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Micrococcus yunnanensis and *Psychrobacter* sp. as Potential Producers of Polymers from Hot Spring

Micrococcus yunnanensis dan Psychrobacter sp. *sebagai Penghasil Potensial Polimer dari Sumber Air Panas*

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Abstract

Polyhydroxyalkanoates (PHAs) and exopolysaccharides (EPSs) are biopolymers bacteria under nutrient-limiting conditions. In this study, bacterial strains were isolated from hot springs. Soil samples were collected from Tatta Pani, Azad Kashmir, Pakistan. Bacterial strains AJ2 and AJ3 were selected due to their ability to produce PHAs and EPSs. Phylogenetic analysis showed that strain AJ2 was *Micrococcus yunnanensis* and AJ3 was *Psychrobacter* sp. Three carbon sources (glucose, glycerol, and molasses) were used for polymer production. The effect of high pH (8) and high temperature (55 °C) was checked on PHAs and EPSs production. The highest yield of PHAs was given by strain AJ3 (89.43%) with molasses. When grown at 55 °C for 24 hours, strain AJ3 showed the highest PHAs accumulation, 79% with glucose. At alkaline pH 8, strain AJ3 gave 34% PHAs with molasses. The highest EPSs production was observed for strain AJ3. AJ3 gave 70g/L of EPSs with both glucose and glycerol. The amplification of the *phaC* gene was done to confirm the genetic basis of PHAs production. FTIR analysis showed clear bands at 1722 cm^{-1} and 2925 cm^{-1} representing the carbonyl and alkyl groups of PHAs, respectively.
Keywords: exopolysaccharides, Kashmir, *Micrococcus yunnanensis,* polyhydroxyalkanoates, *Psychrobacter* sp.

Abstrak

Polihidroksialkanoat (PHA) dan eksopolisakarida (EPS) adalah biopolimer yang diproduksi oleh bakteri yang hidup pada kondisi nutrisi yang terbatas. Dalam penelitian ini, strain bakteri diisolasi dari sumber air panas. Sampel tanah dikumpulkan dari Tatta Pani, Azad Kashmir, Pakistan. Strain bakteri AJ2 dan AJ3 dipilih karena kemampuannya menghasilkan PHA dan EPS. Analisis filogenetik menunjukkan bahwa strain AJ2 adalah Micrococcus yunnanensis dan AJ3 adalah Psychrobacter sp. Tiga sumber karbon (glukosa, gliserol, dan molase) digunakan untuk produksi polimer. pH tinggi (8) dan suhu tinggi (55 °C) diperiksa pengaruhnya terhadap produksi PHA dan EPS. Hasil tertinggi PHA diberikan oleh strain AJ3 (89,43%) dengan tetes tebu. Saat ditumbuhkan pada suhu 55 °C selama 24 jam, strain AJ3 menunjukkan akumulasi PHA tertinggi, 79% dengan glukosa. Pada pH basa 8, strain AJ3 memberikan 34% PHA dengan molase. Produksi EPS tertinggi diamati untuk strain AJ3. AJ3 menghasilkan 70g/L EPS dengan glukosa dan gliserol. Amplifikasi gen phaC dilakukan untuk mengkonfirmasi dasar genetik produksi PHA. Analisis FTIR menunjukkan pita yang jelas pada 1722 cm^{-1} dan 2925 cm^{-1} masing-masing mewakili gugus karbonil dan alkil PHA.
***Kata kunci**: eksopolisakarida, Kashmir, Micrococcus yunnanensis, polihidroksialkanoat, Psychrobacter sp.*

INTRODUCTION

Hot spring is the natural habitat of thermophilic bacteria. Even though high-temperature environments are generally associated with Archaea, the main constituents of many high-temperature populations comprise hyperthermophile bacteria (Sohail et al., 2020). Thermophilic bacteria are considered essential because they can produce thermostable products of industrial importance. It was assumed that the extreme conditions of the hot spring must lead bacteria to the production of exopolysaccharides (EPSs) and carbon and energy storage in the form of polyhydroxyalkanoates (PHAs) granules.

