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Isolation, Screening and Characterization of Exopolysaccharide Producing *Pseudomonas* Sp. from The Soil Sample

Jadhav V. G.[1*], Baisthakur P. O.[2], Joshi R. D.[3], Jadhav A. S.[4]

[1]*Department of Microbiology, Yashwantrao Chawhan Arts, Commerce and Science College, Lakhandur, Bhandara, Maharashtra*

[2]*Department of Microbiology, Late Baburao Patil Arts and Science College, Hingoli*

[3]*Department of Microbiology, Yogeshwari Mahavidhyalay, Ambajogai, Beed, Maharashtra*

[4]*Department of Microbiology, Yeshwantrao Chavan Institute of Science (Autonomous), Satara, Maharashtra*

**Corresponding author's E-mail: vishnujadhav77@gmail.com*

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Abstract

Exopolysaccharides (EPS) are vital metabolites that specific environmental microorganisms produce and expel. The goal of the current investigation is to identify bacteria that produce exopolysaccharides in soil samples from the Buttenath Dam region, At. Chanai, Tq. Ambajogai, Dist. Beed situated in the Marathwada region of Maharashtra, India. Two methods, such as gravimetric analysis of exopolysaccharide dry weight and quantification as, instance, for total carbohydrate content using phenol sulphuric acid technique, were used to screen the EPS-producing capacities of the chosen isolates. Post preliminary screening by selecting thick, ropy-like colony formers on agar medium, the results showed that thirteen (13) distinct colonies were marked to manufacture EPS. Tentative identification of these isolates was done based on morphological and biochemical tests. Three (03) isolates, VJ001, VJ003 and VJ010, were identified through secondary screening as the most effective EPS producers (producing precipitates over 1.46 mg/L of total dry weight and 8). This was in comparison to other bacterial colonies that were also isolated. Out of these screened isolates, VJ003 was the isolate with the highest exopolysaccharide produced and this isolated bacterium was identified using 16S rRNA gene sequence and phylogeny study as Pseudomonas aeruginosa VJ003 with NCBI GeneBank accession no. OR196093. The Pseudomonas aeruginosa VJ003 strain can be used in biotechnological sectors because of the ability it has to create significant levels of exopolysaccharides, according to the results of the current study.

Keywords: *Soil, Exopolysaccharide Bacteria, Isolation, Screening, Identification, 16S sequencing, Pseudomonas aeruginosa*

1. Introduction

High molecular weight compounds with water solubility are called exopolysaccharides (EPS). EPS are biomolecules containing long-chain structure with lower amounts of proteins, lipids, nucleic acids, humic chemicals, and organic macromolecules like polysaccharides (Das et al., 2020; Flemming & Wingender, 2010; Moxon & Kroll, 1990; Mukherjee et al., 2019). Although different microbial species' fine structures of EPS varies substantially from one another (Gupta and Diwan, 2017), a kind of structurally distinct exopolysaccharide biopolymer with potential bioactive have recently been reported (J. Liu et al., 2016; Mahapatra & Banerjee, 2016). EPS also helps the microorganisms connect and colony formation at the roots in an irreversible manner because it works with a network of fibrillar material to securely attach the bacteria to the root surface (Tisdall & Oades, 1982). By creating cation bridges, hydrogen bonds, Van der Waals forces, and anion adsorption mechanisms on clay surfaces, soil aggregates are protected by the EPS, which also aids in the release of capsular and slime materials

produced by soil microorganisms. In addition, these substances facilitate the production of biofilms by giving them mechanical stability (Sutherland & Sutherland, 2001) and mediating their attachment to surfaces (Luttge et al., 2005).

Pseudomonas aeruginosa is a Gram-negative, opportunistic pathogen that can cause a variety of infections in humans, including pneumonia, sepsis, and infections of the urinary tract, ears, and eyes. The bacterium is also known to create biofilms, which are intricate bacterial colonies encased in an extracellular polymeric substance (EPS) matrix. The EPS is made up of a variety of biopolymers, including exopolysaccharides (EPS).

