Tech Science Press

Blend of Polyhydroxyalkanoates Synthesized By Lipase Positive Bacteria From Plant Oils

Nasir Javaid, Rida Batool and Nazia Jamil*

Department of Microbiology and Molecular Genetics, University of the Punjab, Quaid-e-Azam Campus, Lahore-54590, Pakistan.
*Corresponding Author: Nazia Jamil. Email: nazia.mmg@pu.edu.pk.

Abstract: A total of 5 biochemically characterized lipase positive bacterial strains were screened for Polyhydroxyalkanoates (PHA) production by Nile blue staining and confirmation was done by Sudan Black B. PHA production ability for all strains was optimized followed by time profiling calculation and comparison via using glucose and two plant oils i.e., canola and mustard oil. Quantitative analysis showed that glucose can serve as a carbon source for maximum biomass (2.5 g/L CDW for strain 5) and PHA production (70.3% for strain 2). PHA produced by strain 2 was further analyzed for its chemical composition and type via Fourier Transform Infrared (FT-IR) spectroscopy. It revealed homopolymer (PHB) and copolymer (PHB-co-PHV) production of PHA (peaks at 1743 cm^{-1} and 2861 cm^{-1}, respectively) with both canola and mustard oil unlike glucose which produced only homopolymer one i.e., PHB (peaks at 1110 cm^{-1}, 1411 cm^{-1} and 1650 cm^{-1}). Crystallinity of FT-IR analyzed PHA was calculated using mathematical formulas which showed decrease from glucose to canola to mustard oil. This study revealed that plant oils can serve as better carbon source to produce better quality (ductile and copolymer) PHA. Moreover, 16S rRNA gene sequencing analysis showed that strain 1, strain 2, strain 3, strain 4 and strain 5 are *Stenotrophomonas* sp. N3, *Exiguobacterium* sp. N4, *Exiguobacterium* sp. Ch3, *Cellulosimicrobium* sp. A8 and *Klebsiella* sp. LFSM2, respectively.

Keywords: Bioplastics; polyhydroxyalkanoates; lipase positive bacteria; plant oils, FT-IR

1 Introduction

Biodegradable plastics are gaining importance; as the problems of using synthetic plastics like solid waste management, increment of CO_2 in the atmosphere, being non-dismantled in soil for longer time and depletion of non-renewable resources could be controlled by using them [17]. This can be realized by the huge increase in bioplastic production, which is expected to touch 1.7 million tons by 2015 (http://www.chem.umn.edu/csp/pdfs/CWJune2012%20article.pdf). Wide varieties of microbes like bacteria, cyanobacteria, plants and yeasts have been reported to synthesize and accumulate PHA [7]. Low yield was observed from plant cells. They are able to produce <10% (w⁄w) of PHA while bacteria canproduce as high as 90% (w⁄w) of the dry cell mass [2]. It is believed that about 300 different prokaryotic microorganisms exhibit the phenomenon of intracellular PHA production and many of them, such as *Ralstonia*sp., *Bacillus* sp., *Pseudomonas* sp. *and Alcaligenes*sp.etc. produce PHAs naturally [14]. All Gram positive and negative bacteria, Archaea, halophiles bacteria are able to produce PHA [28]. Out of these, *Bacillus* has been reported as the first genus to accumulate PHAs while *Haloferaxmediterranei* has the ability to accumulate PHA in large amounts.

Biosynthesis of PHA is activated when there is reduction nofnutrients essential for growth, such as magnesium, sulfur, nitrogen or phosphorus but additional accessibility of carbon source [20,30]. Under nutrient depletion conditions growth rate of bacteria slows down and they start storing their carbon reserves and energy as PHA granules, inside their cells [10] mainly in cytoplasm as water-insoluble

DOI: 10.32604/jrm.2019.00023

granules [29]. More than 150 identified polyhydroxyalkanoates constitute short-chain length and medium-chain length monomers [9,11]. Several PHAs like PHB and Poly(3HB-co-3HV) are produced industrially now a days and one of the examples is KANEKA Biopolymer AONILEX [18]. By structure, PHA granules are subcellular complexes which consist of a polyester core, regulatory proteins and associated proteins such as phasins, PHA synthases and PHA depolymerases [11].

Several carbohydrates including different sugars like glucose, fructose, sucrose, maltose, cellobiose, arabinose, propionate, glycerol, and mannitol has been used as carbon source for PHA accumulation in *Bacillus* sp. [21]. Production cost of PHA remainshigher as compared to conventional plastics when these sugars are used as carbon source. PHA production was also checked by using fatty acids [23]. The production cost can bereduced by trying to produce PHAs from plant oils which are more proficient carbon source [4]. So, now days, plant oils are being used industrially as a potential feedstock for PHA accumulation e.g., Danimer Scientific, USA is using Cold pressed canola oil to produce mcl-PHA (Nodax® PHA) and Kaneka Corporation, Japan is using Plant oils to produce PHB-PHHx (AONILEX®).Different plant oils like linseed, soybean, cottonseed, oilseed radish, and peanut oils have been reported for bioplastic production [13]while this study deals with canola and mustard oil. Canola (*Brassicanapus*) is included in oil seed crop with protein contents of 30-45% (w/w) and oil content of 40-50% (w/w) in defatted meal [12] and considered to be a cheap carbon source for bioplastic production. Mustard oil is considered to be an antibacterial essential oil which inhibits bacterial growth by disrupting the membrane of bacterial cells [5].

In the present study, five bacterial strains were analyzed to produce PHA using three different carbon sources. The goal of this study was to evaluate a) how different carbon sources affect the quantity and quality of produced PHA helping to produce good quality PHA with cheap carbon source and b) how mustard oil affects bacteria in terms of PHA production despite its antibacterial nature.

2 Materials and Methods

2.1 Isolation of PHA Producing Bacterial Strains

Strain 1, strain 2, strain 3 and strain 4 were isolated from soil while strain 5 was isolated from tissue sample and were biochemically characterized. Strains were grown in nutrient rich medium (yeast extract 5 g/L, NaCl 2.5 g/L and trypton 8 g/L) followed by spreading (50 µl) of grown culture on tributyrin agar medium, to confirm lipase presence [6] and PHA detection agar (PDA), to screen PHA producers via visualizing under UV after overnight incubation at 37°C. Intracellular PHA granules were later confirmed by Sudan black B and Acridine orange staining (Kumar 2006). Recipe for 1 liter PDA includes: 2.0 g $(NH_4)_2SO_4$,13.3 g KH_2PO_4, 1.2 g $MgSO_4.7H_2O$, 1.7 g Citric acid, 10.0 mL trace element solution, 10.0 mL Nile blue (0.5 mg/mL in methanol) and 15.0 g agar. Trace element solution is composed of following ingredients (per liter of distilled water): 10.0 g $FeSO_4.7H_2O$, 2.25 g $ZnSO_4.7H_2O$, 1.0 g $CuSO_4.5H_2O$, 0.5 g $MnSO_4.5H_2O$, 2.0 g $CaCl_2.2H_2O$, 0.23 g $Na_2B_4O_7.10H_2O$, 0.1 g $(NH_4)_6Mo_7O_{24}$ and 10.0 mL 35% HCl.