PHAs are biodegradable polymers and have properties similar to petroleum-based plastics (Morgan-Sagastume et al., 2014). They are

considered green polymers of the future, along with polylactic acid (PLA) and polybutylene succinate (PBS). They are likely to substitute conventional plastics steadily (Kourmentza et al., 2017). PHAs are synthesized by a broad range of microorganisms (Muangwong et al., 2016) under conditions where limited nutrients are available (Kalia & Kumar, 2017). PHAs production is reported among various bacteria, including *Bacillus* sp., which has been extensively studied after the discovery of poly-β-hydroxybutyrate (PHB) in a strain of *Bacillus megaterium* (Poli et al., 2011).

PHAs are generally classified into two main categories based on the number of carbon atoms present in their monomer unit (Sohail & Jamil, 2021), i.e., short-chain-length (SCL-) PHAs having 3-5 carbon atoms per monomer and medium-chain-length (MCL-) PHAs having 6-14 carbon atoms per monomer (Ciesielski et al., 2014). Presently, 59 different PHAs synthase genes are cloned and sequenced from 45 different bacteria. PHAs synthases can be grouped into four classes based on their subunit composition and substrate specificity (Sohail et al., 2020). The *phaC* synthase gene is the key gene required in PHA production. PHAs degrade in almost all natural environments (Lim et al., 2021). PHAs can be wholly degraded in aerobic environments to produce H_2O and CO_2. Under anaerobic conditions, PHAs are mineralized to produce H_2O, CO_2, and CH_4 (Ferreira & Åkesson, 2020).

PHAs have a wide range of applications in different fields. PHAs are used as precursors of biofuels (Gumel et al., 2013), as a solid substrate for the denitrification of wastewater and water, in hospitals as surgical swabs, wound dressing, and as a lubricant for the surgeon's gloves in the powder form as drug release system (Shrivastav et al., 2013) and tissue engineering (Ray & Kalia, 2017).

Many microorganisms produce EPSs present outside their cell wall. EPSs may be in capsules, or they can be secreted into the environment as slime. EPSs protect bacteria from drying and changes in water potential. It is vital for immunity and tolerance against antibacterial agents (Hassan & Ibrahim, 2017).

EPSs are extremely heterogeneous polymers, and they contain a species-specific number of different monosaccharides and non-carbohydrate substituents. An oligosaccharide repeating unit forms the polysaccharide chains. The oligosaccharide repeating units can differ depending on the degree and level of polymerization. EPSs have a wide range of applications in pharmaceutical, food, and many other industries (Poli et al., 2011). EPSs are divided into two groups based on their composition. Homopolysaccharides are composed of only one type of monosaccharides, mainly fructose or glucose (Yoshikawa et al., 2021), while heteropolysaccharides are composed of more than one type of sugar monomers. They are produced by the activity of intracellular glycosyltransferases from sugar nucleotides (Galle et al., 2011).

Many bacterial EPS have been studied extensively in recent years. Still, only a few are industrially developed due to their high production cost, mostly downstream processing and substrate cost (Freitas et al., 2011). EPS acts as a stabilizer and texturizer in the dairy industry. It increases the viscosity of the final product (Charchoghlyan et al., 2017). Xanthan and dextran are polysaccharides in dairy products, confectionery, drinks, and bakery products (Patel & Prajapati, 2013).

