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GRAY WATER TO GREEN GOLD: CHARACTERIZATION AND POTENTIAL OF POLYHYDROXYALKANOATE-PRODUCING MICROBES FROM INDUSTRIAL EFFLUENTS

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Abstract *Due to high organic load and potential toxicity, the industrial wastewater (WW) poses significant environmental challenges necessitating effective management strategies. The WW presents a unique prospect for bioprocessing such as for the production of polyhydroxyalkanoates (PHAs) due to the presence of microorganisms. This study aimed to isolate and characterize PHA-producing microbes from industrial WW, evaluating their potential for biopolymer synthesis. The collected WW samples were analyzed physiochemically to determine values of parameters like temperature, pH, and concentrations of TDS, BOD and chemical oxygen demand (COD). Results of this research work shows the temperature 40ºC, pH =6.2, BOD 3900mg/L, COD 6960m/L and TDS 868mg/L, showing a high number of pollutants adequate for microbial growth. Total of nine strains of bacteria were isolated, among which three strains are recognized as PHA producers that are Serratia nematodiphla, Pseudomonas granadensis and Enterobacter cloacae. These strains were identified through staining techniques and molecular characterization using 16S rRNA sequencing, UV Visible spectroscopy determined characteristics. A total indicated characteristic absorbance peaks corresponding to PHA, while FTIR analysis identified functional groups indicative of biopolymeric structures. The investigations determined that WW is an efficient substrate for growing PHA producing bacteria, providing sustainable waste management and non-toxic materials. In future researcher should focus on various factors to create an ideal environment for cultivation and increasing the capacity for PHA production. This approach turns waste into valuable products and advancing circular economy.*

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Keywords: Industrial wastewater; Polyhydroxyalkanoates (PHA); *Serratia nematodiphila; Pseudomonas granadensis; Enterobacter cloacae; 16s RNA*

Introduction

Synthetic polymers are known for their specific properties such as lightness, durability, and high strength (SennaKesavan et al.,2020) Synthetic polymers can modified chemically to make improved and adaptable materials that can be used in various products in almost all industries (Choi et al., 2021). However, the manufacture of petrochemical plastics has caused ecological issues due to their non-degradable nature and accumulation in diverse environments (Acharjee et al., 2023). Worldwide, plastic production is still increasing, reaching in 2018 up to 359 million tons, which is 155 million tons more as compared to the year 2002 (Conti et al., 2021). Despite several efforts to increase the quantity of plastic waste directed for recycling, about 32.5% was recycled in the year 2018 in Europe. The residual plastics were discarded in the landfills, left in natural environment, or in open dumps (Preka et al., 2022). Through their decomposition, microplastics are generated that negatively affect the environment and is a global issue (Wojnowska-Baryła et al., 2022). Microplastic particles can easily enter the body through food or water and transfer via the food chain to other living entities (Al Mamun et al., 2023). Consequently, synthetic polymers need to be replaced by their eco-friendly substitutes (Fagnani et al., 2020). Additionally, biobased alternatives to petrochemical polymers are produced through microbial processes from renewable resources, which can decrease greenhouse gas emissions. Products made from

biopolymers can be easily recycled into virgin polymer and, at the end of functional life, biodegrade in the ecosystem (De Gisi et al., 2022).

The rapid growth of the global population, urbanization, and industrialization have resulted in the generation of excessive wastewater (WW), leading to high pollution loads (Zittis et al., 2022). Interestingly, industrial WW can serve as a nutrient-rich medium for microbial growth, enabling the degradation of organic compounds and elimination of nutrients (Putatunda et al., 2022; Papadopoulos et al., 2022; Abdelfattah et al., 2022). Conventional WW treatment plants employ anaerobic and aerobic processes to reduce organic matter and solid waste (Wahyuni et al., 2023). Polyhydroxyalkanoates (PHA), biodegradable polymers produced by microorganisms, offer a promising alternative to traditional plastics, with potential applications in packaging, disposable products, and food additives (Charves et al., 2012; Priya et al., 2022; Kumar et al., 2023). Biopolymers, generally called polyhydroxyalkanoates (PHAs), were discovered in *Bacillusmegaterium* in the twentieth century (Saravanan et al., 2023). About 300 species of microbes accumulate PHAs; among them, halophiles can produce up to 70% (Rawat and Goswami, 2023). PHAs are a group of biocompatible and biodegradable polyesters of natural origin, manufactured by a range of Gram-positive and Gram-negative bacteria as carbon reserves (Acharjee et al., 2023). PHAs are very important microbial polymers industrially, accumulating in different archaea and bacteria (Pala-Ozkok et al., 2022). Microbes that live in limited nutrients conditions accumulate PHA in their cells that provides energy to them (Kumar et al., 2023). PHAs have many properties, such as good resistance to gases, UV resistance, and insolubility in water, high tensile strength, biocompatibility, and biodegradability (Panou and Karabagias, 2023). Due to theit piezoelectric nature, they can be used for making sensors for electronicapparatuses (Feng et al., 2023). Due to high cost of PHA production it faces commercial disadvantages. PHA productionis widely studied in gram negative bacteria such as Pseudomonas, Alcaligenes and *E.Coli* (che et al., 2023). Gram negative bacteria produces high amount of PHA as compared to Gram positive bacteria. Similarly, some Gram-positive bacterial strains are also PHA producers, such as numerous species of Bacillus (e.g., *Bacillus cereus* and *Bacillus megaterium*) (Rehakova et al., 2023). Some Fungi of genus Streptomyces are also found to produce PHA (Mandie et al., 2019: Marimuthu et al., 2020: Urbanek et al., 2020)Because PHA producing bacteria are lipidic in nature therefore they are isolated on utilizing lipophilic stains like fluorescent oxazone, Nile blue(Ostle and Holt, 1982), Nile red (Spikermann et al., 1999) and Sudan black (Schlegel et al., 1970) the species and genus of PHA producing bacteria and the presence of one or more PHA biosynthesis genes can be determined by PCR-based molecular assays (Ryan et al., 2013). In this respect, the 16S rDNA gene delivers high-grade resolution for taxonomical determination; hence, if two strains show less than 97% identity in the sequence of 16S rDNA, it can be concluded that these species do not belong to the same species (Pan et al., 2023). The current study aimed to isolate and characterize PHA-producing bacterial strains using molecular and microbiological methods to explore native strains useful for biopolymer production on an industrial scale. The identification of PHA-producing microbes can encourage the use of inexpensive substrate derivatives from WW treatment plants. These microbes can be utilized as substitutes in WW treatment, as industrial wastes can serve as settings for biological degradation and bioplastic production, thereby encouraging biotechnological development in the region.