2.2 Utilization of Various Carbon Sources for PHA Production by Bacteria

Reference carbon source i.e., glucose and experimental carbon sources i.e., canola (brand name CanOlive) and mustard oil (brand name Sarsona by Marhaba products) were diluted, adjusted to pH 7.0, filtered (0.45 µm) and added to PHA production medium broth (2.0 g/L $(NH_4)_2SO_4$,13.3 g/L KH_2PO_4, 1.2 g/L $MgSO_4.7H_2O$, 1.7 g/L Citric acid, 10.0 mL/L trace element solution) in such a way that final concentration in solution was 2% i.e., 20 g/Lfor all three carbon sources while a lower concentration (1%) was also used in case of mustard oil due to its antibacterial activity so that bacteria not able to grow at higher concentration (2%) may get a chance to grow at lower concentration (1%). All five strains were inoculated and grown in nutrient rich medium (yeast extract 5 g/L, NaCl 2.5 g/L and trypton 8 g/L) to set up pre-culture via incubating at 200 rpm and 37°C. Once the optical density was reached to 0.5 at 600 nm (approx. within 8 h), 3 mL (3% v/v) pre-culture of each bacterial strain was inoculated into four flasks

each containing 97 mL PHA production medium but with different carbon source (three containing glucose, canola and mustard oil at 2% final concentration and one containing mustard oil at 1% final concentration). All flasks were incubated at 37°C with 200 rpm speed and samples were collected after every 24 h from 0 to 96 h to analyze biomass and PHA.

2.3 PHA Extraction

Cells were harvested from collected samples by centrifugation (20 min at 6000 × g) followed by washing (with acetone) and drying of these cells under vacuum at room temperature. The dried biomass was used for PHA extraction using chloroform extraction method. In this method, 100 mL chloroform and 100 mL 6% sodium hypochlorite were used to homogenize about 12 g of biomass powder. The obtained mixture was shaken for 3 h at 300 rpm and 37°C followed by centrifugation for 20 min at 3000 × g. PHA containing bottom layer was collected. Ice cold methanol and chloroform (methanol: chloroform = 9:1) was used to precipitated PHA. Finally, the obtained precipitates of PHA were filtered by simple filtration followed by evaporative drying at 60°C [8]. PHA content was obtained by dividing weight of extracted PHA by cell dry weight (CDW) and was expressed in terms of percentage of CDW.

2.4 PHA Characterization by FTIR Spectroscopic Analysis

For FTIR analysis, strain 2 was grown with glucose (2%), canola (2%) and mustard oil (2%), each in three different flasks, and cells from each flask were collected after 72 h by centrifuging culture at 4000 × g for 20 min. Cell pellet was washed, lyophilized, mixed (5-10%) with KBr (90-95%) and grinded well to make a transparent pellet. The obtained pellet was processed in M-series MIDAC FT-IR processing system (Model-M2000, Serial-725) with frequency range of 500-5000 cm^{-1}. Different peaks were obtained after data analysis (GRAM/AI software) depicting relative absorbance intensities of ester carbonyl band at 1740 $cm^{-1}$and keto carbonyl band at 1715 cm^{-1}. Relative indices and crystallinity of PHA produced by strain 2 were determined by using following formulas [3]:

Keto carbonyl bond index = I_{1715} / I_{1465}

Ester carbonyl bond index = I_{1740} / I_{1465}

Polymer material was also evaluated in terms of its percentage crystallinity based on method suggested by Zerbi [31]

$$\%\, Crystallinity = 100 - \left[1 - \frac{\frac{Ia}{1.233Ib}}{1 + \left(\frac{Ia}{Ib}\right)}\right] \times 100$$

where **Ia** and **Ib** are the absorbance values determined from the bands at 1474 and 1464 cm^{-1} or from 730 and 720 cm^{-1}, respectively.

3 Results

3.1 Strain Identification

All biochemically characterized strains were Nile-blue positive. Presence of PHA granules was confirmed by Sudan black Band Acridine orange staining (Supplementary Data). All strains were confirmed by Sanger dideoxy sequencing: Isolate N3 was identified as *Stenotrophomonas* sp. (GenBank accession number KM234128) having 99% homology with *Stenotrophomonaspavanii* strain ICB 89 (NR116793); Isolate Ch3 was identified as *Exiguobacterium* sp. (GenBank accession number KM234125) having 99% homology with *Exiguobacterium*sp. AT1b (NR074970). Isolate N4 was identified as *Exiguobacterium* sp. (GenBank accession number KM234126) having 99% homology with *Exiguobacterium* sp. AT1b (NR074970). Isolate LFSM2 was identified as *Klebsiella* sp. (Gen Bank accession number KM234127) having 99% homology with *Klebsiellapneumoniae* strain DSM 30104 (NR117686). Isolate A8 was identified as *Cellulosimicrobium* sp. (GenBank accession number JX870652) having 99% homology with *Cellulosimicrobiumcellulans* strain DSM 43879 (NR119095).

3.2 Time Profiling of Biomass and PHA Production

Time profiling for PHA production was done for all strains with all three carbon sources by analyzing samples at equal intervals of 24 h up to 96 h (Figs. 1-5). Maximum biomass was 2.5 g/L produced by strain 5 with 2% glucose at 72 h (Fig. 5(A)) while maximum PHA was 70.3% produced by strain 2 with 2% glucose at 48 h (Fig. 2(A)).