Some researchers have reported separate production of PHAs and EPSs by different bacterial strains, but attention paid to the coproduction of these biopolymers by certain bacteria is deficient. Although these polymers are not produced through the same biosynthetic pathway, they are usually coproduced because the conditions required for their synthesis are the same (Soto et al., 2021). Due to the intracellular production of PHAs and extracellular EPSs, studying them simultaneously with the methods used in this research was possible. Carbon sources provide power and bio bricks for microorganisms. Their selection is very critical for the production of important commercial compounds. In recent years, glucose, molasses, and glycerol have become bio-smart substrates for bacterial fermentation. These substrates have cost-effective advantages like comparatively low cost and appropriate to function as a liquid, and hydrolysis processes are not required for the fermentation process. Molasses is produced from sugar mills as a valuable coproduct with high sugar content. Increased sugar level makes it a bio-smart waste feedstock for fermentation. Large quantities of crude glycerol formed as bio-waste for biodiesel generation, making it a cost-effective carbon source. The main objective of this research was to isolate PHAs and EPSs producing bacteria from hot springs that can use cheap carbon sources. This

study exploits some agricultural waste the sugar industry produces, like molasses and glycerol from biodiesel plants. Bacteria use these wastes to produce extracellular polysaccharides and PHA through an integrative approach. This integrative system may become of considerable importance, being a commercially helpful approach for reducing the production costs of bioplastic from stored PHAs granules through the associated production of EPSs in the same batch fermentation process.

METHODS

Isolation, Characterization, and Identification of Bacterial Strains

Soil samples were collected in sterile glass containers from hot springs of Tatta Pani, Kashmir, Pakistan (34° 11' 0" North, 73° 27' 50" East) to isolate secondary metabolites producer bacteria. The sample was processed for physical parameters like temperature and pH and stored at -80 °C for further analyses (Sohail et al., 2020).

Bacterial strains were isolated by serial dilution and purified by repeated streaking on N-agar (Sohail & Jamil, 2021) plates and incubated at 37°C. Colony morphology characteristics of the isolated bacterial strains were observed from 24 hours of culture on N agar. Gram staining was done to study the cell morphology of the isolated bacterial strains.

The DNA sequences were aligned using the BLAST tool of the NBCI database. The evolutionary analysis was done with the help of MEGA6 Software. The sequences were submitted to GenBank for accession numbers.

Culture Conditions and Screening of Biopolymer Production

For the screening of PHAs producing bacterial strains, the isolates were grown on PHAs detection agar medium [$(NH_4)_2SO_4$, 2 g/L, KH_2PO_4 13.3 g/L, $MgSO_4{\cdot}7H_2O$ 1.2 g/L, citric acid 1.7 g/L, agar 15g/L, and trace elements solution {$FeSO_4.7H_2O$ 10.0g, $ZnSO_4.7H_2O$ 2.25g, $CuSO_4.5H_2O$ 1.0g, $MnSO_4.5H_2O$ 0.5g, $CaCl_2.2H_2O$ 2.0g, $Na_2B_4O_7.10H_2O$ 0.23, $(NH_4)_6Mo_7O_{24}$ 0.2g, 10 mL 35%HCl}s 10 mL] (Chaudhry et al., 2011). Nile red stain having a concentration of 0.5ug/mL (Sohail & Jamil, 2021) was added to this medium. PHAs producing strains give fluorescence when placed under UV light. Sudan Black Staining was also done to confirm the presence of PHAs granules.

Exopolysaccharide-producing strains were identified by growing the isolated bacterial strains in the same potato dextrose agar (PDA) broth medium for 24 hours at 37°C. No separate medium was used for EPSs production. Ice chilled ethanol was added in equal volume to the culture broth. White precipitates indicated the presence of EPSs.

Extraction and Estimation of Accumulated Intracellular PHA Content

The PHAs produced by the isolated strains were extracted using the chemical digestion method. Bacterial strains AJ2 and AJ3 were grown in 250 ml flasks containing 100 ml of PDA broth with 2% glucose, glycerol, or molasses as a carbon source. The flasks were incubated on a shaking incubator at 200 rpm. The bacterial cultures were then centrifuged at 6000 g for 10 minutes. The non-PHA cellular mass was digested with sodium hypochlorite, and then chloroform was added to it in an equal ratio. This mixture was kept at room temperature overnight, forming two layers. The organic layer was separated by using a separating funnel. Chloroform was evaporated, and the dried PHAs were weighed. The following formula calculated PHA content (Sohail & Jamil, 2021):

$$\text{PHAs \%} = \text{Dry cell mass (g)} / \text{PHAs (g)} \times 100 \quad (1)$$

Extraction of Extracellular EPS

The isolated bacterial strains were grown in a PDA broth medium supplemented with glucose, glycerol, or molasses for 24 hours at 37°C. The cells were centrifuged, and ice-chilled ethanol was added to the supernatant. EPS was detected in the form of white precipitates. These precipitates were then centrifuged and weighed.

