Front. Biol. 2017, 12(3): 210–218
DOI 10.1007/s11515-017-1446-2

RESEARCH ARTICLE

Biosynthesis of polyhydroxyalkanoates from styrene by *Enterobacter* spp. isolated from polluted environment

Arooj Arshad, Bisma Ashraf, Iftikhar Ali (✉), Nazia Jamil

Department of Microbiology and Molecular Genetics, University of the Punjab, Quaid-e-Azam Campus, Lahore-54590, Pakistan

BACKGROUND: Styrene and its metabolites are known to have serious adverse effects on human health and hence, strategies to prevent its release, eradicate it from the environment, and understand its route of degradation are being considered.
METHODS: A total of 18 strains were isolated from 4 samples of diesel contaminated soils. Among them 5 strains were selected for their ability to degrade styrene and use it as a sole carbon source to produce PHA. These strains were identified as *Enterobacter* spp. on the basis of 16S rRNA gene sequencing. Bacteria were screened for their ability to produce PHA by utilizing glucose and styrene as a carbon sources. Screening for PHA production was done by Nile blue A, Sudan black B, and phase contrast microscopy and the selected 3 strains showed positive results. Growth kinetics along with time profiling of PHA was performed for glucose and styrene as carbon sources.
RESULTS: PHA extraction was done at equal intervals of 12 h by sodium hypochlorite method which showed that these strains accumulate maximum amount of PHA after 48 h in glucose (30.60%). FTIR analysis of PHA was done which revealed homopolymer PHB and copolymer (PHB-co-PHV) production in strains by utilizing glucose and styrene. Gas chromatography mass spectrometry was carried out to identify the metabolites produced by bacterial strains grown on styrene. Metabolites of styrene degradation included propyne and phenylalanine. Genomic DNA isolation was carried out to amplify *pha*C gene which encodes PHA synthase enzyme.
CONCLUSIONS: The conversion of styrene to polyhydroxyalkanoates (PHA) provides a new and unique link between an aromatic environmental pollutant and aliphatic PHA accumulation.

Keywords biodegradable polymers, environmental pollutants, PHA, FTIR, recycling, bacteria

Introduction

Petrochemical based plastics are an important environmental concern due to their recalcitrance. They are produced 100 million tons annually; 3 million tons are composed of polystyrene polymers. 2.5 million tons of this polystyrene plastic usually ends up in the land fill. So number of studies is focusing to find alternative methods for polymer recycling which includes thermal degradation to yield energy and heat (Miskolczi et al., 2011). Besides this, polystyrene can be converted back into its monomers of styrene by a process called pyrolysis (Hussain et al., 2010). Pyrolysis is the process of thermal degradation which is done at high temperature without the presence of air and produces oils and gases. Styrene oil is metabolized by some bacteria to produce value added products. PHA producing bacteria have been reported to utilize styrene as sole carbon source for the production of useful metabolites e.g., PHA (Pötter and Steinbüchel, 2005). PHAs are valuable biopolymers with wide range of applications (Younas et al., 2015; Ali and Jamil, 2016). They are polyesters of (R)-3-hydroxyalkanoic acid accumulated inside bacteria in the form of granules under stress conditions. So most of the microbes are capable of styrene metabolism under aerobic and anaerobic conditions (Beltrametti et al., 1997). Styrene degradation pathways are divided into upper and lower pathway. During upper pathway styrene is converted to phenyl acetic acid. It is a multistep reaction. In the first step styrene is converted to styrene epoxide as a result of oxidation of vinyl side chain. This step is catalyzed by flavin adenine dinucleotide dependent enzyme styrene monooxygenase (Hartmans et al., 1990). It is an unstable product so it is isomerizes to phenyl acetaldehyde by an enzyme styrene isomerase. In some bacterial species styrene epoxide can be converted to 2-phenyl ethanol (Beltrametti et al., 1997). After this phenyl acetaldehyde is

Received November 2, 2016; accepted February 2, 2017
Correspondence: Iftikhar Ali
E-mail: iftikhar_ali_iftikhar@yahoo.com

converted to phenyl acetic acid with the help of NAD^+ or phenyl acetaldehyde dehydrogenase (Chanprateep, 2010).

During the lower degradation pathway involves conversion of phenyl acetic acid to phenyl acetyl CoA. Phenyl acetyl coenzyme A then get enters into tricarboxylic acid TCA cycle. Besides this direct ring cleavage also occurs in *Pseudomonas fluorescens* and some other some other styrene degradative strains in which direct ring cleavage results in the release of 1, 2-dihydroxy-3-ethenyl-3-cyclo hexane into the medium. Styrene monooxygenase (SMO) is an important enzyme which determines the rate of styrene degradation in styrene degrading organisms (Hollmann et al., 2003). During the PHA biosynthesis phenyl acetyl CoA oxidizes to acetyl CoA (O'Leary et al., 2002). Two molecules of Acetyl CoA condense to form acetoacetyl CoA with the help of PhaA, a beta ketothiolase enzyme. Next step is the reduction of acetoacetyl CoA to (R)-3-hydroxyacyl-CoA catalyzed by phaB which is NADPH-dependent acetoacetyl-CoA reductase (Zhu et al., 2010). Third and last step is the polymerization of (*R*)-3-hydroxyacyl-CoA to the polyhydroxyalkanoate by the enzyme PHA synthase encoded by *pha*C gene (Chaudhry et al., 2011; Moita and Lemos, 2012).

The study described in this paper focused on the use of styrene oil for PHA production. Since virgin petrochemical plastics are poor due to their resistance for degradation, there is a potential to return waste resource into a useful biomaterial.

