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RESEARCH ARTICLE

Synthesis of *scl*-poly (3-hydroxyalkanoates) by *Bacillus cereus* found in freshwater, from monosaccharides and disaccharides

Tayyaba Naeem, Naima Khan, Nazia Jamil (✉)

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

BACKGROUND: Polyhydroxyalkanoates are a good substitute for synthetic plastic because they are highly biocompatible, ecofriendly, and biodegradable. Bacteria in freshwater bodies such as rivers, tube wells, and canals are exposed to alternating high and low concentrations of substrates that induce PHA production.
METHODS: Fresh water samples were collected for isolation of bacterial strains. Screening of PHA in bacterial cells was performed with Sudan and Nile Red staining. Extracted PHA was characterized by FTIR.
RESULTS: In this study, nine bacterial isolates were selected for PHA production on the basis of phenotypic screening. Their ability to accumulate PHAs was determined using different monosaccharides and disaccharides. Two bacterial isolates Bacillus cereus T1 (KY746353) and *Bacillus cereus* R3 (KY746354) produced PHAs. Optimal growth of the bacterial strain (T1) was observed in the presence of glucose, followed by maximum production of PHAs (63% PHAs) during the logarithmic phase of growth. *B. cereus* R3 (KY746354) accumulated 60% PHAs by dry cell weight.
CONCLUSION: PHA accumulation was relatively less with fructose, but both strains showed increased production (up to 50%) with sucrose. The polymer produced was characterized by Fourier-transform infrared spectroscopy (FTIR), which showed that the compound contains short-chain PHAs.

Keywords ecofriendly, biocompatible, *scl*-poly (3-hydroxyalkanoates), *Bacillus cereus*

Introduction

Polyhydroxyalkanoates (PHAs) are polymers of 3-hydroxyalkanoic acid, which are produced as black granules in the intracellular environment of different bacteria (Rehman et al., 2007). Environmental conditions deficient in phosphorus, sulfur, oxygen, magnesium, and nitrogen lead to the production of polyesters as energy reservoirs (Teeka et al., 2010). PHA-producing bacteria are present in both aquatic and terrestrial environments. The average molecular weight of PHAs ranges from 50 to 1 000 kDa (Agus et al., 2006); PHAs exhibit high polydiversity. Approximately 250 bacterial species, including both gram-positive and gram-negative bacteria, are able to produce PHAs. PHAs are categorized into three major groups based on the number of carbon atoms in each monomeric unit. These groups include short-, medium-, and long-chain PHAs containing 3–5, 6–14, and 15 carbon atoms, respectively. Aquatic bacteria are exposed to alternating high and low concentrations of the substrate. Under these transient conditions, bacterial cells produce different types of storage polymers, the most commonly known biopolymer being PHA. Freshwater bodies such as rivers are nutritionally rich because of industrial effluents, hydrocarbons, and pesticides. As described by Sial et al. (2006), industries in Pakistan discharge their waste into freshwater bodies without considering the long-term effect. Industrial units, including textile, chemical, food processing, pulp and paper, poultry, dairy, plastic, paint, pesticides, and leather industries, as well as tanneries and pharmaceuticals directly discharge their waste into the canal system, thus contaminating the groundwater as well. Hence, the water bodies included in this study were rich in organic manure. Bacterial species present in the rivers were screened for PHA accumulation by staining with Nile Blue A dye and observing under UV light (Nagamani et al., 2015). Bacterial species such as *Alcaligenes, Flavobacterium, Pseudomonas, Micrococci, Achromobacter,* and some types of sulfur bacteria are present in freshwater. As

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Correspondence: Nazia Jamil
E-mail: nazia.mmg@pu.edu.pk

freshwater bacteria lack biochemical activities, spore-forming bacteria consist of 10% total bacteria, 95% rod-shaped bacteria, and < 1% cocci (Collins, 1963).

The four biosynthetic pathways responsible for the production of biopolymers are well studied in *Ralstonia eutropha* because it, in particular, possesses the ability to synthesize PHBs using simple carbon sources (Khanna and Srivastava, 2005). β-Thiolase (PhaA) condenses two acetyl-CoA molecules to form acetoacetyl-CoA, which is later reduced by acetoacetyl-CoA reductase (PhaB). Finally, polymerase (PhaC) polymerizes the monomers to form PHB by esterification. This three-step process occurs on the granule surface. Moreover, biosynthesis of biopolymer 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) as a result of enrichment by propionic acid and valeric acid in the culture medium (Zinn et al., 2001).