Polymerase Chain Reaction (PCR) Amplification of *phaC* gene

The PHA synthase *phaC* gene was amplified using Thermo scientific master mix with forwarding primer (5'-ATCGCTATACGCCAGTAAAAGA-3') and reverse primer (5'-ACCCACTTTTGCATTAGCTTC-3') for that gene. *phaR* gene primers are PhaR4F5'-AAACCGAATCTTACTGGGTAAAG-3' and PhaR4R5'-CTTCCACATTGATGACTAATGACG-3'.
Initial denaturation was done at 95 °C for 5 minutes. The next step was 35 cycles of denaturation at 95 °C for 45 seconds, followed by

annealing at 57.5 for *phaC* and 62.3 °C for the Class IV *phaR* gene for 45 seconds. The extension temperature was 72 °C for 45 seconds, and the final extension was done at 72 °C for 10 minutes. The amplified products were sent for sequencing to First Base Laboratories, Malaysia.

Fourier Transform Infrared Spectroscopy of PHAs and EPSs

Fourier transform infrared spectroscopy (FTIR) analysis was performed to identify functional groups in the extracted PHAs and EPSs. The samples of PHAs and EPSs were prepared by using KBr and scanned at 400/cm to 4000/cm wavelength.

Statistical Analysis

All experimental works were performed in triplicate. For statistical data analysis, Microsoft Excel calculated the standard error of the mean.

RESULTS AND DISCUSSION

Polyhydroxyalkanoates (PHEs) and exopolysaccharides (EPSs) are secondary metabolites bacteria produce under nutrient-limiting conditions. Bacteria produce secondary metabolites that enable bacteria to cope with nutrient availability fluctuation. The soil samples were collected from a hot spring in Tatta Pani, Kashmir, Pakistan, located at the bank of the Poonch river at an altitude of 2,237 feet (682 m). The minimum temperature of this area varies between 3.2 °C to 15.91 °C in winter, and the maximum temperature is between 22.8 °C to 37.61 °C during summer, with an average annual rainfall of 1540.7 mm (Ahmed & Akhtar, 2016). Heavy snowfall is from December to February (Ahmed et al., 2014). The temperature of Tatta Pani hot springs is 100–120 °C and is slightly alkaline (Ahmed et al., 2014). The hot spring is a relatively new environment, so there were more chances to isolate novel bacterial strains.

Biodegradability and biocompatibility are the two significant properties of PHAs that attract several industries. However, the high cost of carbon sources is the main problem for the growth of PHAs-based industries. The purpose of using glycerol and molasses in this study was to check the PHAs and EPSs' ability to produce bacterial strains to utilize these sources as they are relatively cheap carbon sources. Using these carbon sources can reduce the total cost of PHAs production. Biodiesel production generates about 10% glycerol (w/w) as a major byproduct. So, each gallon of biodiesel production will generate nearly 1.05 pounds of glycerol. *Purified glycerol* is a highly valued and commercial chemical having thousands of uses (Yang et al., 2012). So, glycerol can be helpful as the carbon source for producing biopolymers on a large scale. *Molasses* is the final waste matter that is obtained in the production of sugar cane by continual crystallization. The remaining syrup from which simple methods cannot obtain crystallized sucrose. The molasses yield is about 3.0 per ton of sugarcane. Although this percentage is influenced by several factors and can vary from 2.2 to 3.7 percent (Purama et al., 2018), it can be used as a cheap carbon source for large-scale PHAs production by bacteria.