Materials and methods

Chemicals used in study

Styrene oil was used as a carbon source for growth and PHA accumulation by bacteria. The medium used for bacterial cultivation was PHA Detection Medium (Choi and Lee, 1999).

Isolation and selection of polyhydroxyalkanoates producing bacteria

Eight different samples were collected from different localities in Lahore, Pakistan (Three diesel contaminated soil samples from railway line, one sample from the automobile workshop, four samples of contaminated frying oil and one sample of frying oil contaminated soil from Sitara ghee and sugar mills). Soil samples were dug out 1-2 m deep carefully to ensure maximum natural flora and placed in sterile falcon tubes; taken to laboratory and processed further within 12 h after collection.

Samples were enriched by culturing in enriched medium (peptone 5 g/L, beef extract 3 g/L) (Sambrook and Russell, 2001) and were serially diluted up to 10^{-8} in saline. 50 μL pre culture of 10^{-2} and 10^{-6} dilution was spread on PHA detection agar (PDA; glucose 20 g/L, $(NH_4)_2SO_4$ 2.0 g/L, KH_2PO_4 13.3 g/L, $MgSO_4 \cdot 7H_2O$ 1.2 g/L, citric acid 1.7 g/L, trace element solution 10.0 ml/L, agar 15 g/L) containing Nile blue dye (0.5mg/mL) (Choi and Lee, 1999; Ali and Jamil, 2014). The trace element solution contained $FeSO_4 \cdot 7H_2O$ 20 g/L, $ZnSO_4 \cdot 7H_2O$ 2.0 g/L, $CuSO_4 \cdot 5H_2O$ 13.3 g/L, $MnSO_4 \cdot 5H_2O$ 1.2 g/L, $CaCl_2 \cdot 2H_2O$ 1.7 g/L, $Na_2B_4O_7 \cdot 10H_2O$ 10.0 ml, $(NH_4)_6Mo_7O_{24}$ 10.0 ml and 35% HCl 15 g/L (Choi and Lee, 1999). After 24 h incubation at 37°C, plates were observed under UV light. Colonies producing yellow or blue fluorescence were purified. CFU/mL was calculated. Isolates were further confirmed by Sudan Black B staining (Zinn et al., 2001). Alcoholic solution of Sudan black B was applied to stain bacterial colonies and glass slides were kept undisturbed for 30min. The excess dye was then decanted and slides were decolorized by adding xylene. Colonies unable to incorporate the Sudan black B appeared white, while PHA producers appeared bluish black. Phase contrast microscopy was done for rapid detection of PHA producers. To make the smear, 40 μL of the inoculated media was taken on a glass slide. It was flooded with crystal violet and washed after 1 min. PHA granules were observed as large inclusion bodies under phase contrast microscope (Hindré et al., 2008).

Morphological characterization

Morphological characteristics such as size, shape, color, margin, elevation, opacity, texture and Gram-staining of the 32 isolated bacterial strains were observed to characterize the bacterial colonies grown on PDA.

Screening on the basis of styrene degradation

Twenty strains which gave positive results for Nile blue A screening, Sudan Black B screening and phase contrast screening were selected and further screened for styrene degradation. Styrene oil was mixed with benzene (Rossi et al., 2011) to be provided in the media as it is very viscous in its actual form. From PDA plates, single colony was inoculated in 5 ml PHA media containing styrene oil as a sole carbon source for periods of up to 4 days at 37°C on a shaking incubator. A range of styrene oil concentration (2%, 4%, 6% up to 20%) was tested for growth and PHA accumulation. Out of 20 different bacterial strains, only 5 strains named 2A, 3A, 3, 11 and 14 were selected for further experiments. These strains were chosen based on their ability to grow best with the styrene oil (6%).

Growth kinetics and time profiling of PHA production

To study the growth pattern of microbial cultures, growth curve was made. 2% glucose was used as standard carbon source while 6% styrene was used as experimental carbon source to compare it with 2% glucose utilization rate. Incubation was given at 37°C in a shaking incubator. OD at 600 nm was noted at different time intervals for consecutive 4

days and pellet was made for each interval. Bacterial growth on styrene started appearing after 3 days and it increased with time up to a certain limit, after that growth declined. PHA extraction was also done from the biomass taken after regular intervals. Percentage of PHA was calculated to estimate and compare the amount of PHA.

Extraction and quantitative analysis of PHA

PHA extraction was done using sodium hypochlorite digestion method (Mohammadi et al., 2012; Ali and Jamil, 2016). Bacteria were grown in PHA medium containing glucose and styrene oil as two separate carbon sources and were harvested at stationary phase (after 72 h of incubation). Centrifugation was done at 6000 rpm for 15 min, washed twice with saline and lyophilized. Weight of the pellet was measured and sodium hypochlorite was added in such a way that 1 g of pellet contained 12 mL sodium hypochlorite. Mixture was shaken at 300 rpm at 37°C for 3 h; equal volume of chloroform was added and centrifuged at 3000 r/min for 20 min. Three separate phases were obtained. Upper phase contained sodium hypochlorite solution, undisrupted cells reside in the middle phase and chloroform layer containing PHA was in the bottom phase. Chloroform layer was transferred into pre-weighed glass vial and chloroform was allowed to evaporate. Resulting crude extract was preserved for further analysis.

FTIR spectroscopy to record IR spectra

Previously extracted PHAs were oven-dried and potassium bromide (KBr) pellets were prepared (Xiao and Jiao, 2011). Pellets were processed in M-series MIDAC FT-IR processing system (Model-M2000, Serial-725) with a spectral range of 500-5000 wavenumber (cm^{-1}). Different peaks were obtained for data analysis (GRAM/A1 software). Most of the sharp peaks were present in range of 1000–3000 wavenumber (cm^{-1}) which is actually specific for particular functional groups of PHA.