Materials and methods

Collection, Transportation, and Physiochemical Analysis of Wastewater Samples

The WW sample was obtained and preserved in sealed plastic bottles from cardboard industrial waste in Peshawar. The sample was packed in a sterile plastic bottle, labeled properly, and immediately taken to the laboratory, where it was kept at 4°C for further examination. All test samples were analyzed for pH, BOD, temperature, and COD at PCSIR (Khan et al., 2023a; Khan et al., 2023b; Farid et al., 2023; Ali et al., 2023; Hamayoon et al., 2024). The analysis of mineral was conducted by the NIFA (Nuclear Institute for Food and Agriculture), Nowshehra.

Serial Dilution and Culturing of Bacterial Isolates

PHA-producing bacteria were isolated by means of the serial dilution method. Total seven test tubes were cleaned and sterilized, and then numbered from one to seven. Dilutions were performed up to 10^{-7}. The test tubes were autoclaved for 15 minutes at 121 °C with distilled water (9.0 ml) inside. The sample (1.0 g) was added to the first test tube and thoroughly mixed using a vortex mixer. From first test tube, sample (1.0 ml) was shifted to second test tube and then mixed by vortexing. This process of transferring 1ml was continued up to the last one i.e seventh test tube on nutrient agar medium, containing beef extract (3g/l), NaCl (5g/l), peptone (5g/l) and agar (15g/l). Culture plates were placed in an incubator for 24 hrs at 28-37^{o}C. After incubation, the culture plates were properly checked for bacterial growth. Single species colonies of bacteria were grown by subculturing bacterial colonies. These single species cultures were kept on nutrient agar plates at 4^{o}C for future usage (Aljuraifani et al., 2019).

Isolation and Identification of PHA-Producing Bacteria

The culture plates were tested after incubation for intracellular lipids by staining bacterial colonies using Sudan Black B (ethanolic solution) at 0.02% level for the identification of PHA producing bacteria. Culture plates were stained Sudan Black B and then left for 1/2hr. These were then washed with 100% ethanol to

remove excess color. PHA producer appeared bluish black while other appeared white (Kanknkar and Khandeparker, 2022). The positive isolates for Sudan Black B were then checked using Nile Blue Dye. About 0.5g/mL of Nile Blue was added to media with higher level of carbon and nutrient required for bacterial cell growth. After 20miutes, the bacterial colonies were examined under UV light (wavelength: 235 nm). Positive colonies displayed pink or bright orange fluorescence (Ratnaningrum et al., 2019).

Morphological Characterization of PHA-Producing Bacteria

PHA positive isolates were further subcultured and were observed for morphological features. The bacterial isolates were identified using the technique of Gram staining. For optimal results, 24-hrs the cultured plates were utilized. Various biochemical tests, including catalase, starch hydrolysis, oxidase, hemolysis of blood, Voges-Proskauer (VP), and NaCl (6.5%) growth, were accomplished (Tyagi and Sharma, 2021; Foreman et al., 2021).

Molecular Identification of PHA-Producing Bacteria

To identify the bacterial strain, 16S rRNA sequencing was performed. The bacterial culture grown on nutrient agar was sent to Macrogen Korea for sequencing. The obtained sequences were then analyzed using the BLAST algorithm to find the most similar matches in the NCBI GeneBank database. The top hits from the BLAST search were recorded. MEGA 7 software was used to construct a phylogenetic tree based on the sequencing data. The sequences were assembled into contigs using CodonCode Aligner version 8.0.2. The contigs were then matched to known 16S rRNA sequences in the NCBI database using the BLAST algorithm. Universal primers were employed for sequencing, following established protocols (Martínez-Gutiérrez et al., 2018). The nonessential vector sequence from the forward 5'-AGA GTT TGA TCM TGG CTC AG-3' and reverse 5'-GGT TAC CTT GTT ACG ACT T-3' sequences using Finch TV software. The reverse and forward sequences align properly with the help of Codon Code Align tool. The complete correct sequence was uploaded to NCBI Nucleotide database to find closely related species (Idris et al., 2020). Then the sequences with high identity percentage to those in database were obtain and stored in FASTA format (Martinez-Gutierrez et al., 2018). Sequences were then submitted to NCBI Gene Bank for accession number. To find evolutionary conserved areas of 16S ribosomal RNA gene among species, MEGA7 software (Molecular Evolutionary Genetic Analysis, version 7.0.18) was used to align specific sequences from 10 to 15 different species using FASTA sequence tool (Mahmoodi et al., 2018). MEGA 7 was utilized to create phylogenetic trees on the basis of nucleotide sequences (Mahmoodi et al., 2018).

PHAs Extraction from Bacterial Cells

The PHA positive bacteria were introduced into nutrient broth (250mL). After incubation period of 72hrs, culture broth were poured in Eppendorf tubes and were centrifuged at 8000rpm for about 15minutes.Then 500uL of sodium hypochlorite was added in each Eppendorf tube. Pellet was then incubated in water bath incubator at 50^{o}C for 1 hour for cell lysis. Then the pellets were removed from water bath and cell extract was centrifuged again at 12000rpm for 30 minutes.Sodium hypochlorite was removed from test tubes and the pellets were washed with the distilled water, then with acetone and at the end washed with absolute ethanol. Then 1ml of chloroform was added to each test tube and incubated overnight at 50^{o}C to evaporate at room temperature. The dried pellets were then collected into two Eppendorf tubes. In one of the tube, 1ml sulphuric acid was poured and incubate in water bath at 100^{o}C for 10minutes to convert PHAs into crotonic acids, which provide maximum absorbance at this stage (Kurian and Das, 2021).

Fourier Transform Infrared (FTIR)

FTIR spectroscopy was used for identification and characterization of PHAs. From bacterial strain dried PHA polymer was obtained and mixed with powdered KBr to form discs. The Spectra between 400 and 4000cm^{-1} was obtained by MRL spectrophotometer.

UV-Visible Spectroscopy

The maximum absorbance and the wavelength of crotonic acid were noted using UV-visible spectroscopy. For around 20 minutes the UV-Vis spectrometer was turned on, allowing the lamps to warm up for stabilization. An absorbance spectrum was performed. The sample was filled into the cuvette and placed in the spectrometer, scanning absorbance from 200 to 800 nm (Kucharska and Karpinska, 2020).

Results

Physiochemical Analysis of Samples

The WW sample collected was blackish-brown in color with a sour and unpleasant odor. The values of physiochemical parameters analyzed for the WW were pH (6.2), temperature (40.5 °C), total suspended solids (TSS; 656 ± 0.61 mg/L), total dissolved solids (TDS; 868 ± 0.95 mg/L), volatile suspended solids (VSS; 50.8 ± 1.46 mg/L), biological oxygen demand (BOD; 3900 ± 2.44 mg/L), chemical oxygen demand (COD; 6960 ± 3.21 mg/L), BOD_5/COD ratio (0.57), total Kjeldahl nitrogen (TKN; 6.26 ± 0.11 mg/L), phosphate (9.5 ± 0.03 mg/L), total organic carbon (TOC; 198.1 ± 2.91 mg/L), total nitrogen (0.02%), phosphorus (0.07 ± 0.01 mg/L), and potassium (20.9 ± 0.02 mg/L) (Table 1). The BOD_5/COD ratio of 0.57 was suitable for the dark fermentation process.

