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Separation of Polyhydroxyalkanoates-Producing Bacterial Strains Using PHA Synthase Gene and Their Evaluation for PHA Deposition

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ABSTRACT

In this study, a variety of samples were screened for the presence of PHA synthase gene. Results showed that 16 out of 102 isolated were positive for PHA respective genes. The highest prevalence was observed in Pseudomonas aeruginosa. The capability of PHA production was also shown by growing these strains on the defined medium and subsequent analysis using intracellular granules staining and Fourier transform infrared spectroscopy (FT-IR). The microscopic analysis showed that the positive strains accumulated PHA in the cell. The FT-IR analysis showed the presence of PHA peaks in the dried cells as well as in extraction product. P aeruginosa strain P7 showed higher concentration of PHA compared to the others as demonstrated by the highest respective peaks in FT-IR.

Key words: Polyhydroxyalkanoates, Biodegradable polymers, PHA synthase gene, FTIR

INTRODUCTION

The accumulation of petrochemical polymers in the surroundings and growing awareness about the environmental pollution throughout the world has triggered the search for new products that are compatible with the environment. Currently, most polymer products are designed and prepared synthetically and very limited consideration is paid for their ultimate disposal. Of particular concern are throw-away plastic products, which are very suitable to use due to their physical properties. They have replaced wood, glass and other packaging materials since their emergence in 1940s (Ojumu 2004). However, these non-degradable plastics are building up in the environment at the rate of 25 million tons per year, which may persist for hundreds of years (Voaides 2010). It is worthy under these circumstances to design and develop biodegradable materials, which ensures an appropriate disposal with reference to the environment and eco system. In this regard, materials such as polyhydroxyalkanoates (PHAs), polylactic acid and others environment-friendly biopolymers are especially useful.

Polyhydroxyalkanoates (PHA) are a class of naturally occurring polyesters that are produced and accumulated as inclusion bodies of carbon and energy reserve material in different microorganisms (Jendrossek 2009). It has been estimated that over 300 bacterial species and more than 90 genera, including Gram-negative and Gram-positive organisms accumulate various

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PHAs (Kim 2007; Razzaq 2010). These polyesters are generally synthesized in two forms depending on the conditions and strains. Short-chain length hydroxyalkanoic acids (SCL-PHAs) with 3-5 carbon side chains and medium-chain length PHAs (MCL-PHAs) having carbon ranging from 6-14 atoms (Kim 2007; Jendrossek 2009). In addition, numbers of copolyesters carrying these SCL-PHAs and MCL-PHAs have also been reported (Lutke-Eversloh 2001; Steinbuchel 2003). There is a considerable attraction in PHAs due to their biodegradability and thermoplastic properties. However, the high cost of production compared to cheap petrochemical polymers, prevents their use on industrial scale. Continuous efforts are being made and several studies are going on to develop a cost-effective strategy by using inexpensive substrates as a carbon source and high yield of biopolymers (Cromwick 1996; Verlinden 2011).

The aim of this study was to screen various samples of different origin for PHA producing bacterial strains using PCR for PHA synthase gene and other conventional methods. Subsequently, positive microorganisms were evaluated for PHA accumulation on defined medium.

MATERIALS AND METHODS

Samples Collection and Isolation of Bacterial Strains

A total of 35 samples of different origin were collected to isolate the bacterial strains for PHA production, and 102 isolates were included in this study (Table 1). Garden Soil, waste water, tap water and drinking water samples were collected from different part of Karachi; air samples were collected by plate exposure method. These samples were subjected to total aerobic count by pour plate method, except air samples isolates, which were directly obtained through the plate exposure method in different parts of the laboratory premises. Briefly, the samples were homogenized in Butterfield phosphate buffer (stock solution: 34g of KH_2PO_4 dissolved in 1000ml of distilled water, pH 7.2±0.5; working solution: 1.25ml of stock solution/1000ml of distilled water). Serial dilutions, i.e. 1:10 to 1:10000 were prepared and 1.0 ml of sample from all dilutions was inoculated on Petri plates. Molten Plate Count Agar was added, allowed to solidify and incubated at 35°C for 48 h. The bacterial isolates were picked on the basis of their colonial morphology and streaked on Tryptic Soy Agar for pure culture.

Table 1 - Samples Description.