GC-MS Analysis

Sample of 5 days old cultured broth containing 6% styrene was taken and mixed with equal volume of cyclohexane (Park et al., 2005). Control was taken by adding styrene into uncultured PHA broth without glucose and vortexed. Upper layer was transferred to glass vials and left overnight to saturate the solution. Eight milligram of purified PHAs was methanolysed by heating at 100°C for 140 min in a mixture of 1.0 mL chloroform, 0.85 mL methanol and 0.15 mL concentrated sulfuric acid. 1.0 μL of the sample was injected in GCMS-QP2010 (Shimadzu) via split injection mode and nitrogen was used as the carrier gas at a flow rate of 3ml per minute. The oven temperature was programmed as follows: 60°C for 2 min at the start and then ramped at a rate of 5°C per minute to 260°C, and held for 15 min. The temperatures of the injector was set at 260°C and column flow rate was 0.57 cm/s. the column (DB-5 MS) had a length of 30 m with thickness of 0.25 μm and diameter of 0.25 mm. Gas chromatography system was coupled with mass spectrometry system (GCMS-QP2010 with D1), in which temperature of ion source was set at 200°C, to analyze the metabolites of styrene produced in PHA sample.

Genomic DNA Extraction, ribotyping and phylogenetic analysis of 16S rRNA genes from bacterial strains

Genomic DNA was successfully isolated from selected 5 strains using CTAB/NaCl method (15). Agarose gel electrophoresis was done for visualization of DNA bands. Isolated colonies of 5 purified strains were sent for 16S rRNA sequencing to Macrogen Korea. Method used for sequencing was Sanger dideoxy method. Resulting sequences were sorted for sequence homology by basic local alignment search tool (BLAST). To check the evolutionary relationship, phylogenetic trees of strains were constructed using MEGA software (Tamura et al., 2013) and aligned by ClustalW. Neighbor joining tree was constructed and reliability was checked by bootstrapping.

Results

Isolation and morphological characterization of PHA producing strains

PHA producing bacteria were isolated from 9 samples with frying oil and diesel contamination. CFU/mL calculated showed that diesel contaminated soil samples contained high bacterial count as compared to other samples. A total of 32 strains were isolated and morphologically characterized by colonial morphology and gram staining. Results showed that most of the colonies observed were small in size, circular shaped, opaque, smooth, off white colored and have raised elevation and entire margin and gram negative rods were excessive among strains however gram positive cocci and gram positive rods were also observed.

Selection of PHA producing strains

PHA production was detected by Nile blue A, Sudan Black B and phase contrast screening and 20 strains showed positive results for all the three types of screenings which indicate the PHA production within these strains. Yellow and blue fluorescence was observed in case of Nile blue screening when observed under UV light. Black PHA granules were observed as a result of Sudan Black B staining which were at pole regions, central region and in some cases have occupied the major portion of the bacterial cell. PHA inclusions as hollow circles in the cytoplasm were observed under phase contrast microscope. Screening results are shown in Fig. 1.

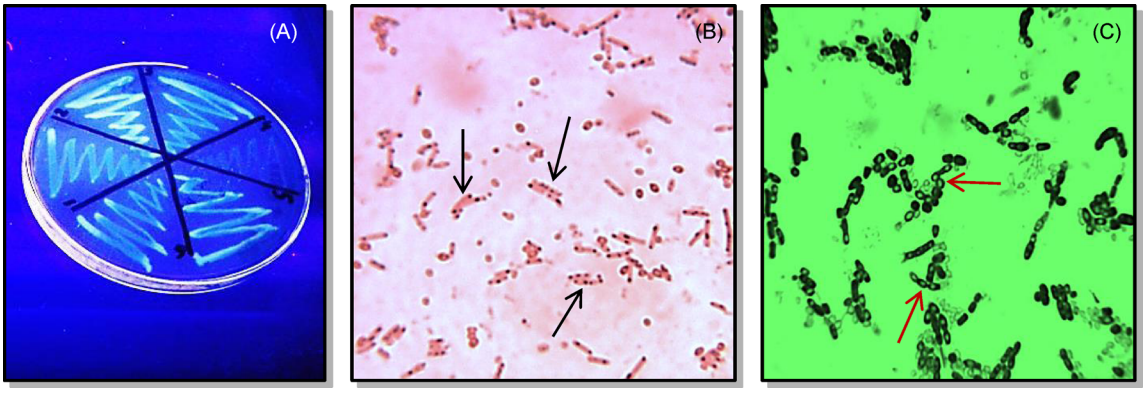

Figure 1 Selection of PHA producers. (A) Blue and yellow fluorescence observed under UV light in plates containing Nile blue. (B) Black granules observed at the poles, central region and occupying the maximum space within the cell when stained with Sudan Black B. (C) PHA inclusions as hollow circles observed within the cytoplasm of cell under phase contrast microscope.

Screening for styrene degradation bacteria

Out of the 20 strains, only 5 strains named 2A, 3A, 3, 11 and 14 degraded styrene oil when provided as a carbon source in the media at different concentrations. 6% styrene was considered to be the appropriate concentration as bacteria grew well on this concentration and it does not show any inhibitory effects on growth. These 5 strains were then selected for further experiments on the basis of their styrene degradability which is the main purpose of this study.

Growth kinetics and time profiling for PHA production

Growth curve of the selected 5 strains was made and it was observed that lag phase of strains inoculated in media containing glucose was short of about 4 h but in styrene this phase was much longer of 48 h. Stationary phase in glucose started after 18 h while in styrene it started after 64 h; after which decline phase started as shown in Fig. 2. Results of time profiling for PHA production showed that the percentage of PHA extracted from strains that were grown on glucose was more (30.60%) than that of styrene (19.50%) and percentage of PHA gradually increased with time. In glucose maximum cell mass and PHA % were obtained at 48 h and started decreasing after it while in styrene maximum biomass and PHA % were obtained after 96 h and started decreasing after this time. It was noted that strain 11 showed promising results as percentage of PHA was high (30.60%) as compared to other strains.