Isolation, Characterization, and Identification of PHA and EPS Producing Bacteria

Ten bacterial strains were isolated from the soil samples. In the isolated strains, five were cocci, and the other five were rods. Among the isolated strains, three were anaerobic while seven were aerobic. Only one isolated strains was Gram-negative, while the remaining nine were Gram-positive. The two selected strains were positive by Nile red and Sudan black staining. Granules of PHAs were seen inside their cells after Sudan Black staining. The selected strains (AJ2 and AJ3) gave white precipitates with ice-chilled ethanol, which showed that they are also positive for producing EPSs.

The selected strains were identified by 16S rRNA analysis. It was revealed that strain AJ2 has a 100% resemblance with *Micrococcus yunnanensis* (MF496374), recently discovered in China (Yunnan referring to the province of China). While strain AJ3 showed 96% similarity with *Psychrobacter* sp. (MF496375). These bacteria live in cold and warm habitats (Tribelli & López, 2018). They are found in a variety of natural environments. The sampling site (hot spring) is present in cold weather, so the presence of *Psychrobacter* sp. in that soil is not unexpected.

Effect of Glucose, Glycerol, and Molasses on PHAs Synthesis

The highest PHA production of PHAs was observed for strain AJ3 (89.43%) when molasses was used as a carbon source under standard incubation conditions. While strains AJ2 did not show any significant production of PHAs with molasses. Its PHAs percentage was 3.20%.AJ3

gave the highest yield (44.8%) when glucose was used as a carbon source. AJ2 gave a 14% PHAs yield, and strain AJ3 gave the highest yield (23.52%) of PHAs when glycerol was used as the carbon source. AJ2 gave a 19.29 % PHAs yield. It seemed that the strains quickly used molasses to produce high PHA content. Better PHA yield obtained with molasses is due to its high sugar content, making it easy for the bacteria to utilize it (Acosta-Cárdenas et al., 2018) (Figure 1(a)).

Effect of High pH on PHAs Synthesis

When the isolated strains were grown at pH 8, after 48 hours of incubation at 37°C, strain AJ3 gave 22.02% PHAs with glucose, 19.52% with glycerol, and 33.61% with molasses. Strain AJ2 gave 5.09% PHA with glucose, 4.31% with glycerol, and 3.49% with molasses (Figure 1(b)).

Effect of High Temperature on PHAs Synthesis

The selected strains were grown at a high temperature (55°C) for 24 hours, and the PHAs yield was measured. Strain AJ3 gave the highest yield (79.0%), while strain AJ2 gave a 15.56% PHA yield, respectively, using glucose (Figure 1(c)).

EPS Production by the Selected Strains

The production of EPSs by the isolated strains was also estimated in this study. Only 30% of the isolated strains were able to produce EPSs. PDA broth medium was used to check the EPSs production of the selected strains as PDA medium provides nutrient limiting conditions, so it was expected to give a high yield of EPSs. Using the same agar medium, i.e., PDA for PHAs and EPSs production, reduces manifolds' cost. Ice chilled ethanol was used to precipitate EPSs. Using different carbon sources did not significantly affect production of EPSs. EPSs production was almost the same in all the carbon sources. After 24 hours of incubation, the production of EPSs by strain AJ2 was 6.92 g/100ml with glucose, 7.12 g/100ml with glycerol, and 7.02 g/100ml with molasses. Strain AJ3 produced 7.16 g/100ml EPSs with glucose, 7.17 g/100ml with glycerol, and 6.79 g/100ml with molasses.