Table 1. Physiochemical Parameters of Wastewater

Parameters	Wastewater	SD
Ph	6.2	0.32
Temperature (^{o}C)	40.5	0.12
TSS (mg/L)	656.0	0.61

TDS(mg/L)	868	0.95
VSS(mg/L)	50.8	1.46
BOD(mg/L)	3900	2.44
COD(mg/L)	6960	3.21
BOD5/COD ratio	0.56	---
Phosphate (mg/L)	6.26	0.11
TKN(mg/L)	9.5	0.03
TOC (mg/L)	198.1	2.91
Nitrogen (%)	0.02	---
Phosphorous (mg/L)	0.07	0.01
Potassium (mg/L)	20.9	0.02

Keys: pH (potential of Hydrogen), BOD (Biochemical Oxygen Demand), COD (Chemical Oxygen Demand), TKN (Total Kjeldahl Nitrogen), TSS (Total Suspended Solids), TDS (Total Dissolved Solids), TOC (Total Organic Carbon), VSS (Volatile Suspended Solids).

Identification and Characterization of PHA-Producing Bacteria

By culturing on nutrient agar plates, a total of 9 bacterial strains were isolated from WW samples (Figure. 1a). The results of the Gram staining and microscopic observation are summarized in Table 2. The Gram staining results showed that 6 (66.66%) isolates were Gram-negative, while the other 3 (33.33%) were Gram-positive of the total 9 isolates. All identified isolates underwent qualitative PHA production testing using Sudan Black B dye (Figure. 1b). Three colonies appeared dark blue in color and were noted to be producing PHA. To further confirm the formation of polyhydroxyalkanoic acids (PHAs), Nile Blue dye was employed (Figure. 1c). By screening with Sudan Black and Nile Blue, a total of 3 PHA-positive strains were isolated: Rj-4, Rj-7, and Rj-8.

Table 2. Morphological Characteristics of Isolates

Sample Number	Shape and size	Gram staining	Sudan Black	Nile Blue
Rj-1	Bacilli, small	Negative	Negative	Negative
Rj-2	Cocci, small	Negative	Negative	Negative
Rj-3	Cocci, small	Positive	Negative	Negative
Rj-4	Basilli, small	Negative	Positive	Positive
Rj-5	Streptococcus, small	Positive	Negative	Negative
Rj-6	Bacilli, small	Negative	Negative	Negative
Rj-7	Basilli, small	Negative	Positive	Positive
Rj-8	Basilli, large	Negative	Positive	Positive
Rj-9	Bacilli, large	Positive	Negative	Negative

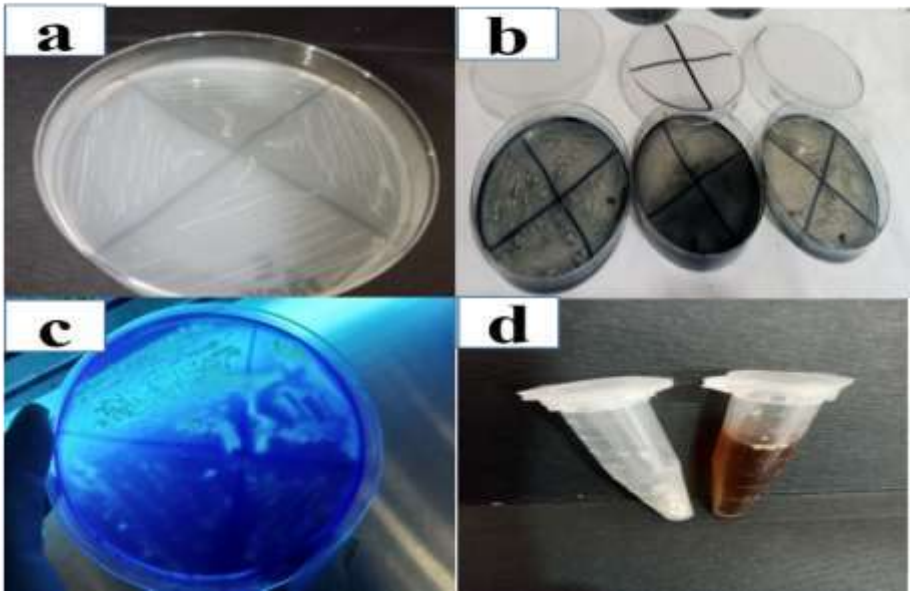

Figure. 1(a) Bacteria on nutrient agar (b) Identification with Sudan Black (c) characterization with Nile Blue (d) Extraction of PHA producing bacteria

Biochemical Identification

The Gram staining results showed that all three PHA-producing bacteria were Gram-negative. Various biochemical tests were performed to identify the isolates according to Bergey's Manual. The results of the biochemical tests are mentioned in Table 3. The isolate Rj-4 showed positive results for catalase, citrate, Voges-Proskauer, and hemolysis of blood tests, while showing negative results for oxidase, 6.5% NaCl growth, and starch hydrolysis. Thus, Rj-4 was identified as *Serratia spp.* Similarly, the isolate

Rj-7 exhibited positive results for citrate, 6.5% NaCl growth, and hemolysis of blood tests, while showing negative results for catalase, oxidase, Voges-Proskauer, and starch hydrolysis; it was identified as *Pseudomonas spp.* The isolate Rj-8 showed positive results for all the tests applied (catalase, oxidase, 6.5% NaCl growth, citrate, and Voges-Proskauer) and was identified as *Enterobacter spp.*

Table 3. Biochemical Identification of Isolates

Biochemical tests	Bacterial Isolates		
	Rj-4 (*Serratia*)	**Rj-7 (Pseudomonas)**	**Rj-8 (*Enterobacter*)**
Catalase Test	Positive	Negative	Positive
Oxidase Test	Negative	Negative	Negative
Citrate Test	Positive	Positive	Positive
6.5 NaCl growth	Negative	Positive	Positive
Voges Proskauer	Positive	Negative	Positive
Starch Hydrolysis	Negative	Negative	N/A
Hemolysis of Blood	Positive	Positive	N/A

Molecular characterization of Isolates

Among the 9 isolated strains, three different PHA producing bacterial species were obtained (*Serratia nematodiphila, Pseudomonas granadensis and Enterobacter cloacae*). The species were evaluated further for *16S* rRNA sequencing and the results were submitted to NCBI. After processing, accession numbers were allotted to the samples. Later, phylogenetic tree wasconstructed with similar sequences by using neighbor joining method for each isolated bacterial strain (Figs 2–4). The bacterium J2 was identified as *Serratia nematodiphila* with 99% sequence identity and high coverage (98%) (Figure.2). The bacterium J3 was identified as *Pseudomonas granadensis* (Figure.3) with 99% sequence identity to the reference sequence LT629778.1, using specific 16S rRNA primers, with high coverage. Another bacterium was identified as *Enterobacter cloacae* (Figure. 4) with 99% sequence identity to the reference sequence CP001918.1, using specific 16S rRNA primers, with complete coverage. Phylogenetic analysis placed the query sequence (3F) within the Enterobacter genus, confirming its close relationship with other *Enterobacter cloacae* strains.