Bacterial cells use sugars such as glucose, sucrose, and fructose for synthesizing PHA. Sugars are converted into two molecules of acetyl-CoA, which is a major substrate of Krebs cycle. Subsequently, the enzymatic activity of 3-ketothiolase, also known as PhaA, converts acetyl-CoA to acetoacetyl-CoA, which, in turn, is converted into 3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase (PhaB). Consequently, PHAs are formed with the help of PHB synthase (PhaC), and Coenzyme-A is liberated (Verlinden et al., 2007). Various gram-positive bacteria are known to produce PHAs, such as *Micrococcus, Microcystis, Corynebacterium, Bacillus, Rhodococcus, Clostridium, Staphylococcus, Caryophanon*, and *Streptomyces* (Lu et al., 2009*)*. Gram-positive bacteria produce less PHAs compared to gram-negative bacteria. However, the PHAs produced by gram-negative bacteria are known to be strongly immunogenic in humans, and therefore, they cannot be used for medical causes. However, macro-amphiphiles synthesized by gram-positive bacteria are complementary to the lipopolysaccharides present in gram-negative bacteria in terms of immunogenicity. These macro-amphiphiles are lipid-based in nature, and they include lipoteichoic acids produced by *Bacillus*, *Staphylococcus*, and *Clostridium* and lipoglycans produced by *Nocardia, Corynebacterium,* and *Rhodococcus* (Sutcliffe et al., 2010; Ray et al., 2013). According to the published studies, *Bacillus cereus* is reported to produce large amounts of PHAs by using various unrelated carbon sources such as gluconate, fatty acids, sucrose, fructose, and glucose. Various kinds of PHAs such as 3-hydroxyvalerate (3HV), 3-hydroxybutyrate (3HB), and 4-hydroxybutyrate (4HB) are produced by *Bacillus* species (Valappil et al., 2007). PHAs have wide range of applications in various fields such as packaging material, disposable products for personal hygiene, and medical products (Smith, 2005). PHAs can be used in numerous applications such as molded goods, performance additives, films, foils, paper coatings, and diaphragms. Poly 3-hydroxybutyrate-co-3-hydroxyvalerate, a copolymer of PHAs, is less stiff, and is, therefore, suitable for packaging applications.

Materials and methods

Isolation and characterization of bacteria

Bacterial strains were isolated from the River Ravi (30° 34′ 59.99 N, 71° 48′ 59.99 E), a tube well, and a canal, and their physical characteristics were observed. Nutrient medium (Cappuccino et al., 1996) was used to isolate the bacterial strains. The isolates were further characterized on the basis of Gram staining, spore staining, and biochemical tests, including catalase and oxidase tests.

Phenotypic screening of isolates for PHA production

Bacterial strains capable of producing PHA under specific conditions were identified. Nile red was added during the preparation of the medium. All strains were inoculated on nitrogen-limited minimal salt agar containing nitrogen-limited mineral medium [$(NH_4)_2SO_4$, 2 g/L; KH_2PO_4, 13.3 g/L; $MgSO_4.7H_2O$, 1.2 g/L; citric acid, 1.7 g/L; and trace elements solution, 10 mL] with different carbon sources (2% glucose, sucrose, and fructose) (Ali and Jamil, 2014). The trace element solution contained (g/L): $ZnSO_4.7H_2O$, 0.2; H_3BO_3, 0.6; $MnCl_2.4H_2O$, 0.06; $CoCl_2.6H_2O$, 0.4; $CuSO_4.4H_2O$, 0.02; and $NaMoO_4.2H_2O$, 0.06. Two-percent solution of each carbon source was separately autoclaved at 110°C for 10 min. After incubating for 24 h, the plates were observed under a UV-transilluminator. PHA-producing bacterial strains emitted fluorescence because of Nile red. However, Nile red screening helped in differentiating PHA producers from the non-producers (López-Cortés et al., 2008). Subsequently, intracellular PHA granules were observed under 100× by Sudan Black A staining (Venkateswar Reddy and Venkata Mohan, 2012).

Growth kinetics of PHA-producing strains by using different carbon sources

The rate of PHA production was determined based on the method described in previous studies, with some modifications (Borah et al., 2002). The biomass of isolates was collected after equal intervals of 24 h, and PHA was extracted. The growth on different carbon sources was monitored based on the increase in optical density. Finally, the results were recorded by drawing a growth curve for each bacterial stain grown on selective nutrient. Thus, comparative analysis of the growth of each strain was performed based on a specific nutrient.