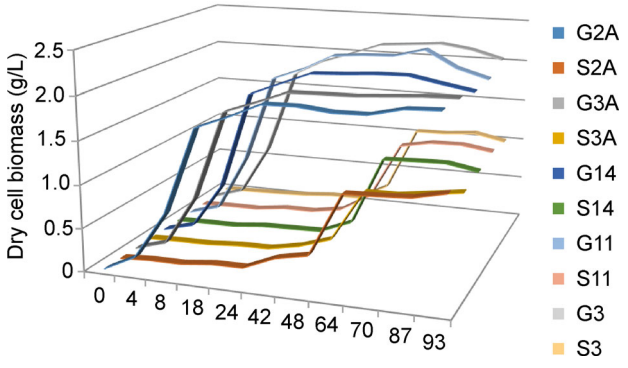

Figure 2 Growth curve of PHA producing strains. Growth on media containing glucose and also on media containing styrene as a carbon source. "G" represents glucose and "S" represents styrene.

Fourier transform infrared spectroscopy (FTIR)

IR spectra contained peaks which indicate the position of $-CH_2$, $-C=O$, $-C-O$ and many other functional groups. Shifting of functional group was also noted as the carbon source changed from glucose to styrene. Detailed results of selected strains are shown in Fig. 3(A-D). Most of the peaks were observed at exact position of PHB (1750-1735 cm^{-1}) which confirms the presence of PHB. All strains showed peaks which corresponds to ketones, esters, aromatics and aliphatic compounds. Also peaks for PHB and PHVs copolymers (2990-2850 cm^{-1}) were observed. All these bands show contribution to PHB which indicates that both *Enterobacter* strain 3A and *Enterobacter* strain 11 are able to accumulate PHB by using glucose as well as styrene as sole carbon source.

GC-MS analysis

GC for *Enterobacter* strain 3A resulted in five peaks, two of cyclohexane and rest three were of Epoxyhexanol, Benzene-dicarboxylic acid and Bromododecane (Fig. 4A). While for *Enterobacter* strain 11 resulted in four peaks cyclohexane, propyne, benzimidazole and phenylalanine (Fig. 4B). Control sample just contained peaks for cycloheaxane and styrene (Fig. 4C). Peak for styrene was not observed in samples containing styrene which shows that styrene is degraded and converted into its metabolites. These results indicate the utilization of styrene as carbon source by the *Enterobacter*

