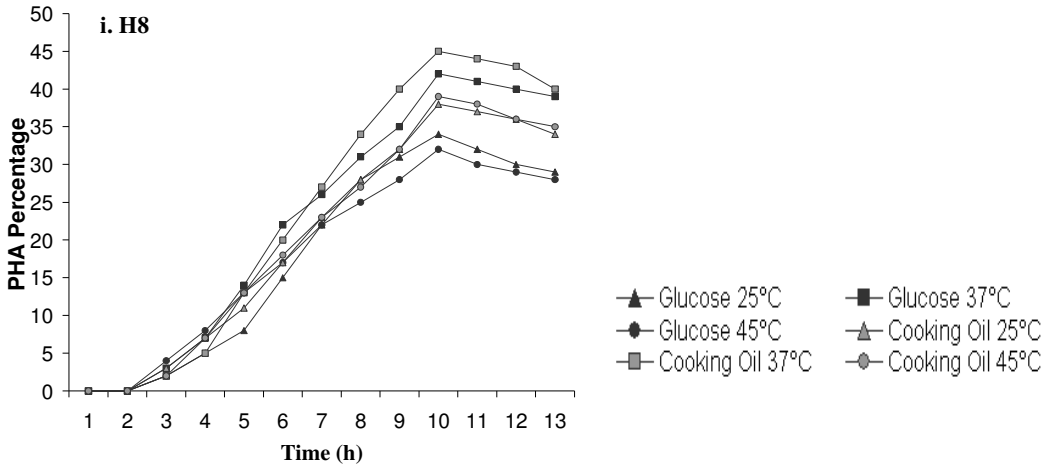


Figure 1i.

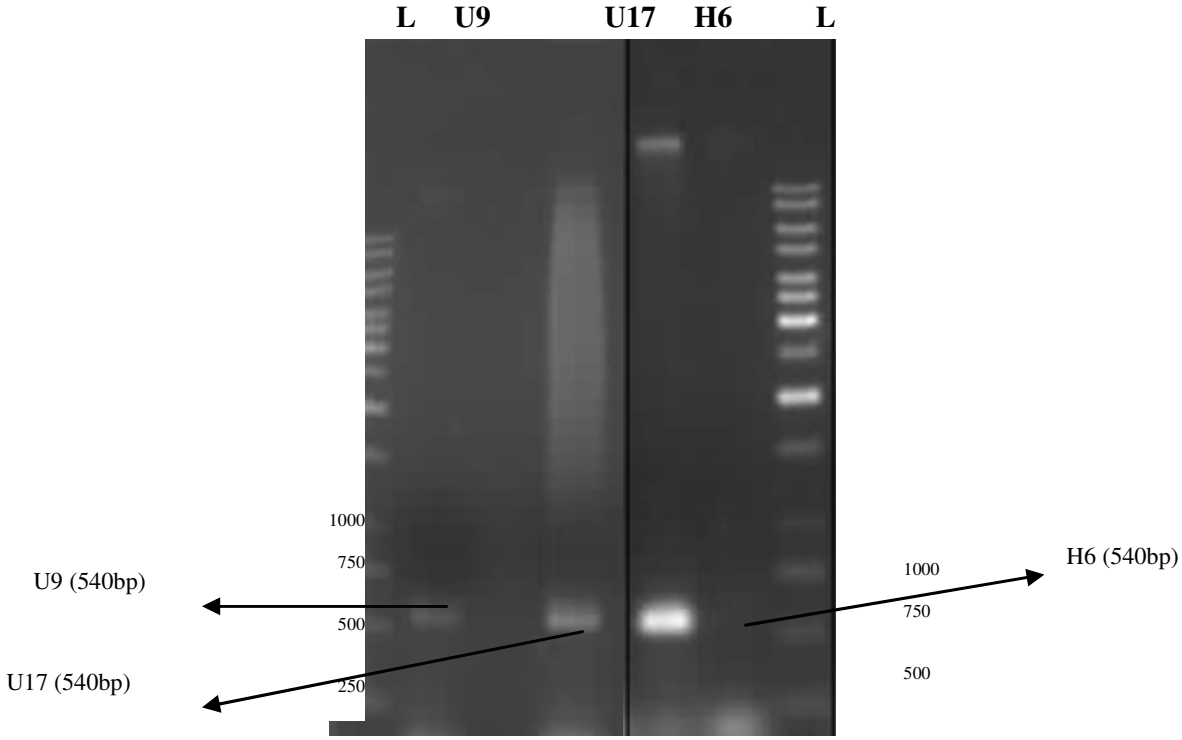


Figure 2. Amplification of phaC gene fragment from U9, U17 and H6 bacterial isolates.

cluster, it has been demonstrated that both of these polymerases are functional proteins which are able to catalyze PHA formation independent of each other; that is, one of the polymerase-encoding genes is enough to produce mcl-PHA in heterologous hosts (Prieto et al., 1999). produce PHA than those of contaminated hilly areas. The reason behind it is that the environment in urban areas fulfills the nutrient depletion requirement in a better way for the production of PHA.

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