Exopolysaccharides are high molecular-weight carbohydrates that are produced by bacteria and released into the surrounding environment. They play a variety of roles in bacterial physiology, including adherence to surfaces, protection from environmental stresses, and quorum sensing. Three main exopolysaccharides *Pseudomonas aeruginosa* produces three main exopolysaccharides: alginate, Psl, and Pel (S. Singh et al., 2021). Alginate is a linear, anionic polysaccharide composed of alternating mannuronic acid and guluronic acid residues. It is the most abundant exopolysaccharide produced by *P. aeruginosa* and is responsible for the structural integrity of the biofilm matrix (Chung et al., 2023) and such biopolymers enhances the plant growth (Rekadwad et al., 2016). Psl is a neutral, heteropolysaccharide composed of glucose, galactose, and mannose residues. It is thought to play a role in the regulation of biofilm formation and antibiotic resistance (Balducci et al., 2023). The biofilm matrix contains a cationic exopolysaccharide called pel that cross-links extracellular DNA (Reichhardt et al., 2020). Biodegradable polymers having industrial significance (Singh et al., 2020).

The biopolymers produced by *Pseudomonas aeruginosa* have a variety of potential applications, including (Bose et al., 2023; Cano-Vicent et al., 2022; Eslami et al., 2020; J. Singh et al., 2023):

- Bioremediation: The EPS can be used to remove pollutants from the environment.
- Drug delivery: The EPS can be used to deliver drugs to specific tissues or cells.
- Tissue engineering: The EPS can be used to create scaffolds for tissue regeneration.
- Food additives: The EPS can be used as thickeners, stabilizers, and emulsifiers in food.

The study of the biopolymers produced by *Pseudomonas aeruginosa* is an active area of research. As our understanding of these biopolymers grows, we will be able to develop new and innovative applications for them. Hence, in the present study, the biopolymer-producing soil bacteria were isolated and screened for the extracellular biopolymer production ability. The ability of biopolymer production was checked by estimating the produced exopolysaccharide grams/litter. The potent isolates were identified on the basis of morphological, biochemical and molecular techniques.

2. Materials And Methods

Site description and soil sample collection

An experimental field 'Buttenath Dam area' was situated at Chanai, Tq. Ambajogai, Dist. Beed (18° 73 20.24′ N latitude, 76° 35 11.21' E longitude; 462 m above sea level), in the Marathwada region of Maharashtra, India. The experiment used (50-day duration), a widely used local variety in these ecologies that is photoperiod insensitive. (Bhattacharyya et al., 2012). Composite soil samples were collected from four corners and center spotting of the selected area of the agriculture field using a clean plastic scrapper and sterile spatula. The soil samples were collected in sterile plastic collection bags. Just after collection of the sample, the soil samples were transported to the research laboratory and stored in the refrigerator at 4 °C for further experimentation and microbial analysis.

Isolation and purification of EPS-producing bacteria

The soil samples collected were subjected to serial dilutions up to 10^{-5} (A. Khan et al., 2023). Tryptic Soya Agar (TSA) (casein peptone; 15.0 gm/l, soya peptone; 5.0 gm/l, sodium chloride; 5.0 gm/l, and agar; 15.0 gm/l) was used to isolate the EPS-producing bacteria using standard spread plate procedures (Sandhya et al., 2009). The plates were incubated for 24 to 48 hours at 28 (±2) ºC. Following incubation, the four-quadrant technique was used to filter and subculture typical mucoid colonies into TSA plates.

The isolated pure colonies were kept in pure culture on TSA media and then further processed based on morphological, biochemical, and molecular procedures for identification.

Screening and selection of EPS-producing bacteria

The ability of isolates to create exopolysaccharides was screened quantitatively using the phenol sulphuric acid method and gravimetrically by setting the dry weight of the exopolysaccharide obtained from the isolates. Each inoculum was generated as a cell suspension containing 1.5 x 108 bacteria per milliliter of overnight-grown culture, which was then added to 50 ml of nutritional medium supplemented with 2% sucrose and incubated in a rotary shaker at 30 oC for three days at 120 rpm. Exopolysaccharides were isolated from the culture media's cell-free supernatant using two volumes of cold ethanol, and they were then precipitated after being chilled at 4°C overnight. After centrifuging at 15,000 rpm for 20 min, the precipitates were recovered and dried at 60ºC to constant weight. Pellets in the form of EPSs were measured for total dry weight and their total carbohydrate contents were determined using a phenol sulphuric acid test (Abdulrazack et al., 2017). For further research, the bacterial strains with the highest yields based on the values for EPS dry weight and carbohydrate content were chosen.