S. No.	Type of samples	Number of isolates	S. No.	Type of samples	Number of isolates
1	Garden Soil	13	8	Lens Solution (Washing)	08
2	Domestic Waste water	17	9	Milk Products	03
3	Air (Laboratory)	15	10	Spinach	01
4	Tap Water	11	11	Meat	05
5	Rice	11	12	Fish meal	06
6	Wheat	05	13	Drinking water	03
7	Spices	04		**Total**	**102**

Type of samples tested with number of bacterial strains isolated from these samples.

Polymerase Chain Reaction

To carry out the PCR experiment, a single colony was picked into molecular biology grade water and heated to 95°C for 10 minutes. All the isolates were screened for *phaC1* and *phaC2* genes using I-179L and I-179R primers and PCR conditions as reported in the literature (Solaiman 2000; Jamil 2007). The sequences of the primers were;

Forward: I-179L: 5'-ACAGATCAACAAGTTC TACATCTTCGAC-3',

Reverse: I-179R: 5'-GGTGTTGTCGTTGTTCC AGTAGAGGATGTC-3'.

Briefly, 2.5µl bacterial suspension was added to 12.5µl of GoTaq® Green Master mix (Promega), 0.5 µL of each primer and 9.0 µL of molecular biology grade water. All the PCR tubes were placed in Biorad thermal cycler and PCR was started by initial denaturation at 94°C for 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. The thermal cycling was terminated with a final extension of 72°C for 5 minutes. Agarose gel electrophoresis was performed using 1% agarose and ethidium bromide staining to analyse the PCR products. Gels were exposed to UV light and photograph was taken after electrophoresis.

Identification of PHA Positive Strains

All the bacterial isolates positive for *phaC1* and *phaC2* genes were subjected to Gram staining and subsequent identification. RapID identification strips (*remel*) were used to identify these strains. Bacterial strains were sub-cultured to obtain a single colony and preceded according to manufacturer's instruction. Results of RapID strips were interpreted and reports were generated using online Eric web software of *remel*.

PHA Production

Experiments were carried out to confirm the production of PHA by the strains positive for respective genes. For the evaluation of PHA, the isolates were grown in 10ml of Tryptic Soy Broth (TSB) at 35°C for 24 h. Cells were harvested by centrifugation and re-inoculated in medium "E" prepared as described earlier with slight modification (Cromwick 1996). The medium "E" was prepared by adding K_2HPO_4 (5.8g/l), KH_2PO_4 (3.7g/l), $MgSO_4.7H_2O$ (0.25g/l), $(NH_4)_2SO_4$ (0.03g/l), and 1.0ml of micronutrient solution prepared in 1M HCl having $FeSO_4.7H_2O$ (2.78g/l), $MnCl_2.4H_2O$ (1.98g/l), $CoSO_4.7H_2O$ (2.81g/l), $CuCl_2.2H_2O$ (0.17g/l), $ZnSO_4.7H_2O$ (0.29g/l), and $CaCl_2.2H_2O$ (1.67g/l). Glucose (2%) was used as a sole source of carbon. The flasks were incubated at 30°C and 150rpm shaking for 48 h after inoculation.

Staining for PHA Granules

Intracellular PHA granules were observed by staining with Sudan black-B as reported earlier (Santhanam 2010). After incubation, heat-fixed smears were prepared and dipped in 0.3% Sudan Black-B prepared in ethylene glycol for 20 minutes. Slides were then decolorized in xylol and after drying, counter-stained with safranin solution for 30 seconds. Slides were washed and observed under the microscope.

Extraction and Characterization

Incubated culture medium was centrifuged at 5000 rpm; cells were harvested and washed with deionized water to extract the PHA. Cell pellets were dried at 100°C in the oven. Dried cell mass was initially checked for characteristic PHA peaks by FT-IR (Thermo Nicolet Avatar 320 spectrophotometer USA) using ATR technique. Subsequently, dried cell mass was used for PHA extraction by refluxing in 100ml chloroform for 4 h as described earlier (Annuar 2008). The chloroform extract was slowly added to stirred cold methanol to precipitate the PHA, which was re-dissolved in chloroform and precipitated again in cold methanol. Precipitate was then separated and air-dried for characterization.