Extraction of the polymer

Solvent extraction method was used for PHA recovery. Sodium hypochlorite (inorganic solvent) and chloroform (organic solvent) was used in equal proportion (Rehman et al., 2007; Jacquel et al., 2008). The upper layer (inorganic)

was separated from the lower chloroform layer containing the polymer. After the chloroform was evaporated, the percentage of PHA was determined using the following formula:

$$\text{Percentage of PHA} = (\text{weight of PHA}/\text{weight of biomass}) \times 100\%$$

Comparative analysis was used to determine the maximum and minimum yields of PHA from the bacterial cells at different time intervals.

Fourier-transform infrared (FTIR) spectroscopy of PHA

FTIR spectroscopy indicates PHA, present in the biomass of microbes and enable a rapid screening of microorganism after lyophilization of cells (Misra et al., 2000). Misra et al. (2000) showed that FTIR spectroscopy is an alternative method for the analysis of PHA/PHB in microbial cells. For qualitative analysis, a semi-transparent, thin pellet of biomass was prepared using potassium bromide, and then scanned by FTIR spectroscopy, at wavelengths ranging from 400/cm to 2000/cm.

Results

Purification and characterization of bacterial strains

Nine bacterial strains were isolated from the River Ravi, a tube well, and a canal located in Lahore, Pakistan. The temperature of the river water (35°C) was higher, compared to that of the canal (30°C) and tube well (28°C) water. The tube well water had the minimum temperature. However, the pH of the water from the canal (7) and tube well (7) was the same, whereas that of the river water was slightly different. The number of bacterial colonies obtained on plating the river water sample (dilutions used, 10^{-1} and 10^{-2}) on nutrient agar was too high to count. The tube well water showed the minimum number of bacterial colonies, compared to other freshwater samples.

Phenotypic screening for PHA production and identification of isolates

Two strains showed fluorescence under the UV-transilluminator, indicating PHA production (Fig. 1). These bacterial strains, namely, R3 and T1, were further confirmed to accumulate dark purple intracellular granules, as shown in Fig. 2. The selected strains (TI and R3) were identified using 16S rRNA gene sequencing; the findings showed that they belong to the genus *B. cereus*, with the accession numbers KY746353 and KY746354, respectively. Figure 3 shows the evolutionary relationships of the taxa.

Substrate utilization and growth kinetics

We observed the growth pattern of PHA-producing strains up to 72 h of incubation. T1 strain grown in the presence of glucose showed the maximum absorbance of 1.325 at a wavelength of 600 nm, while the same strain grown in the presence of fructose showed the minimum absorbance of 1.129. Similarly, the maximum optical densities of 1.3 and 1.08 were observed for strain R3 grown in the medium supplemented with glucose and fructose, respectively (Fig. 5).

Time profiling of PHA accumulation

Both strains produced maximum biomass when supplied with glucose. The *B. cereus* strains T1 and R3 produced 9 and 7 g/L of biomass, respectively, after a 24 h incubation. When sucrose was used, the strains T1 and R3 produced 7 and 9 g/L of biomass after an incubation of 24 h, but relatively less biomass was obtained when fructose was used as the carbon source. After 72 h, the strains T1 and R3 produced 37 and 33 g/L of biomass, respectively, when glucose was used as the carbon source. However, the use of sucrose had a different effect: T1 produced 35 g/L and R3 produced 29 g/L of biomass after 72 h. The strain T1 produced 63% PHA after 24 h, but PHA production gradually reduced to 19% after 72 h of incubation in the presence of glucose. Similarly, the strain R3

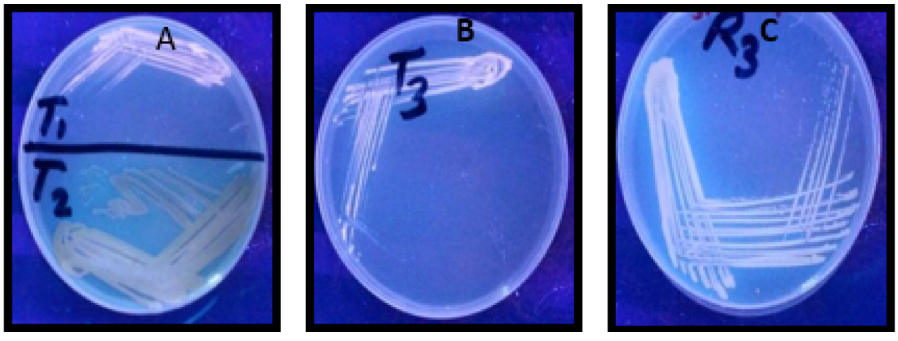

Figure 1 T1, R3, and T3 show fluorescence in plates containing PDA with glucose source during Nile Red screening