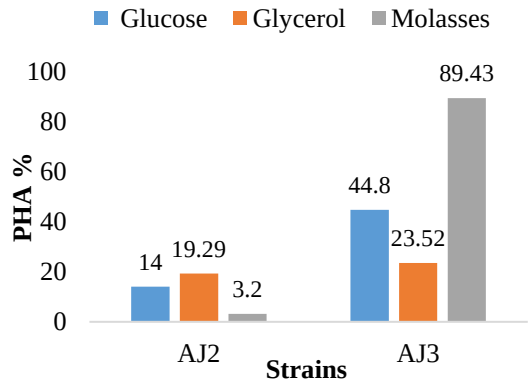

Figure 1(a). PHAs Production by using Different Carbon Sources under Normal Conditions

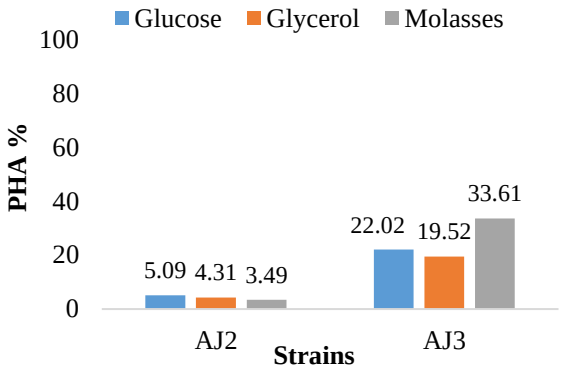

Figure 1(b). PHAs Production by the Isolated Strains at pH 8

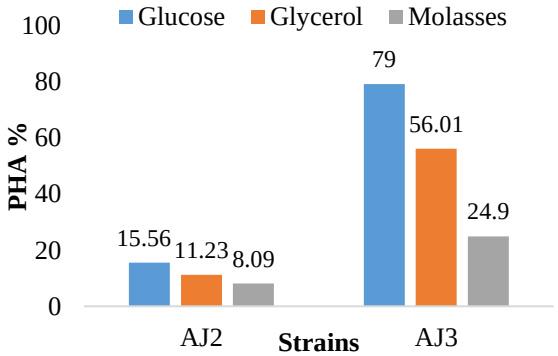

Figure 1(c). PHAs Production at 55 °C using Different Carbon Source

Phylogenetic Analysis of *phaC* and *phaR* Gene

Amplification of partial *phaC* gene from strain AJ3 and AJ2by PCR. Amplification of *phaC* gene products of about 400 kb, 586 kb, 400 kb, and 220 kb, respectively (Figure 2). The DNA sequence of the *phaC* gene was aligned with the NCBI database of the representative bacterial gene, and phylogenetic trees were constructed, which showed that the *phaC* genes of strain AJ3 and AJ2 resemble *Bacillus megaterium* and *Roseobacter* sp., respectively.

Chromosomal genes present on *phaC AB* operon encode the bacterial PHAs. Amplifying the *phaC* gene is crucial to confirm the PHAs producing ability of bacterial strains. Sometimes, the phenotypic approaches, i.e., Sudan Black B staining and Nile blue-screening, may not give accurate results. As Nile blue is lipophilic, it may detect granules of lipids that differ in composition and nature from PHAs(Chaudhry et al., 2011). The *phaC* gene of the two selected strains was amplified using specifically designed primers, and the partial sequences obtained were submitted in GenBank with accession numbers MK138528 and MK000585. The phylogenetic trees of these genes were constructed using MEGA 6 software by a neighbor-joining method that showed that the *phaC* genes of strain AJ3 and AJ2 resemble *Bacillus megaterium* and *Roseobacter* species, respectively.