RESULTS AND DISCUSSION

Polyhydroxyalkanoates (PHA) is an expression comprising a family of polyesters produced by a diverse group of microorganisms. Variety of intracellular storage compounds, including glycogen, polyamino acids, polyphosphates, etc. have been identified in the microbes during the excess availability of nutrients, and PHA is one of them (Sudesh and Doi 2005). The polymerization of PHA in the cells is mainly dependent on an enzyme, called PHA synthase, which catalyzes the reaction to produce PHA and free CoA from hydroxyacyl-coenzyme A (Rehm 2004). As stated earlier, more than 300 bacterial species are able to accumulate the PHA, but the deposition in most of them are very low (Nobia 2007). The use of mixed cultures are gaining interest and it has been concluded in many reports that mixed microbial cultures could be more favorable for PHA production compared to pure cultures (Dias 2008; Tian 2009). In addition, recombinant organisms having *pha* gene expressions also proved to be a good candidate for mass scale PHA production (Nikel 2006).

In this study, 102 bacterial stains were randomly selected from 35 samples of different origin. PCR was performed to screen these strains for *phaC1/C2* genes. It was observed that 16 strains were positive for respective genes and amplified characteristic ~540bp PCR products (Fig. 1). The size of the product was in agreement with the studies reported earlier (Solaiman 2000; Jamil 2007). Table 2 shows the affiliation of these strains with their respective samples. Strains positive for the respective genes were identified on the basis of their Gram reaction and biochemical characteristics. All the positive strains were Gram negative, non-lactose fermentor rods. RapID identification strips NF plus (*remel*) were utilized to observe their biochemical characters and their identity. It was observed that 12 out of 16 positive strains belonged to *P. aeruginosa* species (Table 2).

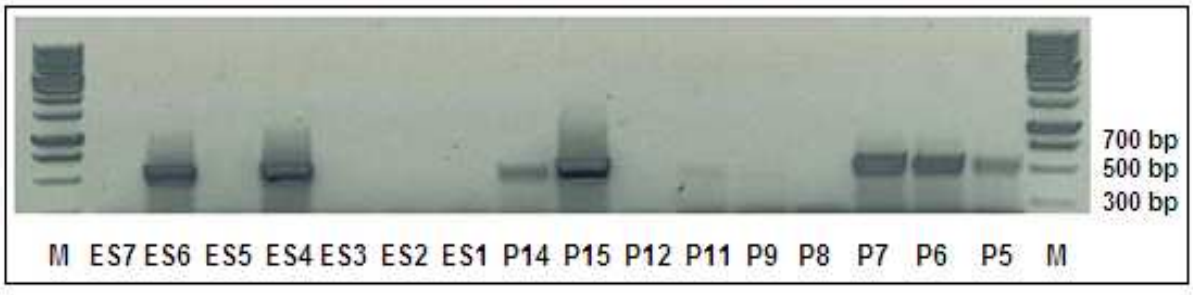

Figure 1 - Polymerase Chain Reaction for *phaC1/C2* genes. PCR products of ~540 bp were obtained in PHA positive strains with primers I-179L and I-179R. M = Molecular weight marker (1kb, Bioron). Bacterial Strains = ES7–P5.

Table 2 - Identification of PHA positive strains using *RapID* NF plus identification strips (*remel*).

S No	ID	Source	Gram Reaction	Organism Identified
1.	ES4	Air	G-ve, rods, scattered	*Comamonas acidovorans*
2.	ES6	Air	G-ve, rods, scattered	*Pseudomonas aeruginosa*
3.	TR11	Tap Water	G-ve, rods, scattered	*Pseudomonas aeruginosa*
4.	TR13	Tap Water	G-ve, rods, scattered	*Pseudomonas aeruginosa*
5.	TR21	Waste water	G-ve, rods, scattered	*Alcaligenes faecalis*
6.	P1	Lens Solution (W)	G-ve, rods, scattered	*Pseudomonas aeruginosa*
7.	P3	Lens Solution (W)	G-ve, rods, scattered	*Pseudomonas aeruginosa*
8.	P4	Lens Solution (W)	G-ve, rods, scattered	*Pseudomonas aeruginosa*
9.	P5	Lens Solution (W)	G-ve, rods, scattered	*Pseudomonas aeruginosa*
10.	P6	Lens Solution (W)	G-ve, rods, scattered	*Pseudomonas aeruginosa*
11.	P7	Lens Solution (W)	G-ve, rods, scattered	*Pseudomonas aeruginosa*
12.	P13	Meat (Beef)	G-ve, rods, scattered	*Pseudomonas aeruginosa*
13.	P14	Soil	G-ve, rods, scattered	*Pseudomonas aeruginosa*
14.	P15	Tap water	G-ve, rods, scattered	*Pseudomonas aeruginosa*
15.	TR49	Tap Water	G-ve, rods, scattered	*Sphingomonas paucimobilis*
16.	TR50	Tap Water	G-ve, rods, scattered	*Stenotrophomonas maltophila*