Identification of the potent EPS-producing isolate:

The potent isolate was identified using morphological and biochemical techniques as mentioned in Bergey's Manual of Systematic Bacteriology. Colony morphology including macroscopic features viz. size, shape, colour, margin, elevation, opacity and consistency were checked whereas in microscopic features; the shape of the cell, gram's nature and motility were checked. In biochemical characterization, Indol, Methyl Red, Voges-Proskeur, Citrate Utilization, different enzymes viz. catalase, urease, nitrate reductase, oxidase etc. production ability was checked using standard methodology. From morphological and biochemical techniques, the isolate was identified up to genus level, for final identification on the species level, a molecular approach was selected in which 16S rRNA gene sequence was studied and phylogeny was constructed for proper identification of the isolate.

3. Results and Discussion

Isolation and screening of EPS-producing bacteria:

After being serially diluted, the collected soil samples were plated on an agar medium and allowed to incubate. Numerous bacterial counts were seen on the plates, and forty distinct colonies were picked out and identified as exopolysaccharide-producing strains based on their propensity to form ropy mucoid on agar medium. Utilizing the criteria of gravimetric assessment of EPS dry weight following cold ethanol precipitation of cell-free supernatant of the cultured medium, these isolates were examined for their ability to produce EPS. Out of these twenty-two isolates, thirteen isolates showed positive results for the production of different amounts of exopolysaccharide as shown in Fig. 1

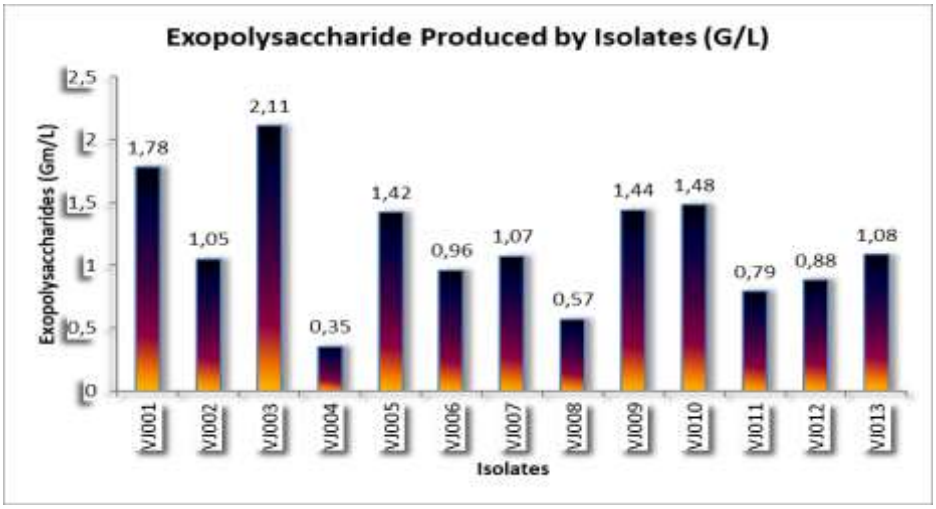

Fig.1 Screening of isolates for exopolysaccharide production

Out of 13 isolates, 3 potent EPS producers namely VJ001, VJ003 and VJ010 screened on the basis of dry weight of exopolysaccharide production as shown in Fig.1.

Identification of potent EPS-producing isolates:

Table. 1 Morphological characterization of soil isolates

Isolates	Colony Characteristics									
	Size mm	**Shape**	**Colour**	**Margin**	**Elevation**	**Opacity**	**Consistency**	**Motility**	**Cell Shape**	**Gram's Nature**
VJ001	03	Round	White	Entire	Elevated	Opaque	Butyrous	Nonmotile	Rod	Negative
VJ002	05	Oval	White	Entire	Concave	Entire	Butyrous	Nonmotile	Cocci	Positive
VJ003	04	Round	Skyblue	Entire	Elevated	Entire	Sticky	Motile	Rod	Negative
VJ004	04	Round	Brown	Entire	Flat	Entire	Butyrous	Motile	Rod	Negative
VJ005	03	Round	White	Entire	Elevated	Entire	Sticky	Nonmotile	Rod	Positive
VJ006	05	Round	Pale	Entire	Concave	Entire	Butyrous	Nonmotile	Cocci	Positive
VJ007	06	Irregular	Brown	Entire	Concave	Entire	Butyrous	Nonmotile	Rod	Positive
VJ008	03	Round	Cream	Entire	Elevated	Entire	Butyrous	Nonmotile	Cocci	Positive
VJ009	02	Round	White	Entire	Concave	Entire	Butyrous	Nonmotile	Rod	Positive
VJ010	04	Round	Skyblue	Entire	Elevated	Entire	Sticky	Motile	Rod	Negative
VJ011	05	Oval	Pale	Entire	Concave	Entire	Butyrous	Nonmotile	Rod	Positive
VJ012	06	Irregular	Pale	Entire	Flat	Entire	Butyrous	Nonmotile	Cocci	Positive
VJ013	01	Round	White	Entire	Elevated	Entire	Sticky	Motile	Rod	Negative