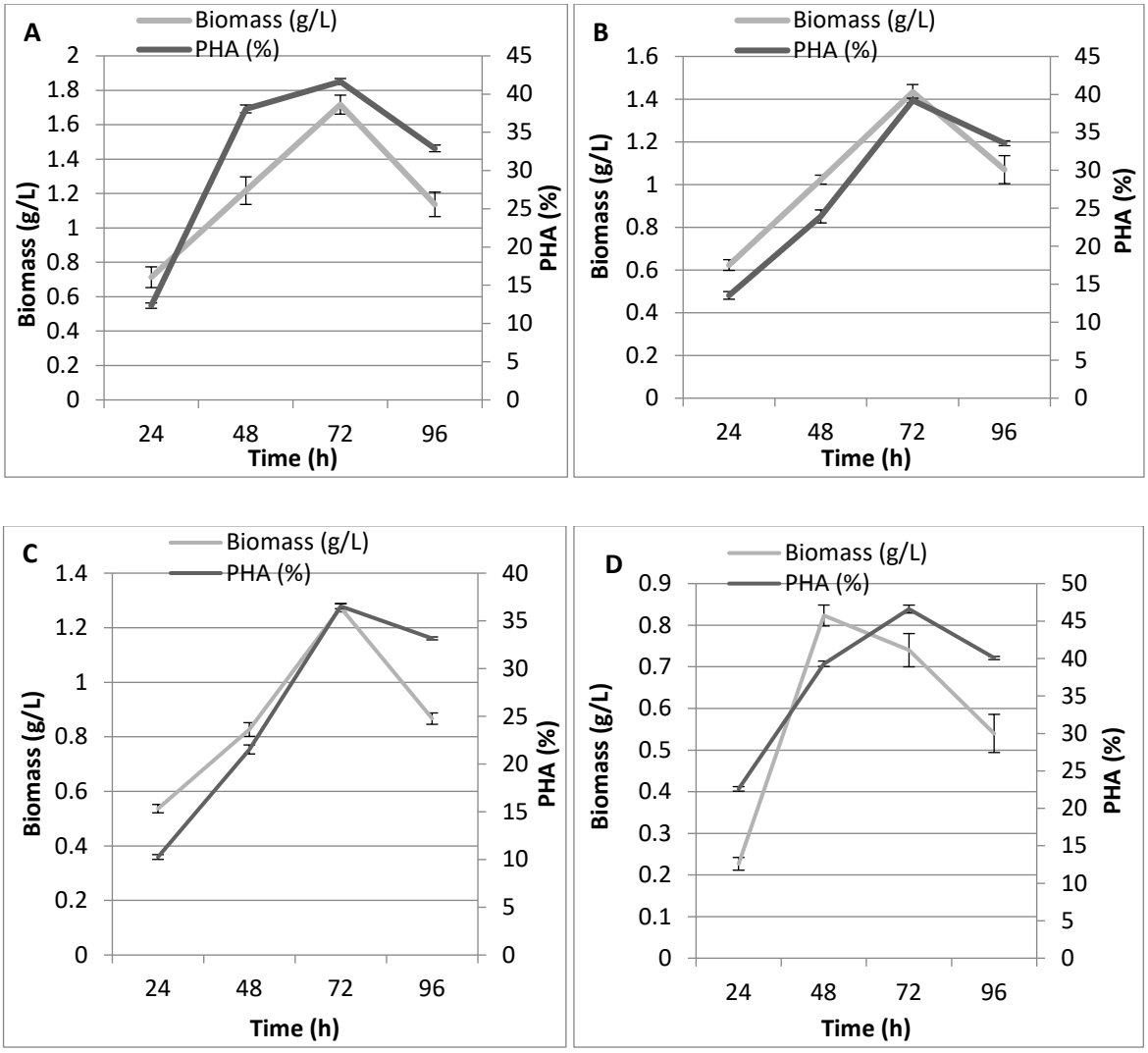

Figure 1: PHA time profiling for *Stenotrophomonas* sp.N3 with **(A)**2% glucose **(B)** 2% canola **(C)** 1% mustard oil and **(D)** 2% mustard oil

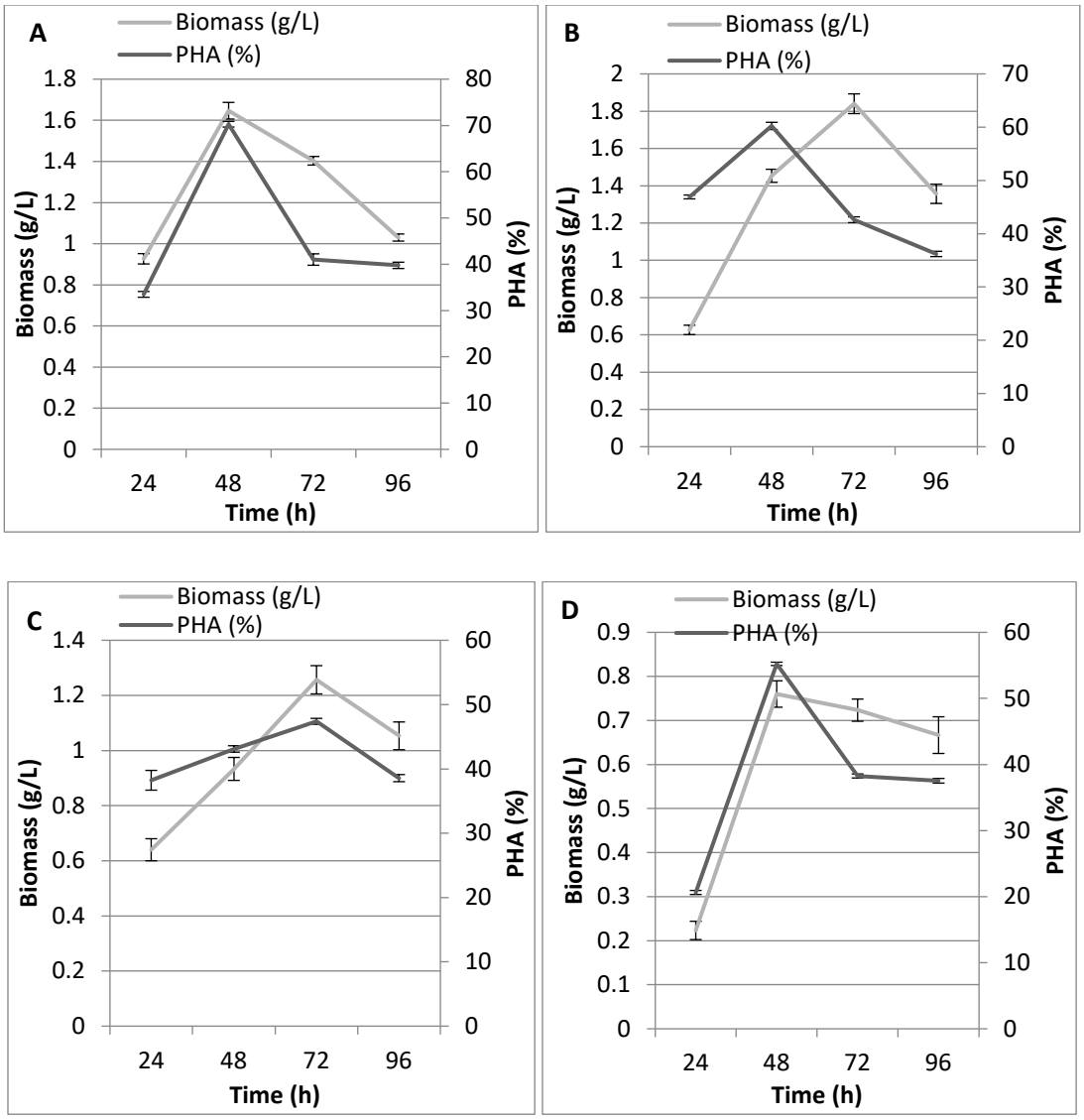

Figure 2: PHA time profiling for *Exiguobacterium*sp. N4 with **(A)** 2% glucose **(B)** 2% canola**(C)** 1% mustard oil and **(D)** 2% mustard oil