PHA production has been widely investigated in Gram-negative species such as members of *Ralstonia*, *Alkaligenes*, *Pseudomonas*, and recombinant *E coli*. In this study, it was demonstrated that *phaC1/phaC2* was commonly distributed among the Gram-negative bacterial isolates from different sources, including air, water, soil, and lens solution washings. Investigation for this gene revealed that around 16% carried PHA synthase gene with the highest prevalence in *P. aeruginosa*. In another study, around 40% of strains isolated from 20 soil samples were positive for PHA production (Chen 2000). This significant difference could be due to the analysis of variety of samples. Fluorescent Pseudomonads including *P. aeruginosa* have been extensively studied and reported to accumulate large amounts of PHAs as carbon storage compounds (Huisman 1989; Pham 2004). They are also probably the most versatile in terms of broad substrate specificity (Sudesh and Doi 2005). All positive strains for respective genes were further screened to determine the intracellular deposition of PHA. Organisms were grown on medium "E" containing glucose as a carbon source. The microscopic examination of the cultures after Sudan black showed that all strains positive for PHA synthase genotype accumulated PHA polymers in the cell (Fig. 2).

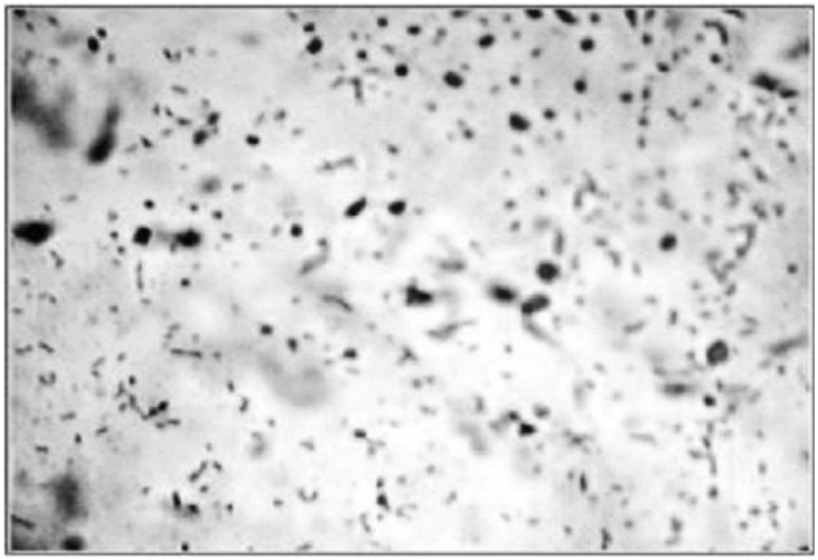

Figure 2 - Staining of PHA. Photograph of stained PHA organism. Intracellular PHA granules are in black and rest is in light colour.

In addition, FT-IR spectroscopy was employed to detect the functional group of PHA in the dried cell mass before and after the extraction. Solvent extraction using chloroform and methanol was performed and extracts were tested using FT-IR technique. PHA extraction can also be done by aqueous phase using sodium hypochlorite, which is comparatively a cheaper method, but it reduces the molecular weight of the polymer (Luzier 1992). A FT-IR using ATR technique was used in this study with the spectra range of 4000-500 cm^{-1}. Several methods have been reported for the qualitative analysis of PHA, including GC, NMR, etc., but FT-IR is reported to be the most rapid and simple method (Hong 1999). It does not require complicated sample preparation and can be used to detect the extracted PHA as well as intracellular PHA in the dried cells (Hong 1999). It was observed that all the strains positive for *phaC1/phaC2* genes also showed PHA in the dried cells before and after the extraction (Figures 3, 4). Strains showed strong peaks ranging from 1728 to 1744 cm^{-1} which were attributed to the stretching vibration of ester carbonyl group C=O peak (Figures 3, 4). In addition, strong peaks were also observed at 2922, 2923 and 2925 cm^{-1}, which represented the stretching vibration of C-H methylene group. Similar pattern of peaks in FT-IR analysis were observed in other studies when glucose containing medium was used for PHA production (Nisha 2012). Although other esters may also generate a similar pattern, but no such interruption was observed when *phaC1/phaC2* negative strains were tested for PHA (data not shown). Moreover, peak shifts were observed when intact cells FT-IR were compared with the FT-IR purified product. It has been reported earlier, which was mainly due to the complex chemical environment in the dried cell mass (Hong 1999).