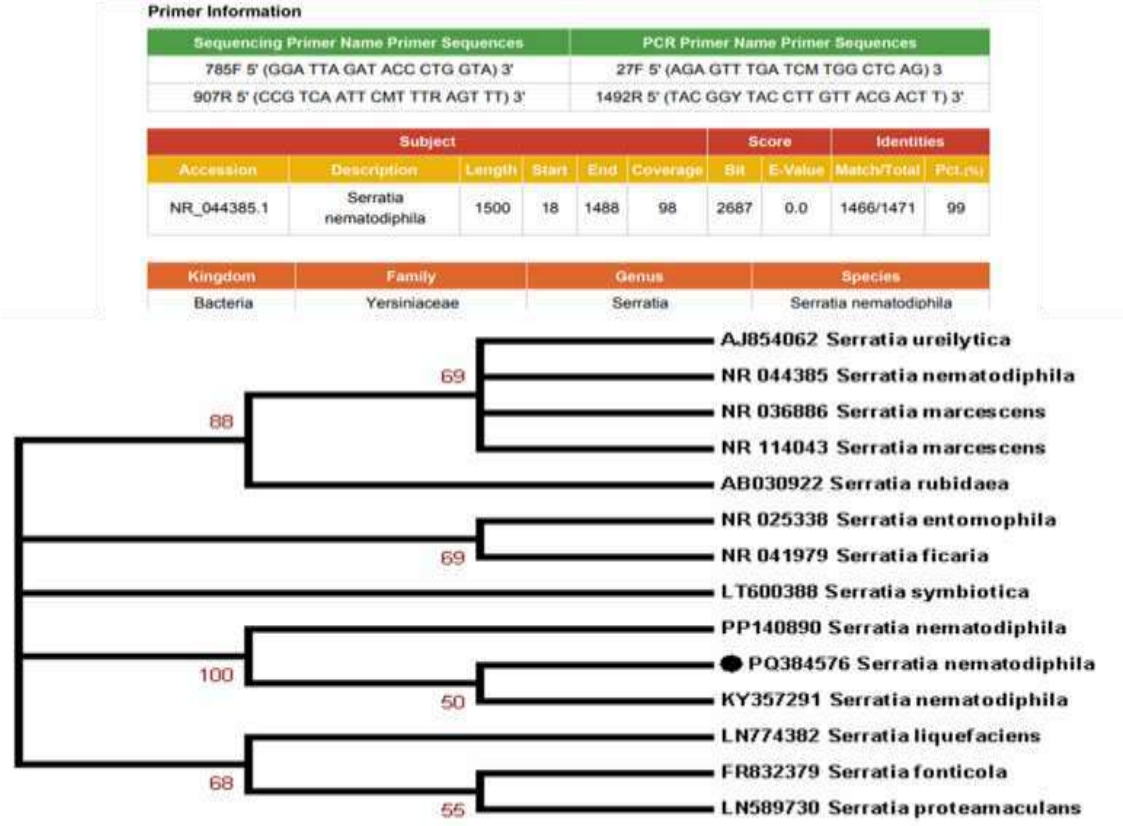

Figure 2. Evolutionary relationships of taxa of *Serratia nematodiphila*.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, (1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004; Kumar et al (2016).

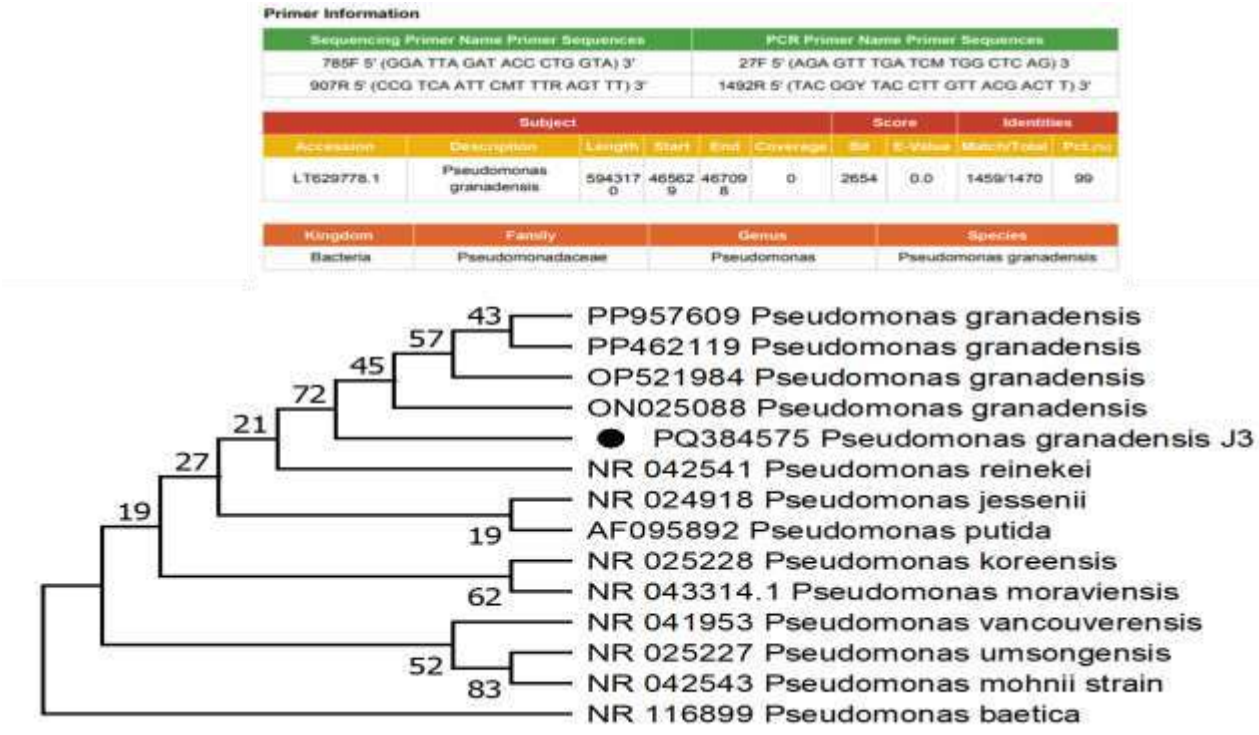

Figure 3. Phylogenetic tree of *Pseudomonas granadensis.* The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987)