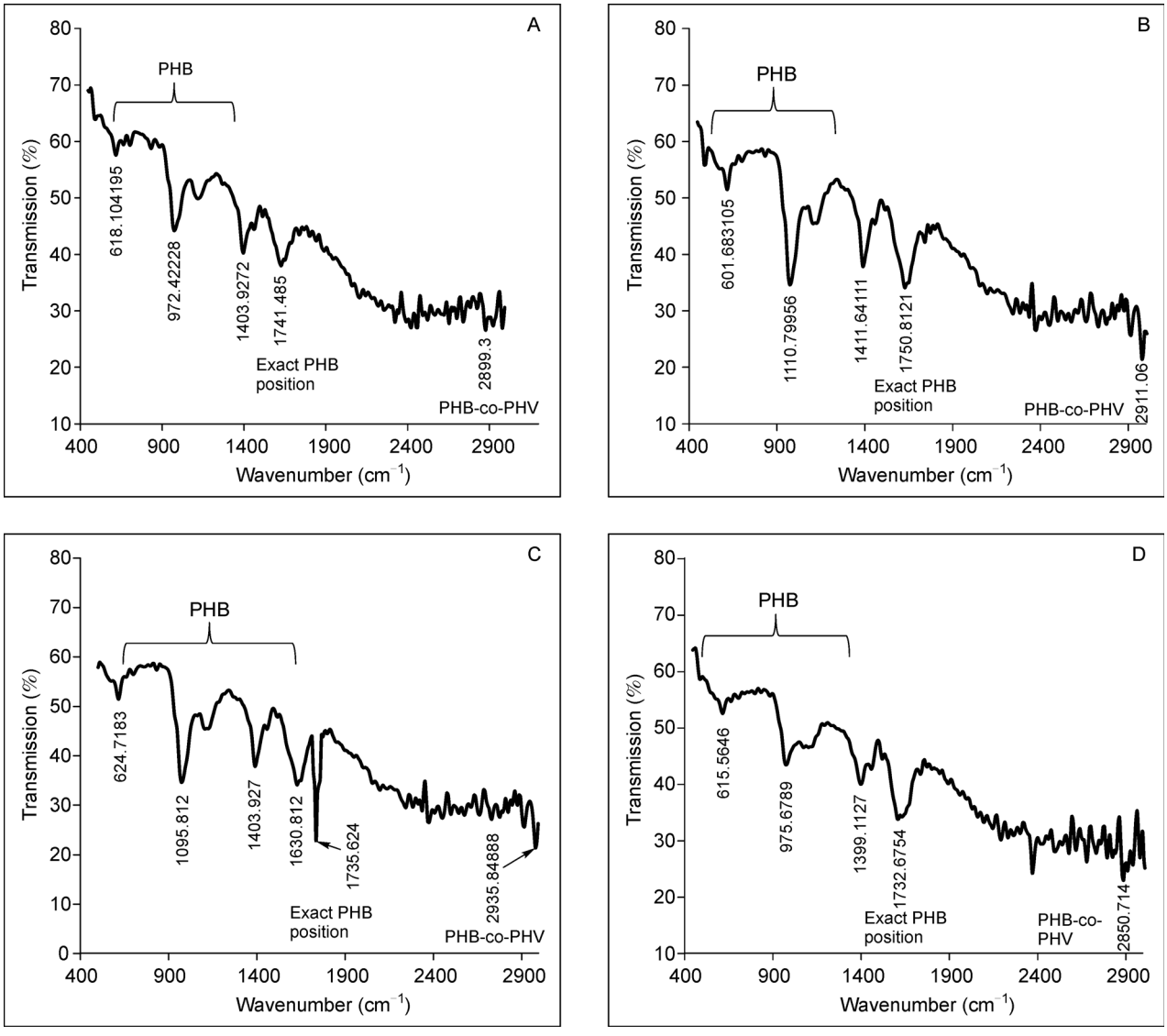

Figure 3 (A) FTIR spectra of PHA extracted from *Enterobacter* strain 3A with glucose as a carbon source. Sharp absorption peaks at 618 cm^{-1}, 972 cm^{-1}, 1403 cm^{-1}, 1658 cm^{-1}, and 2899 cm^{-1} corresponds to ketones, esters, aromatics, CH_3 and CH_2 functional groups respectively. (B) FTIR spectra of PHA extracted from *Enterobacter* strain 3A with styrene as a carbon source. Sharp absorption peaks at 601 cm^{-1}, 1110 cm^{-1}, 1411 cm^{-1}, 1750 cm^{-1} and 2911 cm^{-1} which corresponds to ketones, esters, aromatics and aliphatic compounds. (C) FTIR spectra of PHA extracted from *Enterobacter* strain 11 with glucose as a carbon source. Sharp absorption peaks at 624 cm^{-1}, 1095 cm^{-1}, 1408 cm^{-1}, 1630 cm^{-1}, 1735 cm^{-1} and 2935 cm^{-1} which corresponds to ketones, esters, aromatics and aliphatic compounds. (D) FTIR spectra of PHA extracted from *Enterobacter* strain 11 with styrene as a carbon source. Sharp absorption peaks at 615 cm^{-1}, 975 cm^{-1}, 1399 cm^{-1}, 1732 cm^{-1}and 2850 cm^{-1} which corresponds to ketones, esters, aromatics, primary and secondary amines and aliphatic compounds.

strain 3A and *Enterobacter* strain 11. Different by-products are most probably synthesized during the metabolic degradation of styrene by these bacterial enzymes.

Ribotyping and phylogenetic analysis

The sequencing results of 16S rRNA confirmed that all the 5 strains belong to genus *Enterobacter* (Table 1).

Phylogenetic analysis was done by constructing phylogenetic trees of selected strains which showed graphical representation of evolutionary relationship and reliability was checked by bootstrapping. The identification of bacteria on the basis of 16S rRNA gene sequencing represents a better molecular tool as compared to the conventional biochemical characterization methods. The isolated bacteria belonged to the genus Enterobacter that belongs to the family Enterobacteriaceae. Other members of this genus are more importantly studied from the medical perspective but very little work has been done on the beneficial aspects of these metabolically important genus of bacteria. In this regard our