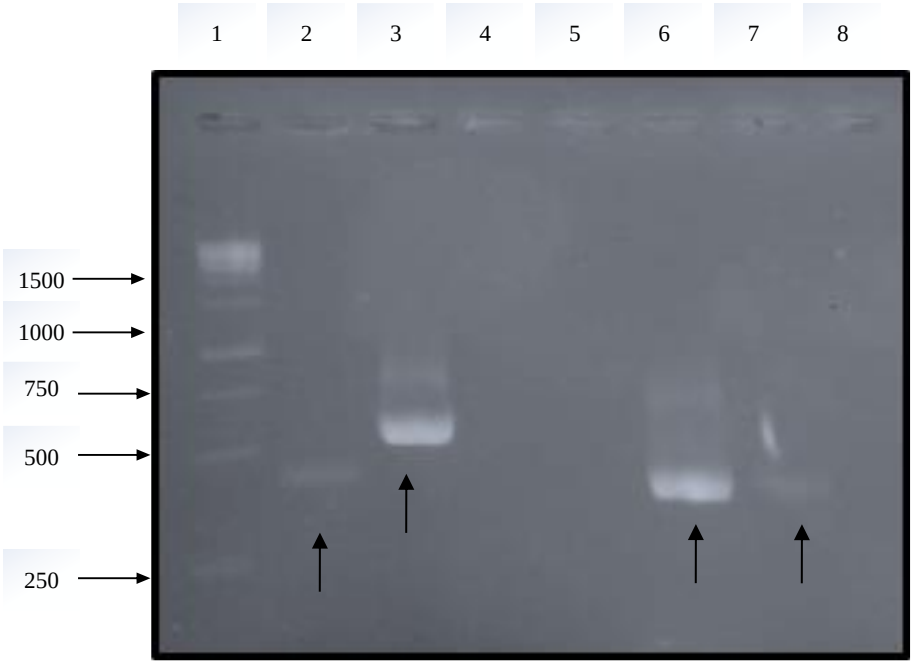

Figure2. Amplification of *phaC* Gene, Lane 1 Contains 1 kb DNA Ladder, Lane Amplification of *phaC* and *phaR* Gene, Lane 1 Contain 1 kb DNA Ladder, Lane 2 (*phaC*), 3 (*phaR*), 6 (*phaC*) and 7 (*phaR*) Products of About 400 kb, 586 kb, 400 kb, and 220 kb respectively

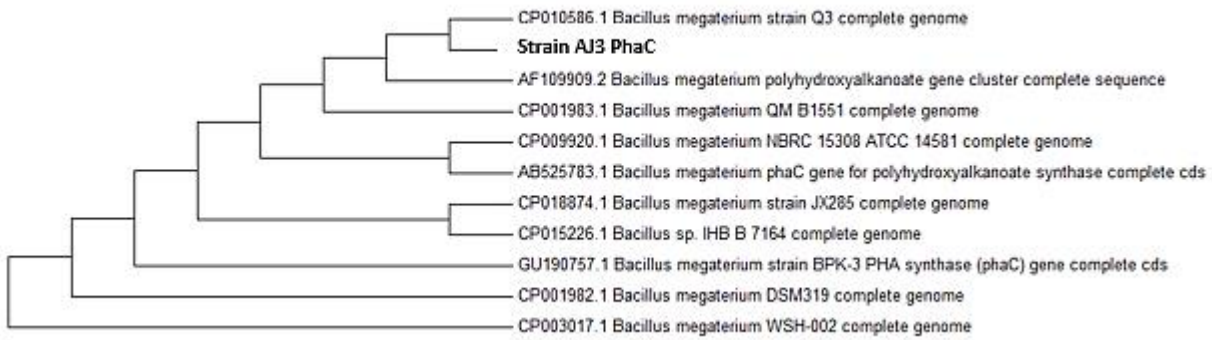

Figure 3. The Phylogenetic Tree of *phaC* gene (MK000585) of Strain AJ3, the Evolutionary Tree, was Constructed using the Neighbor-Joining Tree Method. The Evolutionary Analysis was Done using MEGA6 Software.