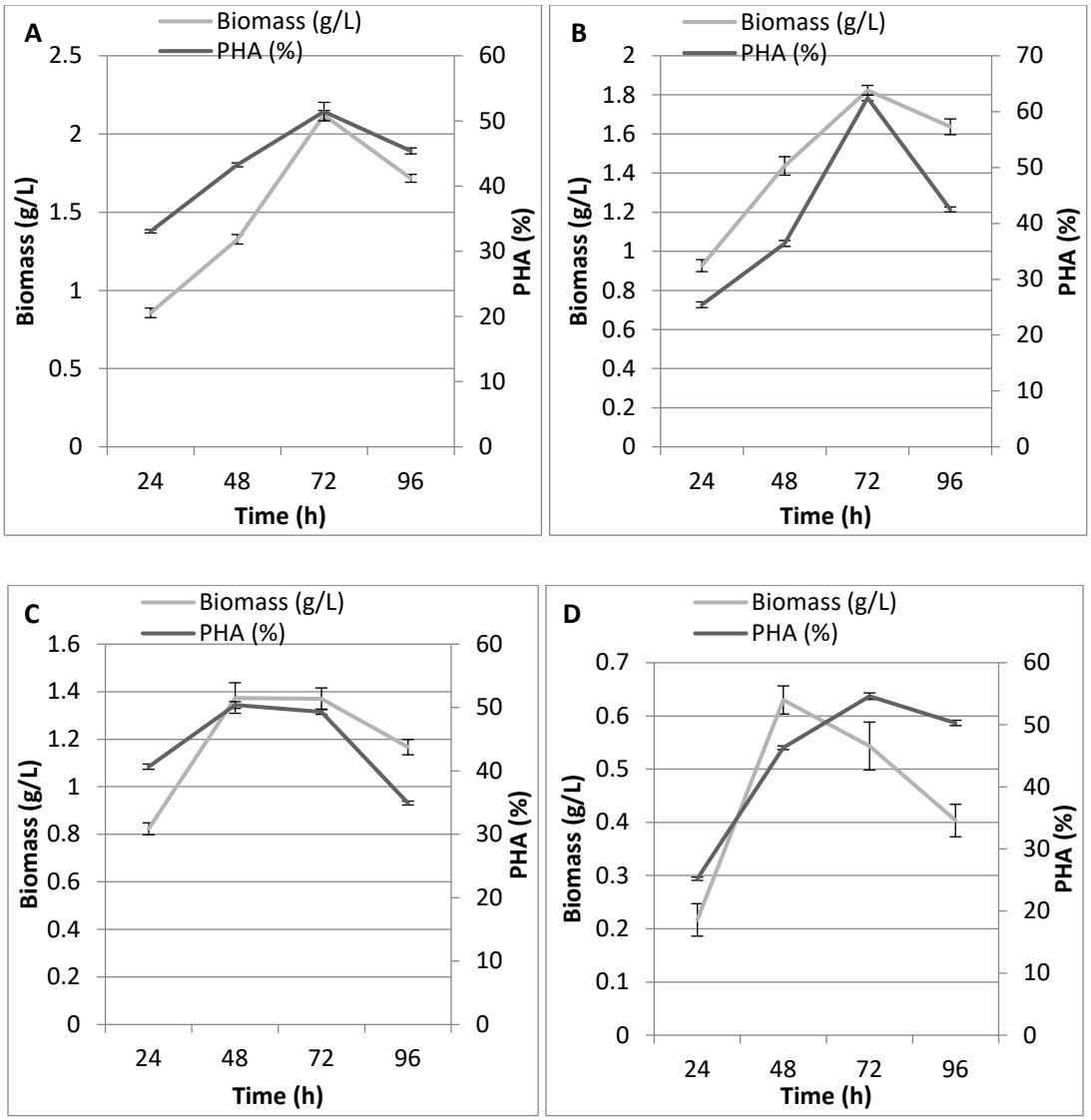

Figure 3: PHA time profiling for *Exiguobacterium*sp. Ch3 with **(A)** 2% glucose **(B)** 2% canola **(C)** 1% mustard oil and **(D)** 2% mustard oil