Table. 2 Biochemical characterization and tentative identification of the isolates

Isolates	Biochemical Characteristics											
	Indole	**MR**	**VP**	**Citrate**	**Catalse**	**Oxidase**	**Nitrate reductase**	**Urease**	**H_2S Production**	**Pigment**	**Tentative Identification**	**Polysaccharide Produced**
VJ001	+	+	-	-	+	-	+	+	+	-	*Escherichia coli*	1.78 Gm/L
VJ002	-	+	-	+	+	-	+	+	+	+	*Staphylococcus aureus*	1.05 Gm/L
VJ003	-	-	-	+	+	+	+	-	-	+	*Pseudomonas aerugonosa*	2.11 Gm/L
VJ004	-	-	+	+	+	-	+	+	-	-	*Klebsiella pneumonia*	1.33 Gm/L
VJ005	-	-	+	+	+	+	+	-	+	-	*Bacillus subtilis*	1.42 Gm/L
VJ006	-	-	+	-	+	-	+	+	+-	-	*Staphylococcus epidermidis*	0.96 Gm/L
VJ007	-	-	+	+	+	+	+	-	+	-	*Bacillus subtilis*	1.07 Gm/L

VJ008	-	+	-	+	+	-	+	+	+	+	*Staphylococcus aureus*	1.11 Gm/L
VJ009	-	-	+	+	+	+	+	-	+	-	*Bacillus subtilis*	1.44 Gm/L
VJ010	-	-	-	+	+	+	+	-	-	+	*Pseudomonas aerugonosa*	1.48 Gm/L
VJ011	-	-	+	+	+	+	+	-	+	-	*Bacillus subtilis*	0.79 Gm/L
VJ012	-	+	-	+	+	-	+	+	+	+	*Staphylococcus aureus*	0.88 Gm/L
VJ013	-	-	-	+	+	+	+	-	-	+	*Pseudomonas aerugonosa*	1.05 Gm/L

The colony morphology of the isolates is shown in Table 1. The isolates were subjected to screening for exopolysaccharide production and these isolates were tentatively identified on the basis of morphological and biochemical tests mentioned in Table 2.

The most potent isolate VJ003 was subjected to molecular identification. 16S rRNA gene of VJ003 was sequenced (Fig. 2) and blasted on the NCBI portal to check the most similar sequence available on the online database. The most related sequences were downloaded and comparatively studied in the MEGA-X program to construct the phylogeny tree using the neighbour-joining method (Fig. 3). According to the NCBI database and Phylogeny, isolate VJ003 showed maximum similarity with *Pseudomonas aeruginosa* MAR species and identified as *Pseudomonas aeruginosa VJ003*. The 16S rRNA gene sequence was submitted to the NCBI database and got GeneBank Accession no. OR196093.