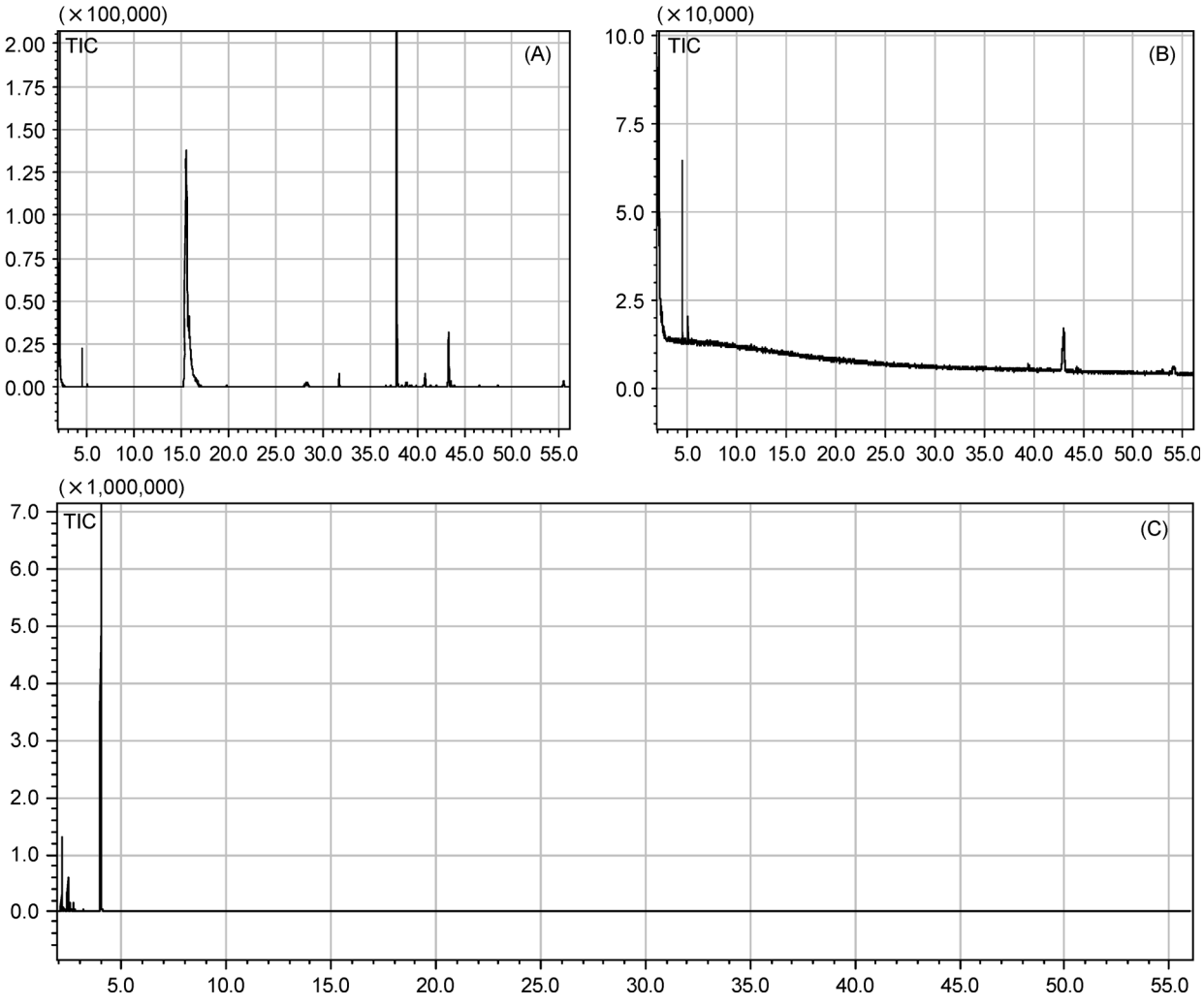

Figure 4 Gas chromatographs of sample from *Enterobacter* strain 3A (A) and from *Enterobacter* strain 11 (B). The control sample reported only styrene peak (C).

Table 1 Identification of isolated bacteria on the basis of 16S rRNA gene sequencing

Code of strain	Identity in BLAST (%)	Name of strain
2A	99	*Enterobacter cloacea* strain 2A
3A	99	*Enterobacter* sp. 3A
3	99	*Enterobacter* sp. strain 3
11	95	*Enterobacter asburiae* strain 11
14	99	*Enterobacter cloacea* strain 14

strains would be very useful for the degradation of complex carbon molecules such as styrene and many more.

Discussion

Samples for isolating PHA producers was selected as frying oil or soil contaminated with oil and diesel because oil provides a substrate for the PHA production. Frying oil contains fatty acids, triacylglycerols and other carbon rich substrates in it which are utilized for PHA production (Peplinski et al., 2010). Fatty acids present in the oils are metabolized to acetyl CoA by the β- oxidation pathway (Obruca et al., 2010). This acetyl CoA is crucial for PHA biosynthesis.

Selection of PHA producing bacteria was performed on the basis of fluorescence microscopy, Sudan black B staining and phase contrast microscopy. Among 30 isolated strains only 20 strains showed positive results for all the three types of screenings. Nile blue allows the fast preliminary analysis to occur, providing a rough insight that whether PHAs are present in the cells or not (Chaudhry et al., 2011). It can be applied at very low concentrations which does not inhibit the growth of bacteria. Sudan black positive strains showed black color inclusion bodies inside the cell after staining with Sudan black dye. Sudan black B is a slightly basic dye and it

combines with the acidic groups in lipid moieties of the compound and thus it stains the phospholipids in the compound. It is due to the physical solubility of the dye in the lipid particles and these lipid particles are present in the PHA granules. So in this way Sudan black stained black the PHA producing bacterial strains (Spiekermann et al., 1999). The third screening technique was Phase contrast screening to confirm PHA producers. PHA exist as discrete inclusions that are typically 0.2–0.5 μm in diameter localized in the cell cytoplasm and may be visualized quite clearly with a phase contrast light microscope due to their high refractivity (Sudesh et al., 2000). PHA inclusions were seen as hollow circles inside the bluish black boundary of the cell. It has been reported that after PHA accumulation cells become enlarges as compare to normal cells (Sathesh and Murugesan, 2010).

Growth curve indicated a long lag phase in styrene carbon source. The reason behind this long lag phase is that bacteria adapt itself to grow in the presence of styrene and prepare enzymes required for styrene degradation. Contrary to this lag phase is much short in glucose as it is easily available carbon source in medium and is present in excess amount and bacteria has priority to use glucose than any other carbon source in medium. Log phase time period was same for glucose and styrene. During log phase bacteria divided in excessive amount and produced PHA due to plenty of available carbon source. After which stationary phase was started in which division and death rate are equal bacteria utilize PHA granule as carbon source for its survival due to depletion of carbon source. During death or decline phase nutrients in media are replenished.

Strains 3, 11, 14, 2A and 3A were optimized for PHA production using different time intervals that on which time PHA production was at maximum level (Luhana and Patel, 2013). Strains were grown in PHA broth containing 2% glucose as well as in PHA broth containing 6% styrene. After regular intervals of time, broth was taken and its biomass was calculated. Time intervals used were 24, 48, 72, 96 and 120 h. PHA extraction was also done from the biomass taken after regular intervals. PHA extraction was done by sodium hypochlorite digestion method which will be discussed in the next section. Product accumulation kinetics revealed that the percentage of PHA extracted from strains that were grown on glucose was more (30.60%) than that of styrene (19.50%) and percentage of PHA gradually increased with time. Bacteria showed maximum PHA percentage at stationary phase. At this phase bacteria store carbon and wait for the limiting nutrients to return back to the medium (Paladino, 2009). PHA percentage increases as the time increases however biomass increased up to 72 h and then either become constant or decreases. Strains grew more efficiently on glucose as compared to styrene. The reason behind may be that styrene is an aromatic compound. Aromatic compounds change the membrane structure by changing the membrane fluidity and protein conformations. This alteration disrupts the membrane integrity, energy transduction mechanisms and membrane associated enzyme activity. This is how it proves toxic to microbes