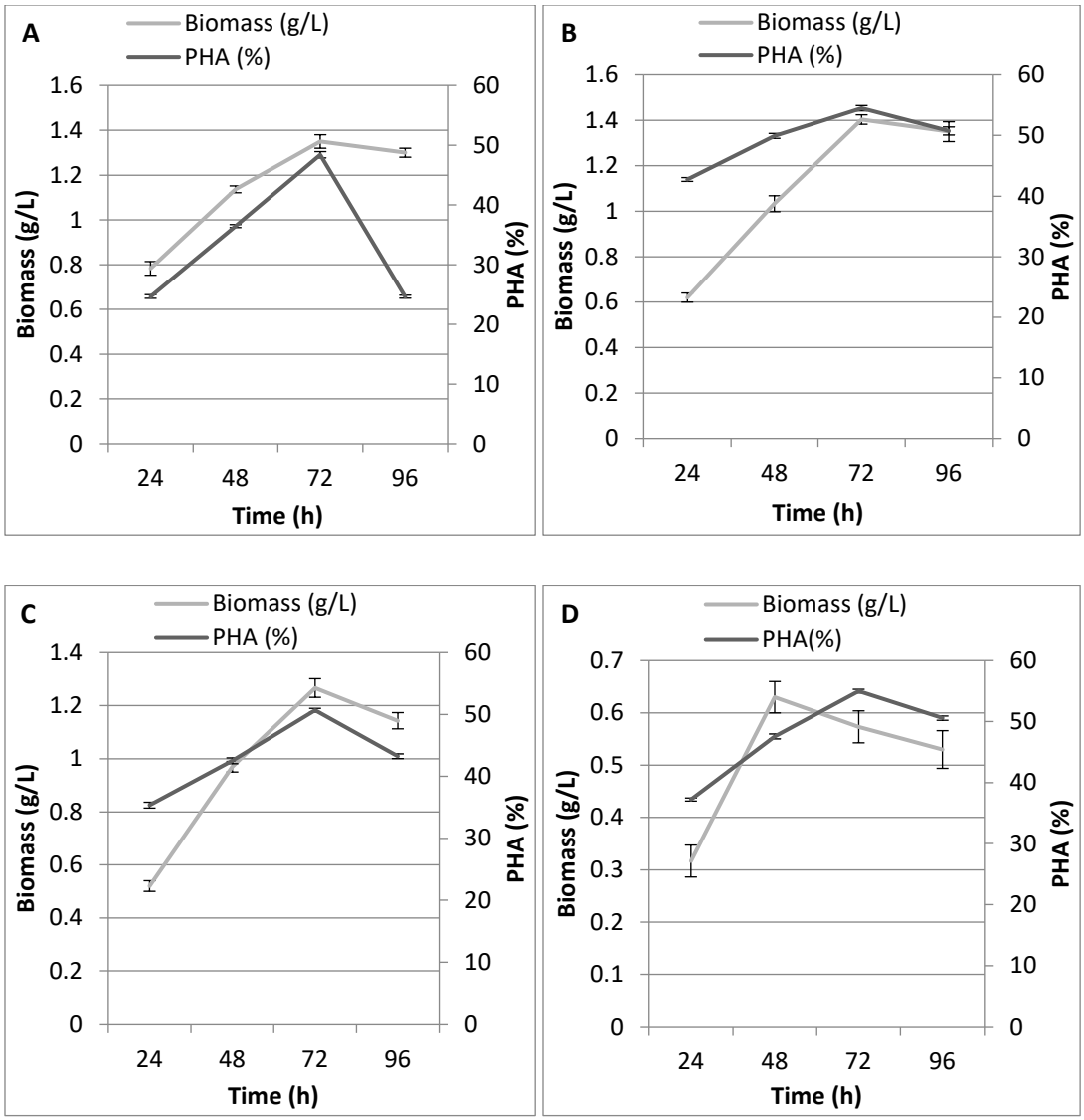

Figure 4: PHA time profiling for *Cellulosimicrobium*sp. A8with **(A)** 2% glucose **(B)** 2% canola **(C)** 1% mustard oil and **(D)** 2% mustard oil

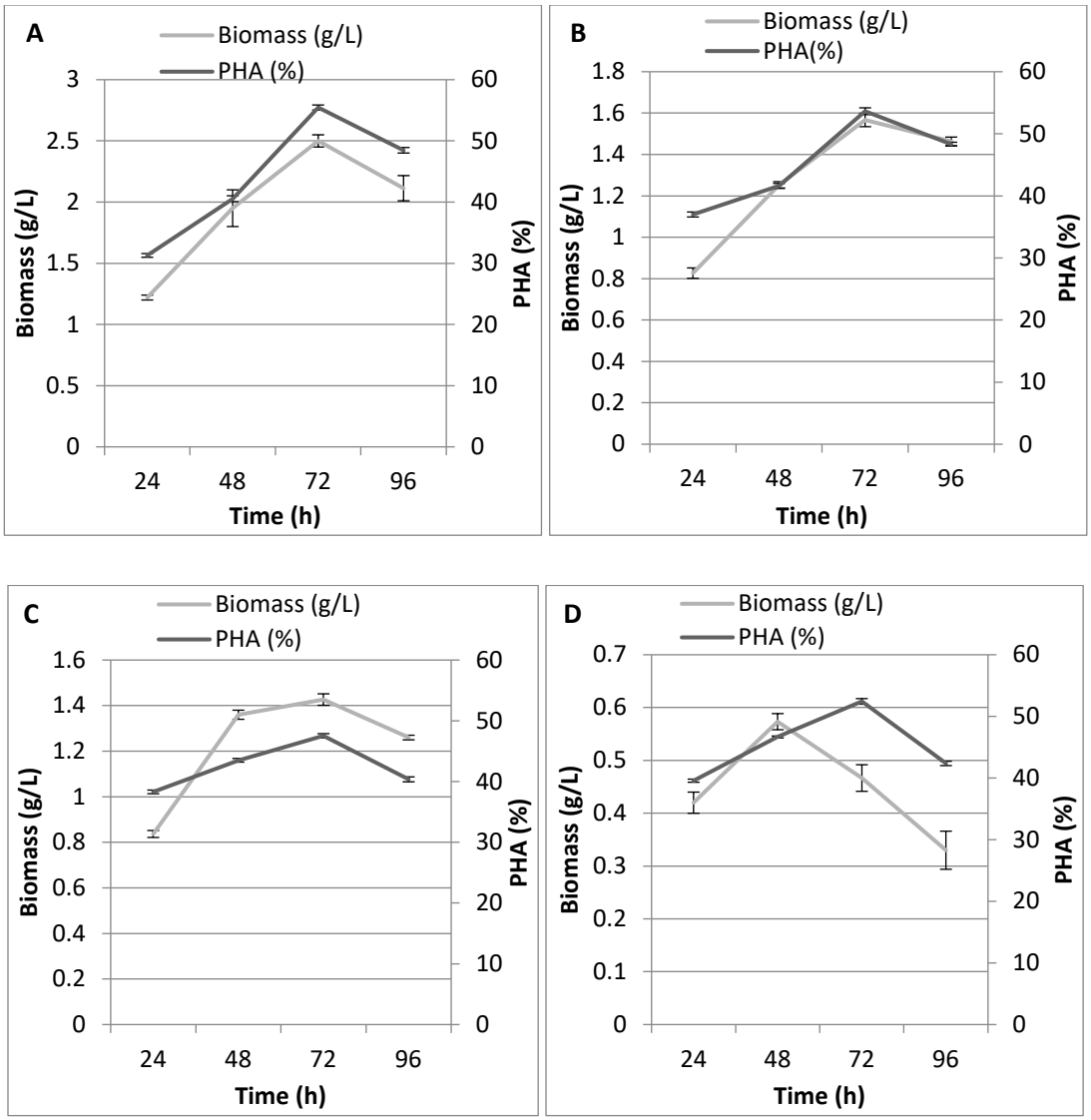

Figure 5: PHA time profiling for *Klebsiella*sp. LFSM2with **(A)** 2% glucose **(B)** 2% canola **(C)** 1% mustard oil and **(D)** 2% mustard oil

Different indices like keto carbonyl and ester carbonyl bonds were also observed in FTIR spectra (Supplementary Fig. 2) to be shifted with the shift of carbon source (Fig. 6, Tab. 1). Shifting was also observed with crystallinity with following trend; Glucose > Canola oil > Mustard oil (Fig. 7).