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
CP001918.1	Enterobacter cloacae	5314581	1015780	1017255	0	2704	0.0	1473/1477	99

Kingdom	Family	Genus	Species
Bacteria	Enterobacteriaceae	Enterobacter	Enterobacter cloacae

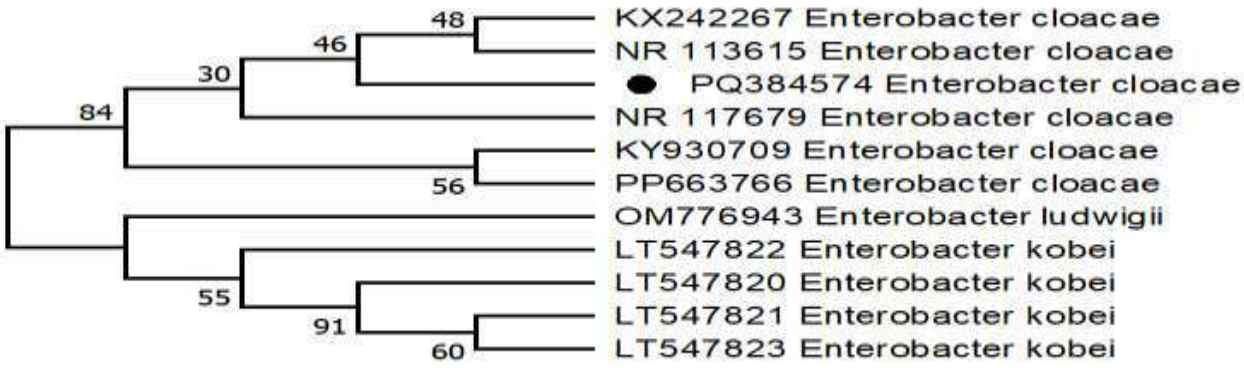

Figure 4. Phylogentic tree of *Enterobacter clocacae.*The evolutionary history was inferred by using the Neighbor-Joining method (Saitou and Nei (1987)

PHA Characterization

UV Spectrum for PHA characterization

The primary validation of the obtained polymer was carried out by determining absorbance that ranged from 200 to 800 nm using a UV–Vis spectrophotometer. The sharp absorbance peaks were detected in the range of 369 to 519 nm for PHA obtained from *Serratia* (Figure.5). The primary confirmation of the PHA obtained from *Pseudomonas* (showed sharp absorbance peaks in the range of 380 to 487 nm, characteristic of PHA molecules Figure.6). Similarly, PHA obtained from *Enterobacter* exhibited sharp absorbance peaks in the range of 380 to 409 nm, which is also PHA characteristic (Figure.7).

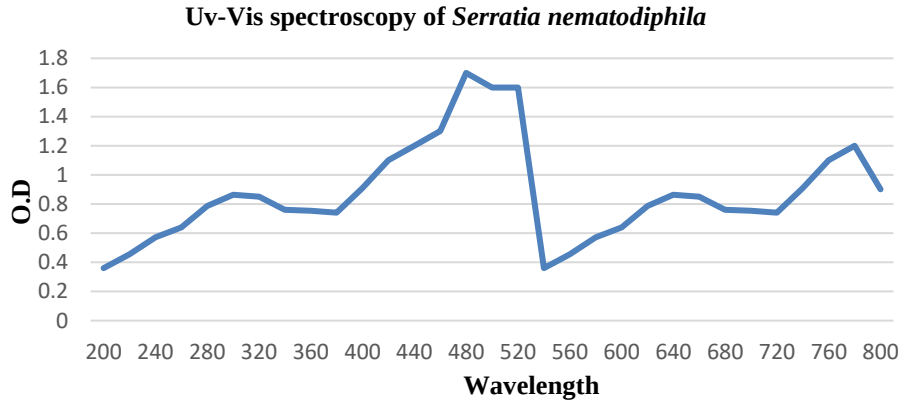

Figure 5. **In this spectrum, you can see a peak in absorbance around 500nm. This peak represents the wavelength at which crotonic acid absorbs the most light**

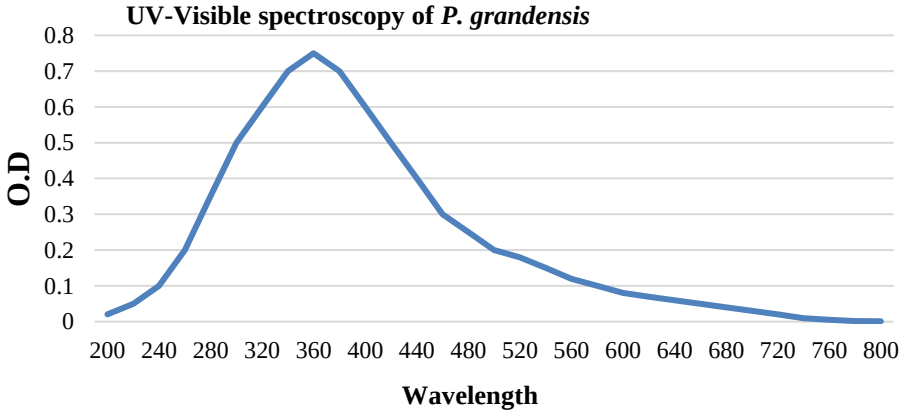

Figure 6. This spectrum peak show absorbance around 280nm. This peak represents the wavelength at which crotonic acid absorbs the most light

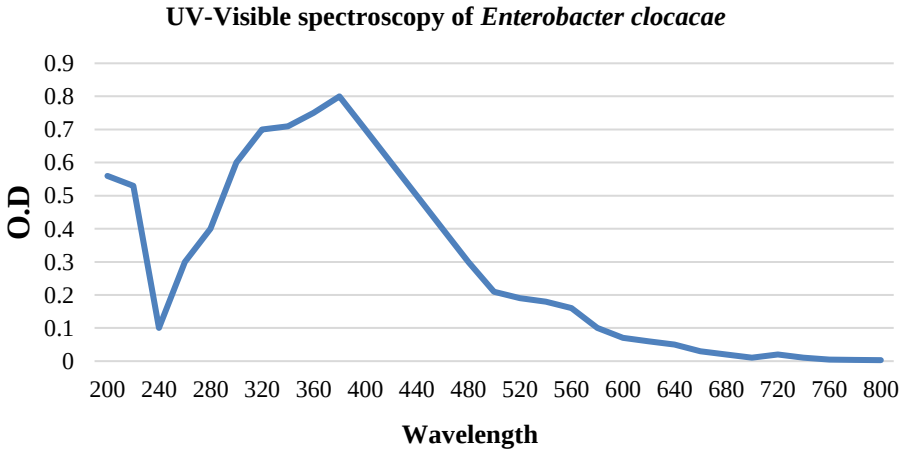

Figure 7. This spectrum peak show absorbance around 380nm. This peak represents the wavelength at which crotonic acid absorbs the most light