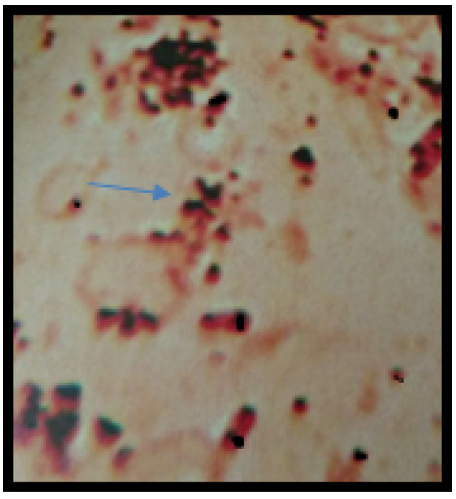

Figure 2 Light microscopy analysis of the black granules produced by PHA-producing bacteria in the presence of glucose, stained using Sudan staining

produced 60% PHA after 24 h, which decreased to 18% after 72 h. The same trend, but relatively lower production of PHA, was shown by both strains in the presence of sucrose and fructose.

FTIR spectroscopy of PHA

FTIR spectroscopy showed that *B. cereus* produced PHA in the medium supplemented with glucose. Figure 4 illustrates the extent of intense absorption at 1700 cm^{-1} corresponding to the C = O stretching group. Other weaker bands and characteristic bands were also observed at different positions in the spectrum produced by the intact cells. The bands produced in the regions of 2300–2400 and 1000–1100 cm^{-1} can be attributed to the stretching vibrations of the –OH- and –COC- groups, respectively.

Discussion

Various bacteria are capable of producing different biological types of polyester, which are collectively known as PHAs (Bugnicourt et al., 2014). These polymers are synthesized intracellularly and accumulate large amount of energy, in addition to carbon. PHAs possess two major characteristics, including biodegradability and biocompatibility, which has drawn the attention of various industries (Verlinden et al., 2007). These thermoplastic granules are usually produced when growth medium is deficient in some of the major nutrients, including nitrogen, magnesium, and phosphorus.

Phenotypic screening revealed two best producers of P HA, namely, *B. cereus* R3 (KY746354) and *B. cereus* T1 (KY746353) (Kumar et al., 2013). These strains were grown in the PHA detection medium containing 2% glucose or sucrose or fructose, for observing the growth kinetics in the presence of different nutrients (Kumar et al., 2014). Our results indicated that glucose is the best carbon source for allowing maximum growth of bacterial strains, specifically *Bacillus* species. However, the strains showed minimum growth in the presence of fructose as the carbon source (Kumar et al., 2009). *B. cereus* T1 strain showed more growth on PDA medium, compared to the R3 strain. The results proved that glucose is the more suitable carbon source that allows maximum growth of the strains within 72 h of incubation. The line graph indicates that minimum growth was observed in the presence of fructose. Subsequently, these strains were grown on a PHA detection medium containing different nutrients, including glucose or sucrose or fructose.