Table 1: Summary of Infra-Red absorption bands and corresponding possible functional groups observed in biomass of *Exiguobacterium* sp. N4 by utilizing different carbon sources

Exiguobacteriumsp. N4 Wavenumber (cm^{-1})	**Assignment and remarks,**		**Stretching**	**Functional groups**	**Contribution for PHA type**
Glucose (2%)	**Canola oil (2%)**	**Mustard oil (2%)**			
625	624	-	C-CO-C bend 630-535 cm^{-1}	C-CO-C in ketones	-
1110	1172	1180	C=O, C-O stretch 1300-1000 cm^{-1}	Esters	PHB
1411	1465	-	Ring stretch 1500-1400 cm^{-1}	Aromatics	PHB
1650	1643	-	C=O, C-O stretch 1690-1630 cm^{-1}	Esters, primary and secondary amines	PHB
-	1743	1743	C=O stretch 1750-1735 cm^{-1}	Esters	Exact PHB Position
-	2861	2861	CH_2, CH Stretch 2990-2850 cm^{-1} - CH_2 2932.2 cm^{-1}	CH_3 and CH_2 in aliphatic compound	PHB-co-PHV

4 Discussion

Bioplastics, in comparison with synthetic plastics, have renewability, sustainability, biodegradability and eco-friendly behavior, because of which these naturally occurring plastics are becoming popular day by day [25]. Till now many different carbon sources have been utilized to get best yield of PHA. Some carbon sources are supposed to be good for PHA production but are much expensive e.g., ethanol which is expensive due to its volatility [25]. Plant oil for PHA production is said to be an economically viable source due to cheapness of plant fatty acid mixtures rather than oily purified acids.

For the estimation of PHA production, PHA detection agar supplemented with Nile blue A dye was used. Nile blue A is responsible to give fluorescence to PHA producing strains under UV illuminator (302-365 nm). It is difficult to discriminate between PHA negative and PHA positive strains of some Gram positive bacteria such as *Bacillus megaterium*or *Rhodococcusruber* [24] so Sudan Black B staining [22] was also carried out. Moreover, granules were also seen by fluorescent microscopy with acridine orange. All the experimental strains were positive for PHA production due to the presence of PHA granules in them, seen by microscope.

Five genetically identified strains (*Stenotrophomonas*sp.N3*, Exiguobacterium*sp. N4*, Exiguobacterium*sp. Ch3*, Cellulosimicrobium*sp. A8 *and Klebsiella*sp. LFSM2) were used in this research work. Their microscopic analysis revealed that *Stenotrophomonas*sp.N3 and *Klebsiella*sp. LFSM2 are Gram-negative while all others are Gram-positive due to variable composition of cell wall. These bacterial strains were further analyzed for their biochemical characterization. All of the strains were lipase positive i.e., they catalyze the hydrolysis or breakdown of the fats or lipids due to the presence of "lipase" [26].

Different microorganisms showed different growth patterns with different carbon sources depending upon ease of utilization. In this research work, glucose (2%) was used as a standard while canola oil (2%), mustard oil (2%) and mustard oil (1%) were used as experimental carbon sources to evaluate their utilization by bacterial strains. From all five strains, *Stenotrophomonas*sp.N3*, Exiguobacterium* sp. Ch3 and *Klebsiella*sp. LFSM2 showed maximum growth with glucose, 1.91, 2.34 and 2.07 O. D at 600 nm respectively, while *Exiguobacterium*sp. N4 and *Cellulosimicrobium* sp.A8 showed maximum growth with canola oil, 2.02 and 2.01 O. D at 600 nm respectively (data not shown). With mustard oil, all strains showed lesser growth with 2% concentration of it as compared to that of 1% due to more inhibitory effect with more concentration of mustard oil. This attribute of mustard oil can be used to control many pathogenic organisms [5].The purpose of using mustard oil was to check that either mustard oil triggers the bacterial organism to produce PHA or not along with growth inhibition as PHA is produced under harsh environment [15].

Among different carbon sources, it was observed that glucose (2%) produced maximum 70.3% PHA with *Exiguobacterium*sp. N4 and 2.5 g/L biomass with *Klebsiella*sp. LFSM2; canola oil (2%) produced maximum 62.4% PHA with *Exiguobacterium*sp. Ch3 and biomass 1.82 g/L with same strain; mustard oil (1%) produced maximum 50.7% PHA with *Cellulosimicrobium*sp. A8 and biomass 1.43 g/L with *Klebsiella* sp. LFSM2; and finally mustard oil (2%) produced maximum about 55% PHA with strains *Exiguobacterium*sp. N4, *Exiguobacterium*sp. Ch3 and *Cellullosimicrobium*sp. A3 while biomass 0.82 g/L with *Stenotrophomonas*sp.N3. From data, it can be evaluated that relatively better carbon source for obtaining maximum PHA (70.3% with *Exiguobacterium*sp. N4) and biomass (2.5 g/L with *Klebsiella*sp. LFSM2) is 2% glucose. Previous data reveals maximum PHA 76% by *Ralstoniaeutropha*with 2% glucose; 90% by *Wautersiaeutropha*with 2% canola [16]; and 81.3% by *Comamonas testosterone* with 1% mustard oil [27]. Our strains can touch these figures by further optimizing the conditions and putting some genetic modifications in them. Most organisms grow happily in glucose unlike other carbon sources so the maximum growth obtained was that with glucose. When there will be high number of cells then the overall percentage of PHA will also be higher which is obvious from our results.

The PHA of all strains was extracted in little glass vials and macroscopic analysis was done. *Exiguobacterium* sp. N4 produced both main types of PHA i.e., thread-like and granular, with different carbon sources unlike other strains (data not shown). Once high yield and seemingly better quality of PHA was noted with *Exiguobacterium* sp. N4, the next aim was to do detailed chemical analysis of PHA produced by this strain. For this purpose, FT-IR analysis of PHA, produced by using 2% glucose, canola and mustard oil, was performed. FT-IR spectra of biomass (Supplementary Fig. 2) have bands which characterize cellular macromolecules like lipids, proteins, carbohydrates, nucleic acids and PHAs. Spectrum of FTIR in actual, is a chemical profile of a sample. With glucose, FTIR spectra of *Exiguobacterium*sp. N4 revealed sharp absorption bands at 1110 cm^{-1}, 1411 cm^{-1} and 1650 cm^{-1} which correspond to ester carbonyl group (C=O), ester group stretching C-O and amine carbonyl group (C=O) respectively and correspond to the exact peak positions for PHBs. With canola oil, it showed peaks at 1172 cm^{-1} (C=O), 1465 cm^{-1} (aromatic ring stretch), 1643 cm^{-1} (C=O), 1743 cm^{-1}, (C=O stretch) and 2861 cm^{-1} (-CH_2) wavenumber and each peak represents particular PHB functional group (showed within respective brackets). Peaks at 1743 cm^{-1} and 2861 cm^{-1} also showed that *Exiguobacterium*sp. N4 can accumulate exact PHB polymer as well as PHB copolymer (PHB-co-PHV) by using canola oil. Particular peaks at 2931.1 cm^{-1} and 1279.5 cm^{-1} shown by poly(3HB-co-3HV), corresponds to typical poly(3HB-co-3HV) which is very close to the value shown by our strain. With mustard oil (2%), it showed peaks at 1180 cm^{-1}, 1743 cm^{-1} and 2861 cm^{-1} which correspond to ester carbonyl group (C=O), exact PHB ester carbonyl group (C=O) and aliphatic compound group (CH_2) respectively. While shifting of functional group has also been noticed as the carbon source changes from glucose to plant oils. Production of poly (3HB-co-3HV) was evident by the presence of peaks at 2861 cm^{-1} positions in our strains (Tab. 1). These observations due to the complex nature of plant oils which make bacteria to switch another metabolic pathway as PHA synthesis is directly associated with the carbon source [23].