FTIR Spectra for PHAs

To analyze PHA produced by *Serratia*, their polymers were extracted from bacterial cells and

cultured in the phenol presence and were characterized by FTIR spectroscopy. (Figure. 8). In the presence of phenol (600 mg/L), the absorption peak at 722 cm^{-1} in FTIR spectrum shows C=C bending associated with alkene groups. The peak at 1243 cm^{-1} indicates C–N stretching related to amine groups, while the absorbance peak at 1181.55 cm^{-1} shows the ester linkage of C–O groups (Figure 5). The absorption peaks at 1710 and 1737 cm^{-1} correspond to the stretching modes of carboxylic acid and conjugated aldehyde groups, respectively. The absorption peak at 2923 cm^{-1} is characteristic of N–H stretching related to amine groups. The highest FTIR peak for *Serratia* was observed at 2923 cm^{-1}, indicating strong N–H stretching associated with amine functional groups, while the lowest peak at 722 cm^{-1} showed strong C=C bending related to alkene functional groups. Bands between 700 and 3000 cm^{-1}, as shown in Table 4, relate to stretching of C=O and C–O of different groups present in the molecule of highly ordered crystalline structure. All these bands confirm that extracted polymer is PHA.

Table 4. FTIR analysis of PHAs produced by *Serratia*

Frequency (Range)	Absorption (cm^{-1})	Appearance	Group	Compound Class
3000-2500	2923	M	C-H stretching	Alkane
1650-2000	1710	S	C=O stretching	carboxylic acid
1600-1300	1463	M	C-H bending	Alkane(Methyl group)
1000-1400	1243	S	C-O stretching	Alkyl aryl ether
650-1000	842	M	C=C bending	Alkene (trisubstituted)
650-1000	722	S	C=C bending	Alkene (disubstituted)

S: Strong, M: Medium

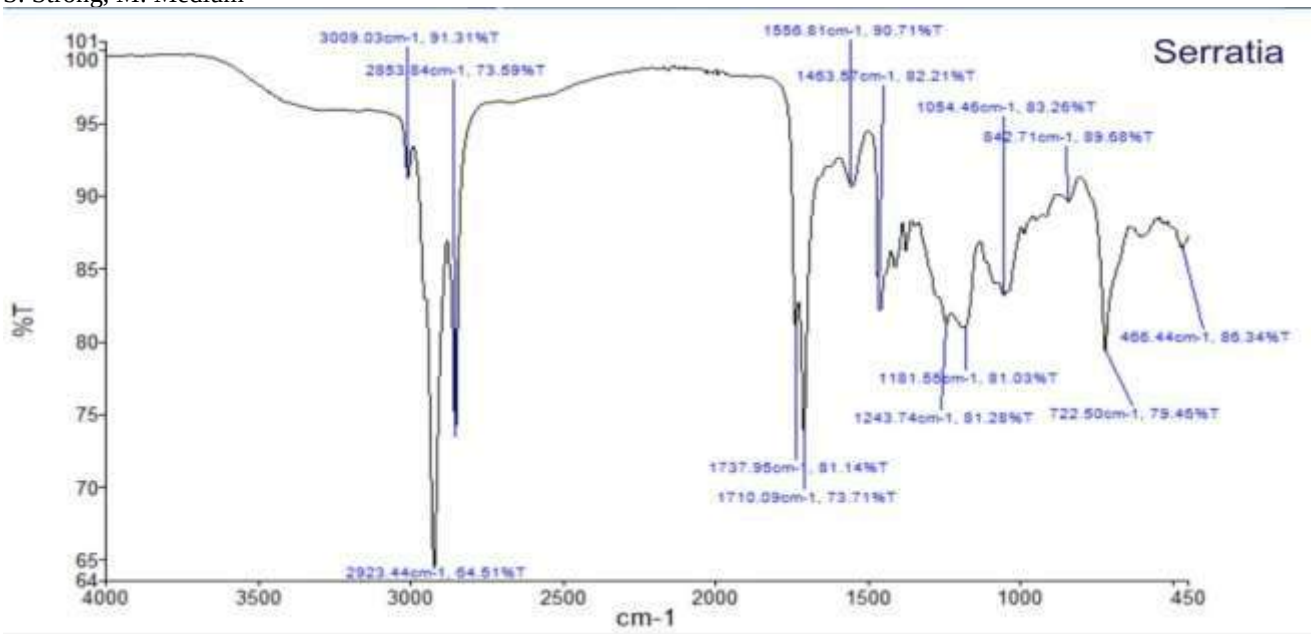

Figure 8 FTIR analysis of PHAs produced by *Serratia*

FTIR analysis of the isolated polymer from *Pseudomonas* revealed absorption bands at 810, 814, and 1638 cm^{-1}, corresponding to C=C bending associated with alkene groups. The absorption peak at 1265 cm^{-1} corresponds to C–O stretching related to aromatic ester groups (Figure. 9). Similarly, the absorption peaks at 1505 and 3457 cm^{-1} correspond to N–O stretching (nitro compounds) and O–H stretching (alcohol), respectively. The highest peak for *Pseudomonas* was observed at 3457 cm^{-1}, indicating strong O–H stretching associated with alcohol functional groups, while the lowest peak showed C=C bending for alkene (tri-substituted) functional groups. The analysis exhibited bands between 700 and 3000 cm^{-1}, as shown in Table 5, which relate to C=O and C–O stretching of various groups present in the molecular chain of the highly ordered crystalline structure. These bands confirm that the extracted polymer is PHA.