Out of 16 positive strains, *P. aeruginosa* P7 strain showed the highest peaks, both in the dried cells and in the extract, and this intensity of PHA region peaks roughly showed the comparatively higher PHA concentration in the respective strain.

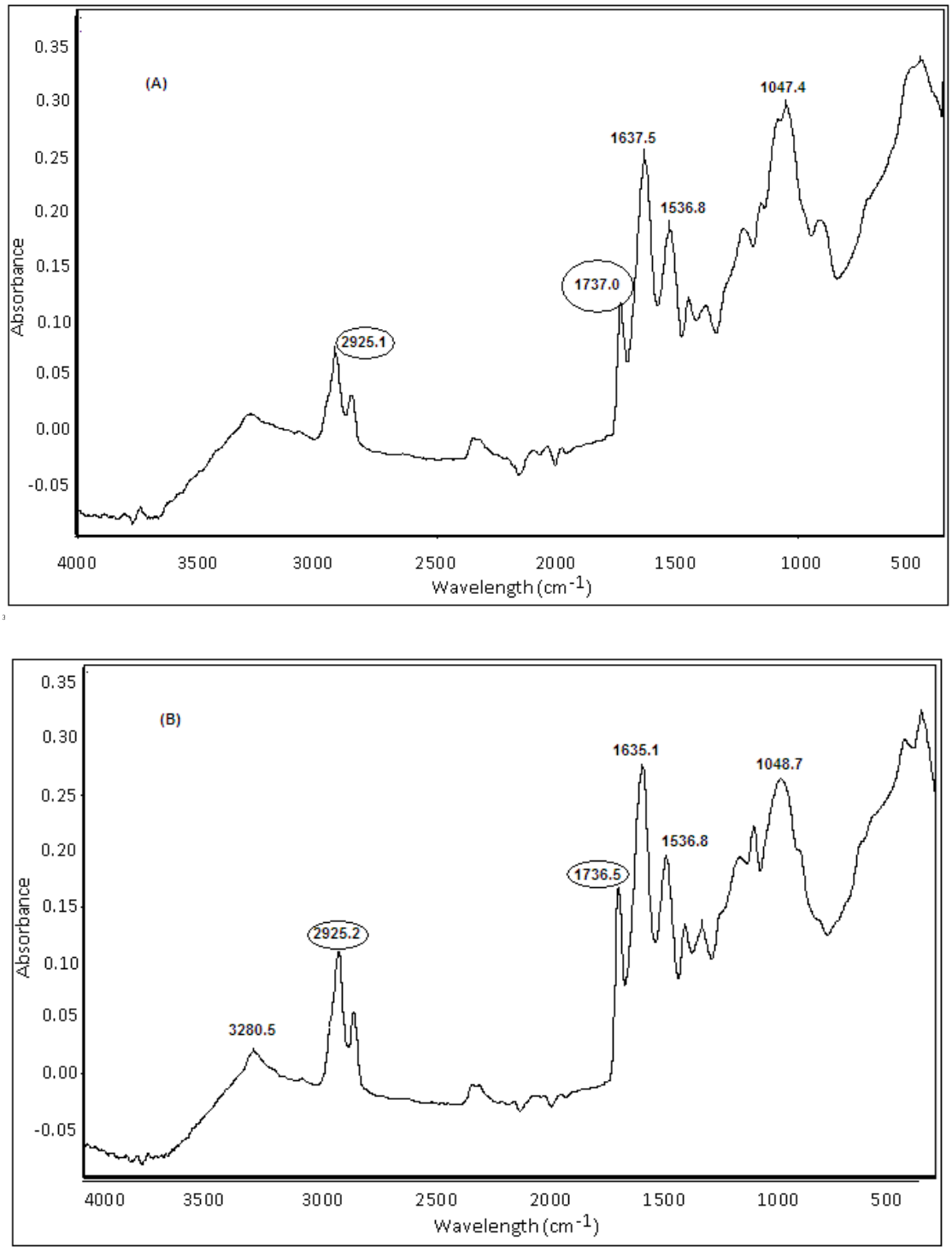

Figure 3 - Fourier-transform infrared (FT-IR) spectra of dried cell mass before extraction of (A) P6 (B) P7.