Results obtained by GC-MS were in the form of peaks, as components of the sample were separated and spectral output was obtained (Yang et al., 2013). GC-MS analysis was done for the PHA accumulated by *Enterobacter* strains 3A and 11 which showed maximum production. Sharp peak of benzene dicarboxylic acid was observed in strain 3A while small peaks of propyne, benzimidazole and phenylalanine were observed in strain 11. Peaks of cyclohexane were observed in both samples as well as in control as it was used as a solvent while peak of styrene was detected only in control but not in rest of two samples which is due to the fact that styrene was degraded by *Enterobacter* sp. Basically *Enterobacter* is involved in anaerobic degradation of styrene. Upper pathway for styrene degradation is same in both aerobic and anaerobic styrene degraders divergence arise only in lower pathway of styrene degradation bacterial cultures but divergence arise in the lower pathway (Velasco et al., 1998). In the upper pathway styrene is converted to styrene oxide, phenylacetaldehyde and then to phenyl acetic acid (O'Leary et al., 2002). *Enterobacter* utilizes the oxygen dissolved in media to undergo through upper pathway of styrene metabolism. But lower anaerobic pathway it converts phenylacetic acid to benzoic acid which structural homology to benzenedicarboxylic acid detected in GCMS chromatogram *Enterobacter* strain 3A structure of both compounds are same but an extra carboxyl group was present in benzenedicarboxylic acid which may come from benzene, toluene or other compounds present in the styrene oil. Bacteria then converts this benzoic acid to acetyl CoA which ultimately leads to the synthesis of PHA (Rosazza et al., 1995). Besides this *Enterobacter* sp. also converts phenyl acetic acid to phenylalanine (an amino acid required for styrene biosynthesis). The propyne is produced by degrading styrene anaerobically which go further in propionate metabolism and it is used as a carbon source by bacteria. It was concluded from GC-MS results that bacteria degrade styrene into phenylalanine and propyne which are used by bacteria for growth purposes by phenylalanine and propionate metabolism. Benzoate degradation results in formation of benzimidazole. Moreover these plasmids play an important role in anaerobic meta cleavage of styrene. The alterations in the pathway may be because of the presence of benzene, toluene and other aromatic compounds in the styrene oil which results in alteration of structures and formation of analogs.

FTIR spectrum is chemical profile of sample (Otari and Ghosh, 2009). Most of the sharp peaks were present in range of 1000–3000 wavenumber (cm^{-1}). This region is actually specific for particular functional groups of PHA. It was noted that in the IR spectra, peaks were present which indicate the position of $–CH_2$, –C = O, –C–O and many other functional groups related to PHA polymers (Michael et al., 2012). Though a number of ambiguous peaks were also seen. Shifting of functional group was also noted as the carbon

source changed from glucose to styrene. By utilizing styrene oil, strains also produced poly (3HB-co-3HV) evident by the peaks present at 2988 cm^{-1} position. These functional groups are characteristics of PHA polymers and further confirmed the PHA accumulation from styrene by our isolated strains.

Conclusion

This study reports the isolation and identification of the potential PHA producing bacteria that belong to the genus Enterobacter. These bacteria could produce about 20% of PHA contents when grown as styrene as sole carbon source, although more PHA contents were produced when glucose was supplied as sole carbon source. In future these bacteria can be further employed in the up-scale fermentations after rigorous optimizations to get better PHA productivity.

Acknowledgment

Authors are thankful to University of the Punjab, Pakistan, International foundation of science, IFS and HEC funded NRPU-3443, Pakistan to support this research

Conflict of interest

Authors declare no conflict of interest.

References

Ali I, Jamil N (2014). Enhanced biosynthesis of poly(3-hydroxybutyrate) from potato starch by Bacillus cereus strain 64-INS in a laboratory-scale fermenter. Prep Biochem Biotechnol, 44(8): 822–833

Ali I, Jamil N (2016). Polyhydroxyalkanoates: Current applications in the medical field. Front Biol, 11(1): 19–27

Ali I, Jamil N (2016).Biosynthesis and characterization of poly3-hydroxyalkanote (PHA) from newly isolated bacterium *Bacillus* sp. AZR-1. Iran J Sci Technol Trans Sci, pp. 1–8

Beltrametti F, Marconi A M, Bestetti G, Colombo C, Galli E, Ruzzi M, Zennaro E (1997). Sequencing and functional analysis of styrene catabolism genes from *Pseudomonas fluorescens* ST. Appl Environ Microbiol, 63(6): 2232–2239

Chanprateep S (2010). Current trends in biodegradable polyhydroxyalkanoates. J Biosci Bioeng, 110(6): 621–632

Chaudhry W N, Jamil N, Ali I, Ayaz M H, Hasnain S (2011). Screening for polyhydroxyalkanoate (PHA)-producing bacterial strains and comparison of PHA production from various inexpensive carbon sources. Ann Microbiol, 61(3): 623–629

Choi J, Lee S Y (1999). Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. Appl Microbiol Biotechnol, 51(1): 13–21

Hartmans S, van der Werf M J, de Bont J A (1990). Bacterial degradation of styrene involving a novel flavin adenine dinucleotide-dependent styrene monooxygenase. Appl Environ Microbiol, 56(5): 1347–1351

Hindré T, Brüggemann H, Buchrieser C, Héchard Y (2008). Transcriptional profiling of *Legionella pneumophila* biofilm cells and the influence of iron on biofilm formation. Microbiology, 154(Pt 1): 30–41

Hollmann F, Lin P C, Witholt B, Schmid A (2003). Stereospecific biocatalytic epoxidation: the first example of direct regeneration of a FAD-dependent monooxygenase for catalysis. J Am Chem Soc, 125 (27): 8209–8217