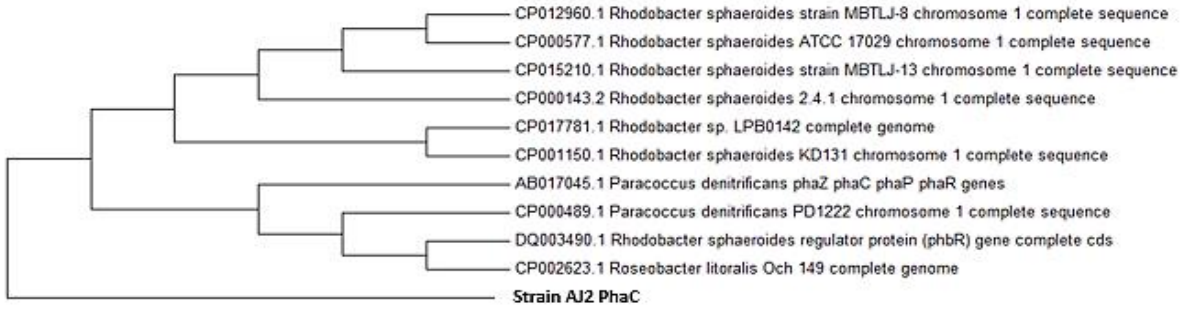

Figure 4. Phylogenetic Tree of *phaC* Gene (MK138528) of Strain AJ2, the Evolutionary Tree was Constructed using the Neighbor-Joining Tree Method, using MEGA6 Software

Table1. FTIR analysis of EPS and PHA of strain AJ3

Sr. No.	Wavenumber (cm^{-1})	Functional Group	Reference
EPS			
1	1541	amide II (C-N-H)	(Forfang et al., 2017)
2	1654	amide I (C = O)	(Forfang et al., 2017)
3	2357 and 2361	C = C	(Mecozzi & Sturchio, 2017)
4	2835- 2915	stretching of CH and CH_2	(Mecozzi & Sturchio, 2017)
PHA			
1	1229- 1057	–CO (backbone carbohydrates)	(Mecozzi & Sturchio, 2017)
2	1381	C-O bend by carboxylate ion	(Brunetti et al., 2020)
3	1459	CH2bending	(Forfang et al., 2017)
4	1722	–C=O carbonyl group	(Mecozzi & Sturchio, 2017)
5	2953	C-H stretching in sCH_2	(Forfang et al., 2017)
6	2925	symmetrical & asymmetrical –CH– vibration	(Hagagy et al., 2021)

Analysis and Characterization of EPSs and PHAs by FTIR

FTIR analysis of the extracted PHA showed clear bands at 1722cm^{-1} and 2925cm^{-1}, representing carbonyl and alkyl groups, respectively. Details of EPS and PHA banding pattern is given in Table 1. FTIR analysis of the extracted PHAs showed clear bands at 1722cm^{-1} and 2925cm^{-1}, representing the carbonyl and alkyl groups, respectively (Mohandas et al., 2018). The band at 1057 cm^{-1}corresponds to –CO stretching (Mecozzi & Sturchio, 2017); this pattern of FTIR spectrum is similar to that of polyhydroxybutyrate (PHB) (Liau et al., 2014).

In the FTIR spectrum of EPS, the bands at 1035 cm^{-1} are of proteins (Orhan-Yanıkan et al., 2020). The band at 1541cm^{-1} shows C-N-H cm^{-1} vibration (amide II). C = O stretching of amide I is seen at wavenumber 1654 cm^{-1} (Forfang et al., 2017). The bands at 2357 cm^{-1} and 2361cm^{-1} indicate the presence of C = C. While, a wavenumber 2835- 2915 cm^{-1} three low bands indicate symmetrical and asymmetrical aliphatic stretching of CH and CH_2 (Mecozzi & Sturchio, 2017). The presence of PHA in psychrophilic bacteria indicates its stability at higher temperatures. Production optimization at increased temperature requests to be concentrated on in the future.

CONCLUSIONS

PHAs and EPSs are produced using three cheap carbon sources (glucose, glycerol, and molasses), and their comparative analysis is done in this study. The best producers were two isolated strains (*Micrococcus yunnanensis* and *Psychrobacter* sp.) were the best producers. Highest PHA (79%) and EPS (70g/L) production were given by strain AJ3 (*Psychrobacter* sp.). Further studies of hot springs can yield novel bacterial strains at different times and seasons. Moreover, in-depth studies of PHA and EPS coproduction at an industrial scale can be conducted.

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