Table 5. FTIR spectroscopy of *Pseudomonas*

Frequency (Range)	Absorption (cm^{-1})	Appearance	Group	Compound Class
3000-4000	3457	S, broad	O-H stretching	Alcohol
1650-2000	1638	S	C=C stretching	Alkene
1300-1600	1505	S	N-O stretching	Nitro compound
1000-1400	1265	S	C-O stretching	Aromatic ester

650-1000	800	M	C=C bending	Alkene (trisubstituted)
650-1000	814	M	C=C bending	Alkene (trisubstituted)

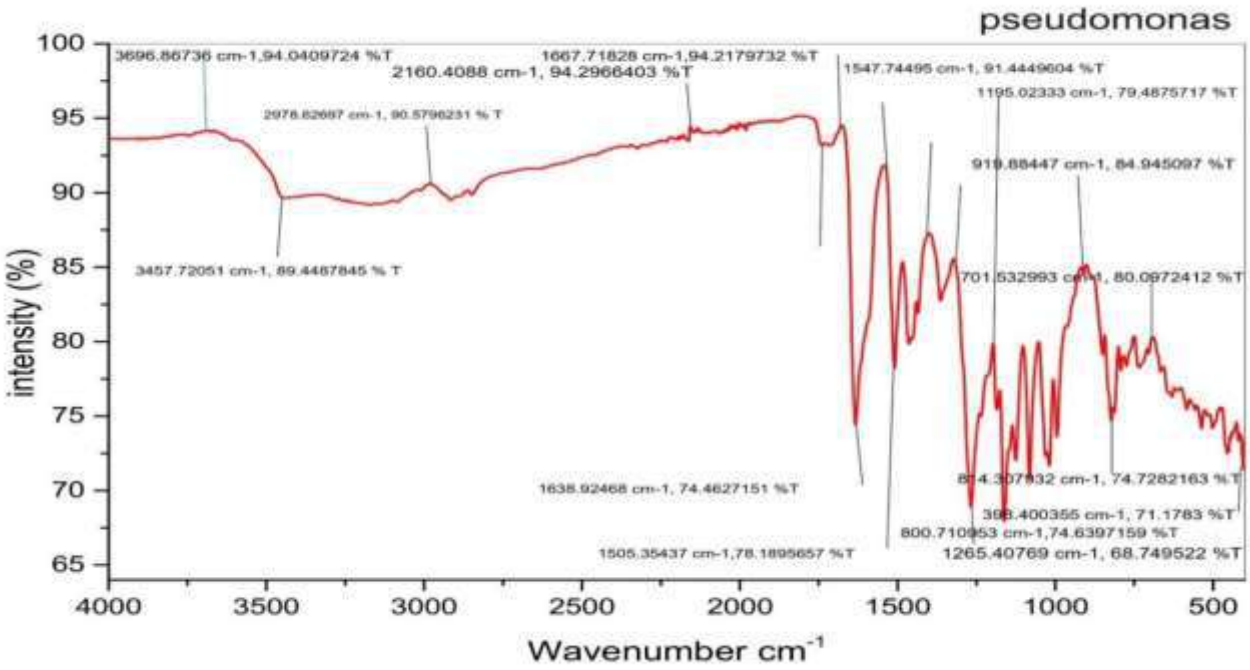

Figure 9 FT-IR analysis of PHAS produced by *Psuedomonas*

FTIR analysis of the isolated polymer from *Enterobacter* revealed absorption bands at 722, 842, and 1710 cm^{-1}, corresponding to C=C bending associated with alkene groups. The absorption peak at 1243 cm^{-1} resembles to C–O stretching related to alkyl aryl ether groups (Figure. 10). Similarly, the absorption peaks at 1463 and 2923 cm^{-1} correspond to C–H (alkane) stretching. The highest peak for *Enterobacter* was observed at 2923 cm^{-1}, indicating medium C–H stretching associated with alkane functional groups, while the lowest peak shows C=C bending for alkene (tri-substituted) functional groups. The analysis exhibited bands between 700 and 3000 cm^{-1}, as shown in Table 6, which relate to C–O and C=O stretching of various groups present in the molecule of highly ordered crystalline structure. These bands confirm that the extracted polymer is PHA.

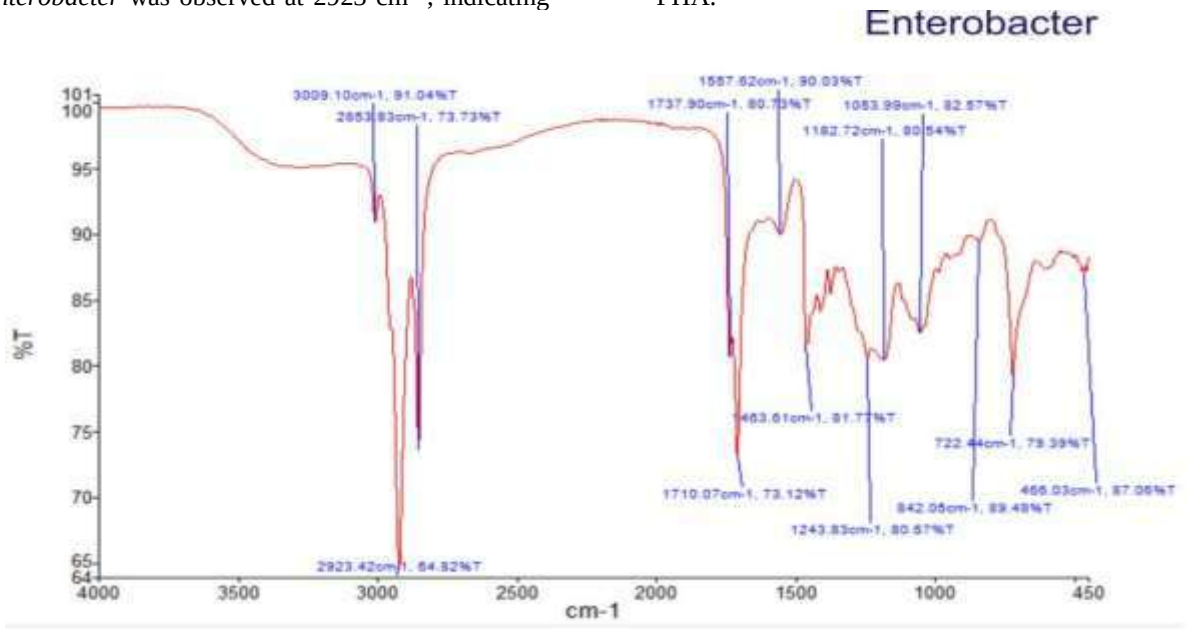

Figure 10. FTIR analysis of PHAS produced by *Enterobactor*

Table 6 FTIR analysis of PHAS produced by *Enterobactor*

Frequency Range	Absorption (cm^{-1})	Appearance	Group	Compound Class
3000-2500	2923	M	C-H stretching	Alkane
1650-2000	1710	S	C=O stretching	carboxylic acid
1600-1300	1463	M	C-H bending	Alkane(Methyl group)
1000-1400	1243	S	C-O stretching	Alkyl aryl ether
650-1000	842	M	C=C bending	Alkene (trisubstituted)
650-1000	722	S	C=C bending	Alkene(disubstituted)