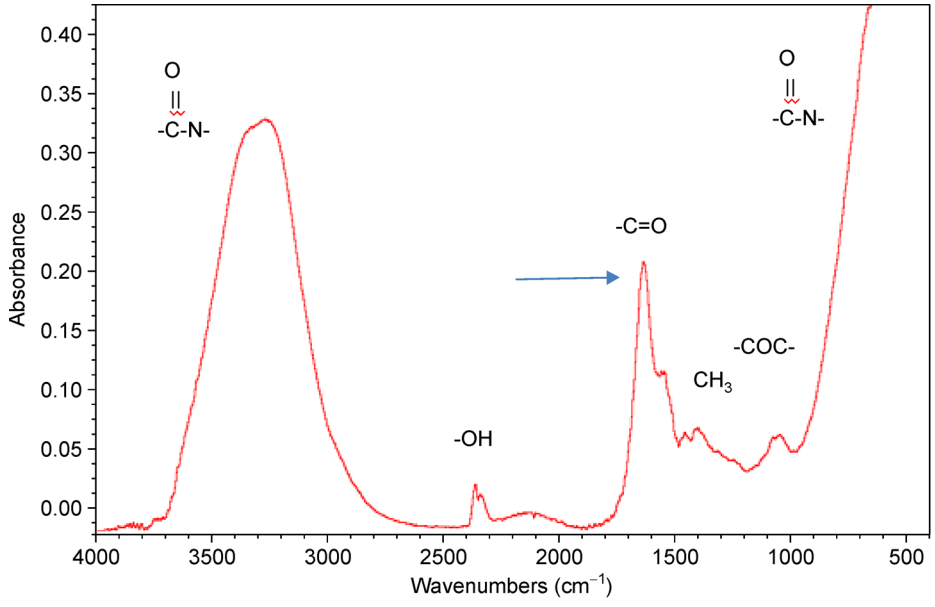

Figure 3 Phylogenetic relationship between *Bacillus* strains and the organism used in this study, namely, T1 (KY746353)

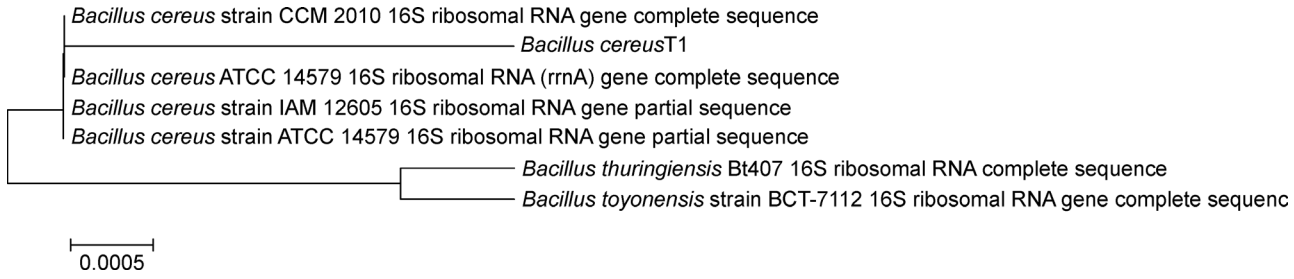

Figure 4 FTIR spectrum of PHA produced by Bacillus cereus T1 (KY746353) in the control medium supplemented with glucose

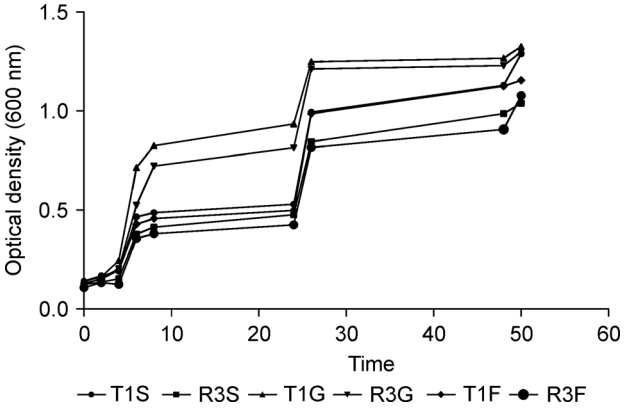

Figure 5 Optical density of T1 and R3 strains at 600nm using different carbon sources. S, G and F are the initials of sucrose, glucose and fructose.

When glucose or sucrose was used as the carbon source, large amount of biomass was produced after incubation for 72 h, as illustrated in Fig. 6. Decrease in biomass production was observed when fructose was used (Chaudhry et al., 2011). The percentage of consumed substrate varied from glucose to fructose, indicating that *B. cereus* preferentially used glucose compared to fructose. According to Kumar *et al.*, analysis of *B. cereus* genome showed the presence of multiple genes encoding glucose membrane transporters, compared to one gene encoding a fructose membrane transporter (Kumar et al., 2009). Both cell growth, determined as the dry weight biomass (DCW), and total PHB content, determined as the proportion of bacterial DCW, were significantly improved when glucose, rather than fructose, was used as the carbon source, irrespective of the nitrogen source. The strain T1 was capable of producing more PHA after 24 h. However, the percentage of PHA production reduced after 24 h and only 20% of PHA constituting higher biomass was synthesized after 72 h on PDA medium containing glucose. Moderate production occurred with sucrose, and minimum production occurred in the presence of fructose (Brandl et al., 1990).