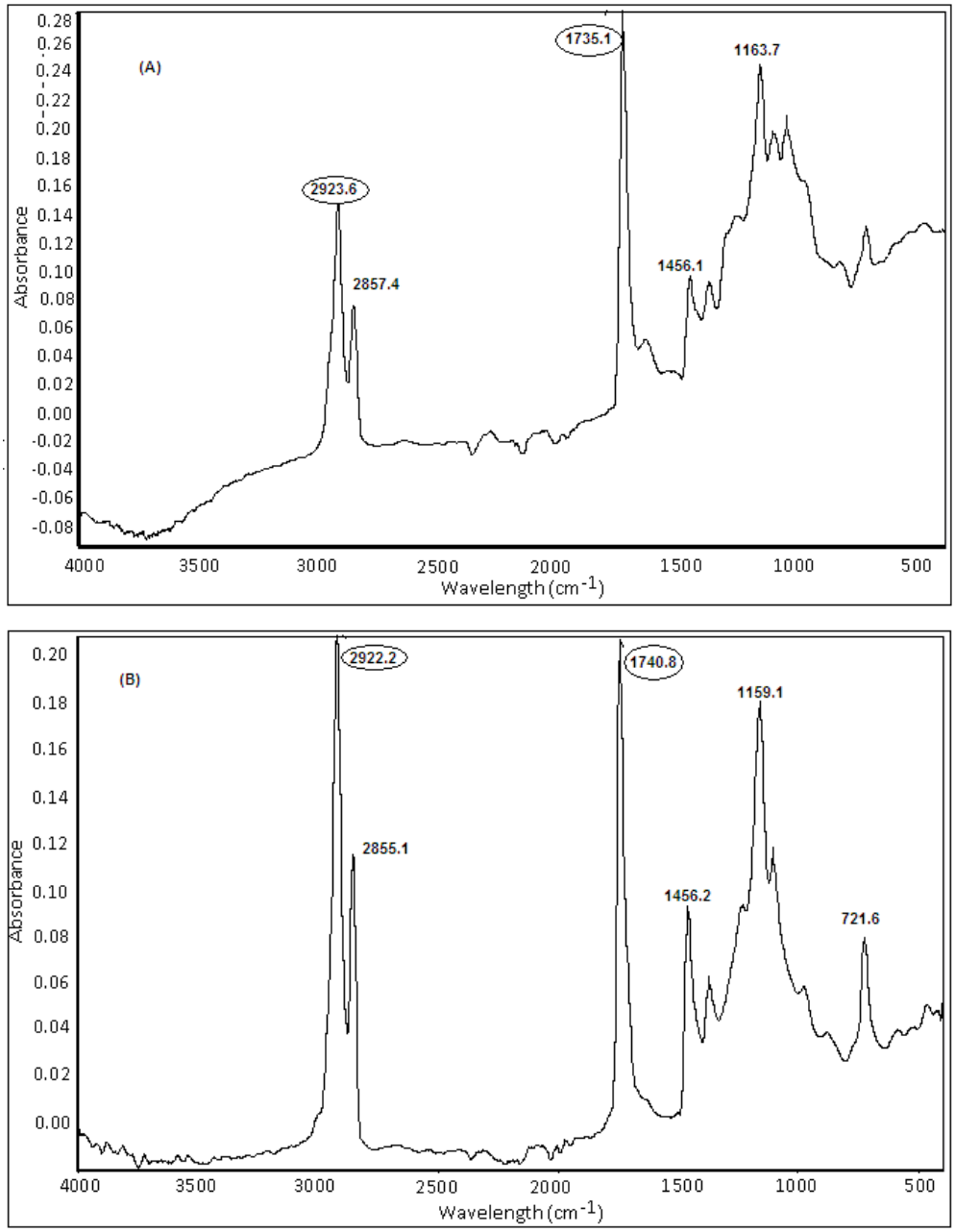

Figure 4 - Fourier-transform infrared (FT-IR) spectra of dried cell mass after extraction of (A) P6 (B) P7.

CONCLUSION

The objective of this study was to see the prevalence of PHA producing organisms in different samples through PCR and their potential to produce the intracellular PHA. It was observed that the genes for PHA synthase were usually distributed in Gram-negative organisms. It was also shown that the rapid screening for PHA producers could be done in the dried cells before and after the extraction through FT-IR.

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