Discussion

Natural resources and processes produce bioplastics, such as microorganisms (bacteria) manipulated to obtain biocompatible and biodegradable polymers (Kumar et al., 2020). The demand for bioplastics in packaging is rapidly increasing among various industries, predominantly the food industry, on a large scale (Muiruri et al., 2022). However, the higher production cost of the biopolymer (PHA) confines its industrial usage due to the limited availability of economical and appropriate substrates (Vigneswari et al., 2021). Therefore, different studies on PHA production have focused on sustainable substrates and growth cultures (Vicente et al., 2023). Previously, Bhuwal et al. (2013) presented that PHA-producing bacteria could be isolated successfully from cardboard, pulp cardboard and kraft industrial wastewater and sludge. The wastewater characteristics from pulp and paper manufacturing may limit microorganism growth due to potentially toxic materials, such as chlorinated organic compounds and lignin derivatives (Toczyłowska-Mamińska, 2017). However, Ghribi et al. (2016) observed that certain bacterial genera, including *Klebsiella spp.*, *Comamonas spp.*, *Pseudomonas spp.*, *Methylobacterium spp.*, and *Ancylobacter aquaticus*, withstand these conditions due to hydrolytic and ligninolytic enzyme activity that biodegrades toxic and lignin compounds (Karigar and Rao, 2011). Research by Jarpa et al. (2012) on PHA biosynthesis from paper mill wastewater treated by a biofilm reactor reported values for pH (6.33–7.67), BOD (441.03 mg/L), and COD (839.00 mg/L), which did not align with the current study. However, the BOD5/COD ratio (0.52) was similar. Baeza et al. (2016) reported values for total nitrogen (0.53 mg/L) and total phosphate (1.01 mg/L) in paper mill wastewater, indicating low nitrogen levels favorable for PHA-producing bacteria. Previous studies have shown that substrate requirements for biopolymer-accumulating bacterial strains include a high organic matter load and low nutrient concentrations (Ben et al., 2011). PHAs accumulate within cells under nutrient-deficient conditions, triggered by cellular stress (Al Rowaihi et al., 2018). Moderate nutrient concentrations, along with other stress conditions (e.g., low oxygen, salinity, and temperature) in industrial processes, may enhance the presence of PHA-producing bacteria in wastewater. The results of the current study indicated that the industrial effluent has adequate organic load and low nitrogen content, demonstrating good conditions for growth of PHA-producing bacteria.

In the present study, industrial wastewater was assessed to isolate strains capable of producing PHA. The PHA-accumulating bacterial strains isolated in this study are *Serratia nematodiphila*, *Pseudomonas granadensis*, and *Enterobacter cloacae*. These strains were identified using various screening methods. Similarly, Munir and Jamil (2015) isolated *Stenotrophomonas*, *Pseudomonas*, *Enterobacter*, *Bacillus*, and *Exiguobacterium* using industrial wastewater as a carbon source for PHA production. The findings of the current study are consistent with those of Valdez-Calderón et al. (2022), who studied polyhydroxybutyrate (PHB) production by *Klebsiella pneumoniae* utilizing media derived from fruit peel residues. Bhuwal et al. (2013) screened PHA-producing bacteria from cardboard, paper, and pulp industry waste, isolating 15 strains positive for Nile Blue staining. Two strains, *Enterococcus sp.* and *Brevundimonas sp.*, produced maximum PHA. In a subsequent study, Bhuwal et al. (2014) isolated 120 bacteria from soil samples, 62 of which tested positive for Nile Blue staining. Of these, 27 isolates produced PHB from cardboard industry effluent. Sinaei et al. (2021) characterized PHA from wheat starch wastewater, isolating 96 bacterial strains—15 from oil wastewater, 24 from starch wastewater, and 32 from activated sludge—capable of producing PHAs. Other studies have isolated PHA-producing bacteria from wastewater. Bhadra et al. (2005) identified a rod-shaped Gram-negative bacterium from the River Torsa in India, affiliated with the genus *Serratia*. Similarly, Pervaiz and Yasmin (2022) isolated *Serratia nematodiphila* from polluted soil in Hattar Industrial Estate, Pakistan, and utilized it for submerged fermentation using simulated wastewater. Pan *et al.* (2021) isolated *Pseudomonas alcaligenes* from food waste oil and studied PHA biosynthesis with energy recovery from fermented wastewater. Zhang et al. (2021) isolated *Pseudomonas sp.* from groundwater in Harbin, China, observing nitrate removal from wastewater at low temperatures via versatile nitrate metabolism pathways. Lam et al. (2017) identified bacterial strains such as *Sphingopyxis terraewere*, *Aeromonas ichthiosmia*, *Yersinia frederiksenii*, and others from wastewater

treatment plants in Hong Kong, exploring PHA production and potential pharmaceutical applications. Similarly, Rakkan et al. (2022) isolated *Enterobacter* strains from wastewater and studied PHA production under batch conditions, while Wan et al. (2017) screened *Enterobacter cloacae* as a phosphorus-accumulating bacterium.

Previous studies have also examined PHA characterization. Umesh et al. (2018) characterized PHA nanoparticles produced by *Bacillus subtilis* using orange peel as a substrate, observing a sharp absorbance peak at 240 nm characteristic of PHA molecules. Senthilkumar et al. (2017) studied PHA optimization from *Pseudomonas aeruginosa* for curcumin delivery nanoparticles, noting crotonic acid peaks between 220–240 nm. The findings of Israni and Shivakumar (2015) on maximum absorption spectra of 235 nm in *Bacillus sp.* corroborate these results. Bhattacharyya et al. (2012) used vinasse for PHA production by *Haloferax mediterranei*, identifying poly-3-(hydroxybutyrate-co-hydroxyvalerate) via UV–Vis spectroscopy, consistent with this study. Gupta et al. (2017) analyzed FTIR spectra of PHA produced by *Serratia sp.*, identifying bands distinctive of biopolymers, similar to the current study. Similar FTIR bands were reported by Singh et al. (2013) and Umesh et al. (2018), further endorsing the obtained polymer as PHA.

Conclusion

The findings of this research demonstrate the promising potential of utilizing industrial WW as a substrate for the cultivation of PHA-producing bacteria. The isolated strains, particularly *Serratia nematodiphila*, *Pseudomonas granadensis*, and *Enterobacter cloacae*, exhibit favorable characteristics for biopolymer synthesis. The physiochemical profile of the wastewater suggests an environment conducive to microbial growth and PHA accumulation, characterized by a moderate organic load and low nutrient concentration. These results underscore the importance of bioremediation and biopolymer production from waste streams, contributing to sustainable practices in waste management and the development of biodegradable materials. Further research should explore optimization of growth conditions and scaling up PHA production processes for commercial applications.

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Declaration

Author contribution

RA, SK, FS: writing original draft, software, data collection, and analysis. FS, AU, SS: supervision, conceptualization, and visualization. RA, FS, SMS, SK and FS: methodology, software, data curation and formatting. FS, AA,: reviewing and editing. AU, MA, QA, AA: final proofreading and funding. All the authors reviewed and contribute in the manuscript.

Data availability

All data generated or analyzed during this study are included in this article.

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Ethics approval and consent to participate

Not applicable

Conflict of Interest

The authors declare that there is no conflict of interest related to this study.

Consent for Publication

Not applicable