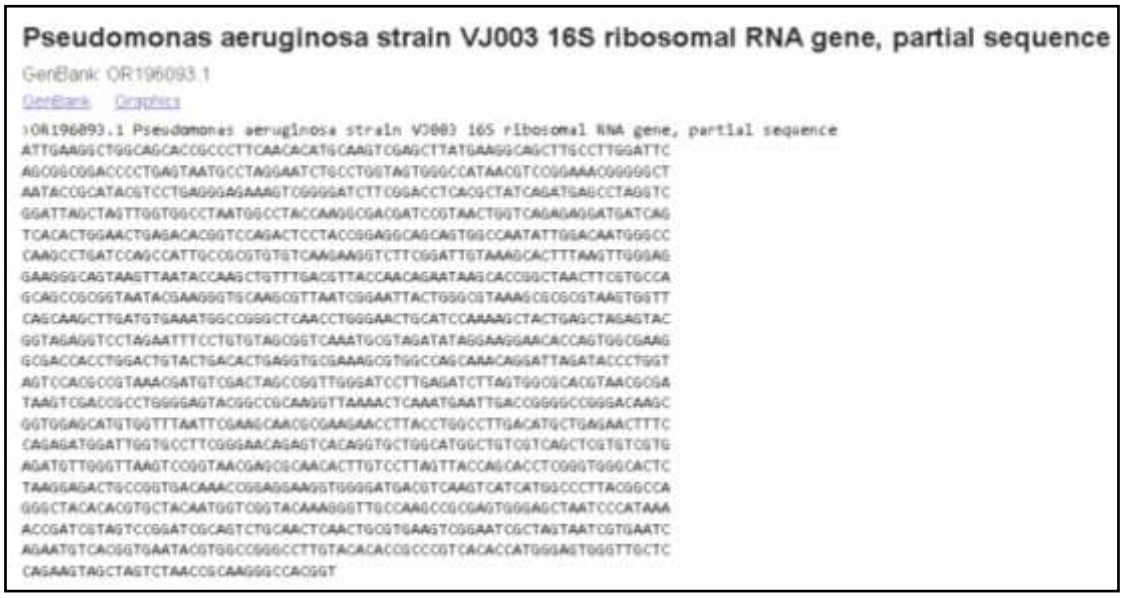

Fig. 2 16S rRNA gene sequence of VJ003 Isolate

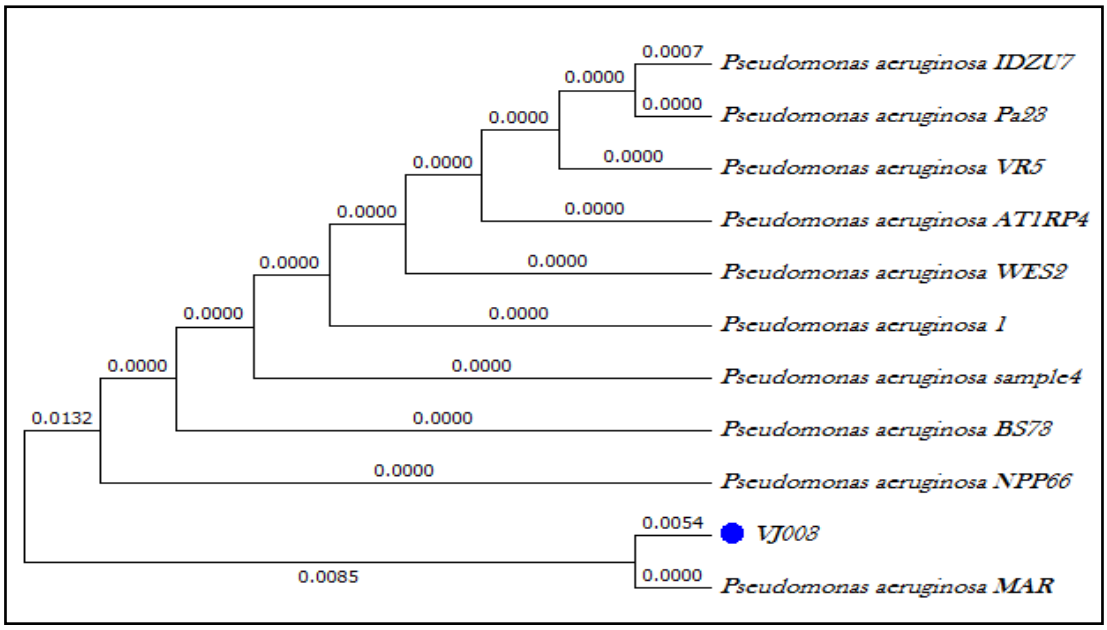

Fig. 3 Phylogeny of VJ003 Isolate

Biopolymers known as microbial exopolysaccharides are released by microorganisms as loosely bound slimes that are associated with the cell surface (Freitas et al., 2011). In contrast to plant polysaccharides, they can be synthesized under controlled fermentation conditions at higher temperatures. Previous research revealed that the amounts and makeup of microbial EPS depended on the species of isolated organism and its cultural conditions (Lynch et al., 2021). The biotechnological applications of these bacteria, including as antibacterial, antiviral, thickening, viscosifiers, suspenders, and gelling agents, can be realized with the successful isolation of bacteria that produce EPS (Abu and Jonathan, 1995). These strains are identified mostly by their phenotypic characteristics, which include colonies on agar medium that glitter, seem slimy, or like rops (Ruas-Madiedo & Reyes-Gavilan, 2005). Out of all the isolates from the two samples in this investigation, forty distinct bacteria that produce exopolysaccharides were chosen based on these traits. Exopolysaccharide synthesis from undiscovered microbial species with this property was the focus of Schmid's (Schmid, 2018) research. (Rühmann et al., 2015) used the development of mucoid and slimy colonies of bacteria cultivated on solid media as morphological criteria for screening and selection of EPS-generating bacteria. Similarly to this, Shukla and Dave's work (P. J. Shukla & Dave, 2018) used the development of mucoid colonies as a phenotypic criterion to screen 99 marine bacterial isolates.

The highest EPS-generating capacity of bacteria was cited by Liu and colleagues as a selection factor for isolates (C. Liu et al., 2010). In this investigation, the highest EPS producers from a battery of forty isolates were chosen using two methods: gravimetric measurement of EPS and determination of the total carbohydrate content present in precipitated EPS by phenol sulphuric acid test. Results from both methods showed that five isolates with the codes VJ001, VJ003, and VJ010 produced copious amounts of EPS that were above 1.46 g/l, confirming their status as the most effective EPS producers. Similar results were obtained by Shukla & Dave (2018), whose isolate PS-47 produced the highest EPS of all the isolates at 1.052 mg/ml. To obtain the efficient strain from the selected isolates that produce the highest amount of EPS, secondary screening is useful.