Hussain Z, Khan K M, Hussain K (2010). Microwave–metal interaction pyrolysis of polystyrene. J Anal Appl Pyrolysis, 89(1): 39–43

Luhana K, Patel V(2013). Quantitative extraction and analysis of bioplastic (PHA) accumulated in bacterial isolates of paint industry effluent. International Journal of Chemtech Applications (INTJCA), 2(3): 52–62

Michael P, Loganayagi R, Nancy D, Ranandkumar S G, Indra A P (2012). Isolation and characterization of indigenous Ralstonia strain, YRF1 for high Polyhydroxy Alkanoates (PHA) production. Elixir Appl Biol, 48(2012): 9424–9427

Miskolczi N, Borsodi N, Buyong F, Angyal A, Williams P T (2011). Production of pyrolytic oils by catalytic pyrolysis of Malaysian refuse-derived fuels in continuously stirred batch reactor. Fuel Process Technol, 92(5): 925–932

Mohammadi M, Hassan M A, Shirai Y, Man H C, Ariffin H, Yee L N, Mumtaz T, Chong M L, Phang L Y (2012). Separation and purification of polyhydroxyalkanoates from newly isolated *Comamonas* sp. EB172 by simple digestion with sodium hydroxide. Sep Sci Technol, 47(3): 534–541

Moita R, Lemos P C (2012). Biopolymers production from mixed cultures and pyrolysis by-products. J Biotechnol, 157(4): 578–583

O'Leary N D, O'Connor K E, Dobson A D (2002). Biochemistry, genetics and physiology of microbial styrene degradation. FEMS Microbiol Rev, 26(4): 403–417

Obruca S, Marova I, Svoboda Z, Mikulikova R (2010). Use of controlled exogenous stress for improvement of poly(3-hydroxybutyrate) production in *Cupriavidus necator*. Folia Microbiol (Praha), 55(1): 17–22

Otari S, Ghosh J (2009). Production and characterization of the polymer polyhydroxy butyrate-copolyhydroxy valerate by *Bacillus megaterium* NCIM 2475. Current Research Journal of Biological Sciences, 1 (2): 23–26

Paladino L P A (2009) .Screening, optimization and extraction of polyhydroxyalkanoates and peptidoglycan from *Bacillus megaterium*.

Park M S, Han J H, Yoo S S, Lee E Y, Lee S G, Park S (2005). Degradation of styrene by a new isolate *Pseudomonas putida* SN1. Korean J Chem Eng, 22(3): 418–424

Peplinski K, Ehrenreich A, Döring C, Bömeke M, Reinecke F, Hutmacher C, Steinbüchel A (2010). Genome-wide transcriptome analyses of the 'Knallgas' bacterium *Ralstonia eutropha* H16 with regard to polyhydroxyalkanoate metabolism. Microbiology, 156(Pt 7): 2136–2152

Pötter M, Steinbüchel A (2005). Poly(3-hydroxybutyrate) granule-associated proteins: impacts on poly(3-hydroxybutyrate) synthesis and degradation. Biomacromolecules, 6(2): 552–560

Rosazza J P, Huang Z, Dostal L, Volm T, Rousseau B (1995). Review: biocatalytic transformations of ferulic acid: an abundant aromatic natural product. J Ind Microbiol, 15(6): 457–471

Rossi G, Monticelli L, Puisto S R, Vattulainen I, Ala-Nissila T (2011). Coarse-graining polymers with the MARTINI force-field: polystyrene as a benchmark case. Soft Matter, 7(2): 698–708

Sambrook J, Russell D W (2001). Molecular cloning: a laboratory manual (3-volume set). Vol. 999. Cold spring harbor laboratory press Cold Spring Harbor, New York

Sathesh Prabu C, Murugesan A (2010). Effective utilization and management of coir industrial waste for the production of poly-Î²-hydroxybutyrate (PHB) using the bacterium *Azotobacter beijerinickii.* Int J Environ Res, 4(3): 519–524

Spiekermann P, Rehm B H, Kalscheuer R, Baumeister D, Steinbüchel A (1999). A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Arch Microbiol, 171(2): 73–80

Sudesh K, Abe H, Doi Y (2000). Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. Prog Polym Sci, 25 (10): 1503–1555

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol, 30(12): 2725–2729

Velasco A, Alonso S, García J L, Perera J, Díaz E (1998). Genetic and functional analysis of the styrene catabolic cluster of *Pseudomonas* sp. strain Y2. J Bacteriol, 180(5): 1063–1071

Xiao N, Jiao N (2011). Formation of polyhydroxyalkanoate in aerobic anoxygenic phototrophic bacteria and its relationship to carbon source and light availability. Appl Environ Microbiol, 77(21): 7445–7450

Yang C, Zhang W, Liu R, Zhang C, Gong T, Li Q, Wang S, Song C (2013). Analysis of polyhydroxyalkanoate (PHA) synthase gene and PHA-producing bacteria in activated sludge that produces PHA containing 3-hydroxydodecanoate. FEMS Microbiol Lett, 346(1): 56–64

Younas T, Ali I, Jamil N (2015). Polyhydroxyalkanotes (PHAs) production by using canola oil as carbon source from bacteria isolated near paper pulp industry. Kuwait Journal of Science, 42(2)

Zhu C, Nomura C T, Perrotta J A, Stipanovic A J, Nakas J P (2010). Production and characterization of poly-3-hydroxybutyrate from biodiesel-glycerol by Burkholderia cepacia ATCC 17759. Biotechnol Prog, 26(2): 424–430

Zinn M, Witholt B, Egli T (2001). Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate. Adv Drug Deliv Rev, 53(1): 5–21