Evaluation of diverse production of PHA can be done by comparing the FT-IR results by *Exiguobacterium*sp. N4. Our results evaluate that canola oil is responsible for producing the PHA with wide variety of functional groups i.e., PHB and PHB-co-PHV. Although, glucose produced maximum amount of PHA but that plastic was containing just homopolymer (PHBs) but not any copolymer (PHB-co-PHV).

It has been reported that a polymeric material which is a blend of PHBs and PHVs is less crystalline, stronger and flexible than that containing just PHBs [19]. Hence, copolymer material (like PHB-co-PHV) is considered to be more suitable for practical applications. In our study, only PHB was produced with glucose while co-polymer was produced with both canola and mustard oil but ratio of PHB to PHB-co-PHV is higher with canola unlike mustard oil so crystallinity of mustard oil is lower as compared to canola. Change in indices and crystallinity of bio-plastic with change of carbon source was also observed (Fig. 6 and Fig. 7).

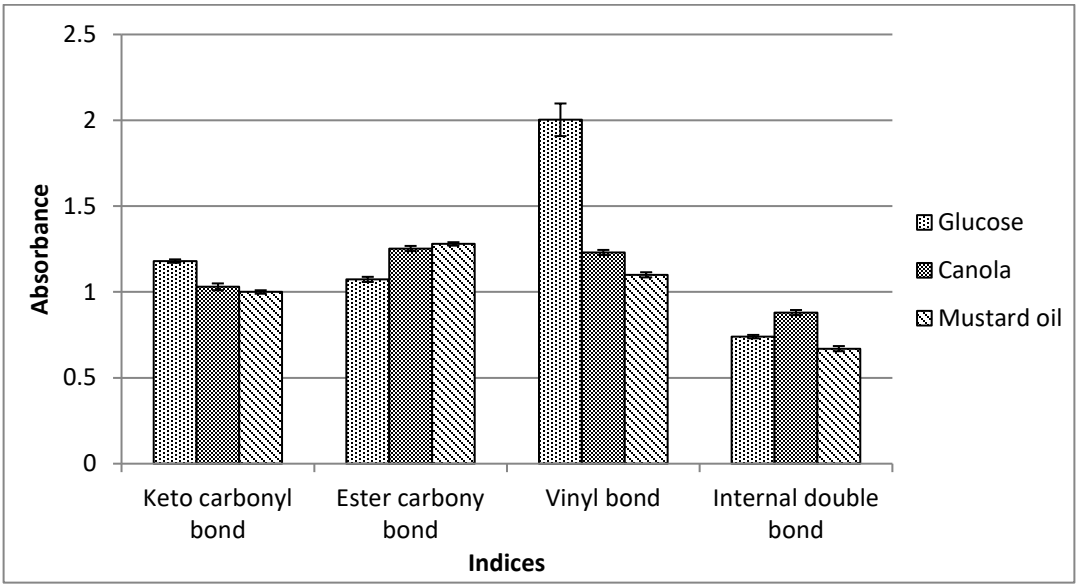

Figure 6: FT-IR spectrum indices of produced PHA by *Exiguobacterium* sp. N4

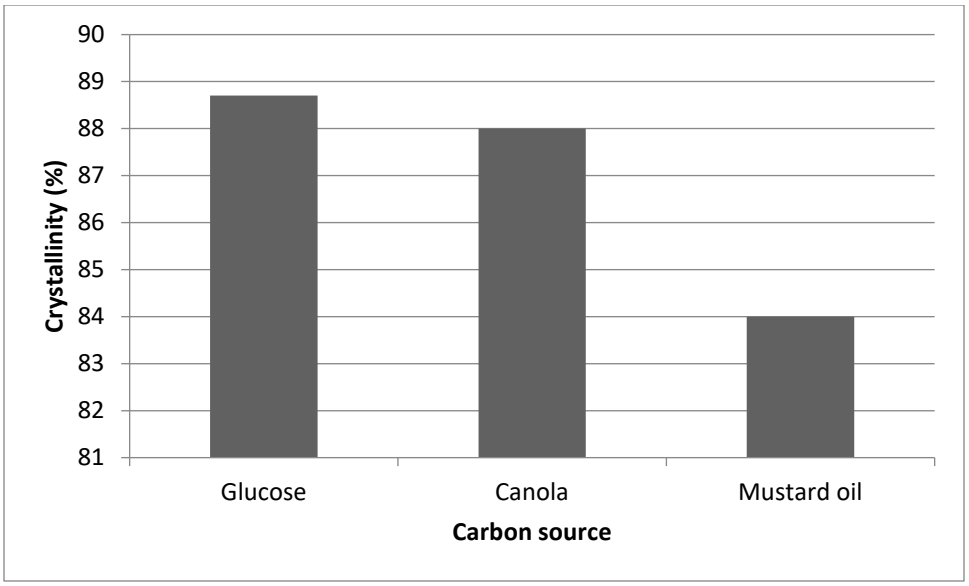

Figure 7: Comparison of change in crystallinity of PHA with change in carbon source

This change is considered to be due to the difference in composition of produced bioplastics. Maximum crystallinity is with glucose (88.7%), then canola (88%) and finally with mustard oil (84%). This change in crystallinity tells about the nature of bioplastic produced by using these carbon sources which can be explained on the basis of FT-IR results. Although, crystallinity difference among carbon sources is not so much high but it could be made considerable via changing the growth conditions like temperature, pH, nutrients, etc.

5 Conclusion

Although maximum amount of PHA was produced by utilizing glucose but the type of PHA was not good. It means that it was highly brittle with maximum crystallinity. Moreover, bacteria under research not only produced PHBs but also produced PHBs and PHVs copolymer by utilizing oils which more strongly states the potential of these bacteria to produce PHA of interested quality. It also showed that utilization of oils not only makes bioplastic material (PHA) economical but also improves its market. The findings of this research work recommend the use of canola and mustard oil as a cheap carbon source for bioplastic production.Future prospects include the optimization of these bacterial strains, either by genetic or growth environment modification, for large scale volumetric production of this Eco-Green PHA with less crystallinity using canola and mustard oil as cheap carbon sources.

Acknowledgement: Authors are thankful to Higher education commission of Pakistan funded project NRPU-3443, to support this research.