Identical rates of PHA production were shown by the strain R3 when grown in the presence of different nutrients for

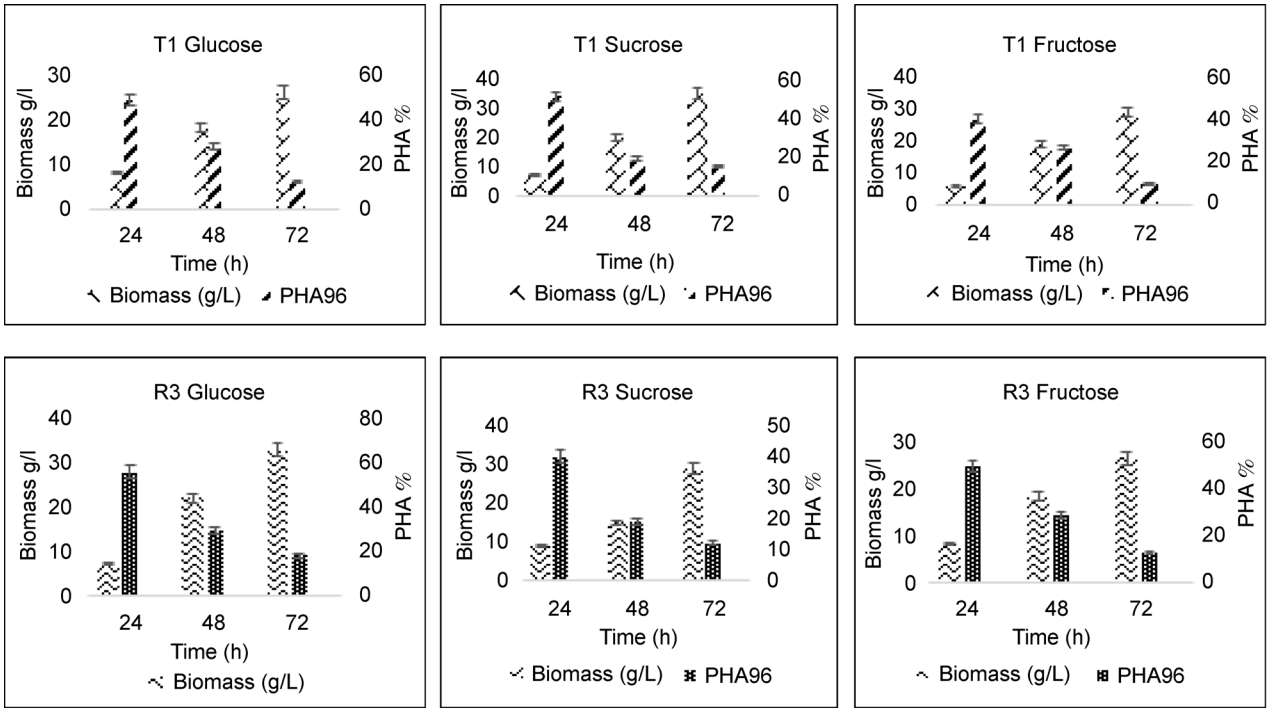

Figure 6 Comparative analysis of the PHA (%) and biomass (g/L) produced by T1 and R3 strains, using different carbon sources

different incubation periods. PHA production decreased after 24 h because sugar sources such as sucrose, fructose, and glucose gradually get exhausted (Fig. 6). High biomass production in the broth led to rapid decrease in PHA accumulation and excess foaming (Kulpreecha et al., 2009). FTIR spectroscopy showed intense absorption at 1400 cm^{-1} that corresponds to the C = O stretching group. The stretching vibrations of the –OH- and –COC- groups were observed at 2300–2400 cm^{-1} and 1000–1100 cm^{-1} (Wu et al., 2001).

Conclusion

B. cereus T1 (KY746353) and R3 (KY746354) produce considerable quantities of PHA when glucose, fructose, and sucrose were used. Comparative analysis of these carbon sources showed different growth rates of bacterial strains. Glucose was found to be the best carbon source for the maximal growth of bacteria and production of *scl*-PHA.

Compliance with ethics guidelines

Tayyaba Naeem, Naima Khan and Nazia Jamil declare that they have no conflict of interest. This article does not contain any studies with human and animals subject performed by any of the authors.

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