References

1. Albertsson, A. C., Andersson, S. O., Karlsson, S. (1987). The mechanism of biodegradation of polyethylene. *Polymer Degradation and Stability, 18,* 73-87.
2. Anderson, A. J., Dawes E. A (1990). Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiology Reviews, 54,* 450-472.
3. Arkatkar, A., Arutchelvi, J., Bhaduri, S., Uppara, P. V., Doble, M. (2009). Degradation of unpretreated and thermally pretreated polypropylene by soil consortia. *International Biodeterioration & Biodegradation, 63,* 106-111.

4. Budde, C. F., Riedel, S. L., Hubner, F., Risch, S., Popovic, M. K. et al. (2011). Growth and polyhydroxybutyrate production by Ralstoniaeutropha in emulsified plant oil medium. *Applmicrobiol Biotech, 89*, 1611-1619.
5. Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods-a review.*International Journal of Food Microbiology, 94,* 223-253.
6. Cappuccino, J. N., Sherman, S. (2002). *Microbiol a laboratory manual*. Pearson Education, Inc. San Francisco, CA.
7. Dalton, D. A., Murthy, G., Strauss, S. H. (2013). Production of traditional and novel biopolymers in transgenic woody plants.*Phytochemicals, Plant Growth, and the Environment*, 59-78.
8. Hahn, S. K., Chang, Y. K., Kim, B. S.,Chang, H. N. (1994). Optimization of microbial poly (3-hydroxybutyrate) recover using dispersions of sodium hypochlorite solution and chloroform. *Biotechnology and Bioengineering, 44,* 256-261.
9. Hazer, B. A., Steinbüchel, A. (2007). Increased diversification of polyhydroxyalkanoates by modification reactions for industrial and medical applications. *Applied Microbiology and Biotechnology, 74,* 1-12.
10. Ince, O., Basak B., Ince, B. K., Cetecioglu, Z., Celikkol, S. et al. (2012). Effect of nitrogen deficiency during SBR operation on PHA storage and microbial diversity. *Environmental Technology, 33,* 1827-1837.
11. Jendrossek, D. (2009). Polyhydroxyalkanoate granules are complex subcellular organelles (carbonosomes). *Journal of Bacteriology, 191,* 3195-3202.
12. Khattab, R. Y., Arntfield, S. D. (2009). Functional properties of raw and processed canola meal. *LWT-Food Science and Technology, 42,* 1119-1124.
13. Kim, J. R., Sharma, S. (2012). The development and comparison of bio-thermoset plastics from epoxidized plant oils. *Industrial Crops and Products, 36,* 485-499.
14. Kumar, B. S., Prabakaran, G. (2006). Production of PHB (bioplastics) using bio-effluent as substrate by *Alcaligens eutrophus.Indian Journal of Biotechnology, 5,* 76-79.
15. Lee, S. Y. (1996). Bacterial polyhydroxyalkanoates. *Biotechnology and Bioengineering, 49,* 1-14.
16. López-Cuellar, M., Alba-Flores, J., Rodríguez, J. Pérez-Guevara, F. (2011). Production of polyhydroxyalkanoates (PHAs) with canola oil as carbon source. *International journal of biological macromolecules, 48,* 74-80.
17. Luckachan, G. E., Pillai, C. K. S. (2011). Biodegradable polymers-a review on recent trends and emerging perspectives. *Journal of Polymers and the Environment*, *19,* 637-676.
18. Osanai, T. A., Numata, K. E., Oikawa, A. K., Kuwahara, A. Y., Iijima, H. I. et al. (2013)Increased bioplastic production with an RNA polymerase sigma factor SigE during nitrogen starvation in *Synechocystis* sp. PCC 6803. *DNA Research, 20,* 525-535.
19. Otari, S. V., Ghosh, J. S. (2009). Production and characterization of the polymer polyhydroxy butyrate-co-polyhydroxy valerate by *Bacillus megaterium* NCIM 2475. *Current Research Journal of Biological Sciences, 1*, 23-26.
20. Poblete-Castro, I., Escapa, I. F., Jäger, C., Puchalka, J., Lam, C. M. C. et al. (2012). The metabolic response of *P. putida* KT2442 producing high levels of polyhydroxyalkanoate under single-and multiple-nutrient-limited growth: Highlights from a multi-level omics approach. *Microbial Cell Factories*, *11(1),* 34.
21. Prabhu, N. N., Santimano, M. C., Mavinkurve, S., Bhosle, S. N., Garg, S. (2010). Native granule associated short chain length polyhydroxyalkanoate synthase from a marine derived *Bacillus* sp. NQ-11/A2. *Antonie van Leeuwenhoek, 97*, 41-50.
22. Schlegel, H. G., Lafferty, R., Krauss, I. (1970). The isolation of mutants not accumulating poly-β-hydroxybutyric acid. *Archives for Mikrobiologie, 71,* 283-294.
23. Shahid, S., Mosrati, R., Ledauphin, J., Amiel J., Fontaine, P. et al. (2013). Impact of carbon source and variable nitrogen conditions on bacterial biosynthesis of polyhydroxyalkanoates: evidence of an atypical metabolism in *Bacillus megaterium* DSM 509. *Journal of Bioscience and Bioengineering, 116,* 302-308.
24. 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. *Archives of Microbiology, 171,* 73-80.

25. Sudesh, K., Iwata, T. (2008). Sustainability of biobased and biodegradable plastics. *CLEAN-Soil, Air, Water, 36,* 433-442.

26. Svendsen, A. (2000). A Lipase protein engineering. *Biochimica et Biophysica Acta (BBA)/Protein Structure and Molecular Enzymology, 1543,* 223-238.

27. Thakor, N., Trivedi, U., Patel, K. (2005). *Bio*synthesis of medium chain length poly (3-hydroxyalkanoates)(mcl-PHAs) by *Comamonas testosteroni* during cultivation on vegetable oils. *Bioresource Technology, 96,* 1843-1850.

28. Van-Thuoc, D., Huu-Phong, T., Thi-Binh, N., Thi-ThoN., Minh-Lam, D. et al. (2012). Polyester production by halophilic and halotolerant bacterial strains obtained from mangrove soil samples located in Northern Vietnam. *Microbiology Open, 1,* 395-406.

29. Verlinden, R. A., Hill, D. J., Kenward, M. A., Williams, C. D., Radecka, I. (2007). Bacterial synthesis of biodegradable polyhydroxyalkanoates. *Journal of Applied Microbiology, 102,* 1437-1449

30. Wong, Y. M., Brigham, C. J., Rha, C., Sinskey, A. J., Sudesh, K. (2012). Biosynthesis and characterization of polyhydroxyalkanoate containing high 3-Hydroxyhexanoate monomer fraction from crude palm kernel oil by recombinant *cupriavidusnecator*. *Bioresource Technology, 121,* 320-327.

31. Zerbi, G., Gallino, G., Del-Fanti, N., Baini, L. (1989). Structural depth profiling in polyethylene films by multiple internal reflection infra-red spectroscopy. *Polymer,* 30, 2324-2327.