The chance to ascertain the relationship between precipitate cell dry weight and their carbohydrate contents is presented by the invention of a process for the selection of the majority prospective producers of exopolysaccharide from groups of selected bacterial isolates. When grown on basal and malt medium, respectively, Bacillus subtilis produced EPS containing 0.91 mg/100 ml and 0.43 mg/100 ml of total carbohydrate (Vijayabaskar et al., 2011). Accordingly, isolates J47 and J1 had the highest carbohydrate contents of 12.76 g/l and 10.95 g/l among other isolates when they were cultivated on an MRS medium (Ayobami et al., 2019). By using gravimetric measurements, Shukla and colleagues found that *Bacillus sp.* produced the most EPS among other bacteria, up to 12 g/l (V. Shukla et al.,

2015). Additionally, Mesorhizobium loti LMG 6125, a chosen rhizobal strain, produced the most exopolysaccharide at a rate of 3.10 g/l (Cristina et al., 2014).

Baisthakur et al. (2022) identified bacterial isolates using morphological and biochemical characteristics. Khan et al. (2019) also reported the morphological and biochemical identification of PHB-producing bacteria isolated from soil samples while Al-Dhabaan, 2019 identified bacterial isolate using the same technique. Chavan et al. (2023) also identified the *Pseudomonas species* using morphological and biochemical characteristics. Each bacteria have different metabolic activities that give specific phenotypic features to the cell. Such cell composition shows positive results in morphological and biochemical tests that can be used for the identification of the isolates up to genus level but for the species level identification, molecular approach is more trustable.

Reddy et al. (2019) reported a molecular approach for the identification of PHB biopolymer-producing bacteria isolated from soil samples and identified as *Acenetobacter nosocomialis*. Baisthakur et al. (2022) identified soil isolates using 16S rRNA gene sequencing and reported the importance of the molecular approach for microbial identification up to a special level. 16S rRNA gene sequence is the most conserved sequence during the evolution and that can give perfect relation with its closest ancestors on the basis of molecular data. Hence molecular identification is one of the most reliable methods for microbial identification.

4. Conclusion

Soil is go down of microorganisms and plenty of industrially important compounds producing microorganisms obtained from soil. Biopolymer is also very important biomolecule having significant application in bioremediation, agriculture, food and beverage industry, medical sector, cosmetics and personal care products. One of a broad and varied group of soil bacteria known for their capacity to create a range of biopolymers is the bacterium Pseudomonas. In the present research work, the Biopolymer producer was isolated and screened. The obtained VJ003 isolate was screened as the best biopolymer producer and identified as *Pseudomonas aeruginosa* VJ003 using morphological, biochemical and molecular techniques with 16S rRNA gene sequencing. Characterization and structure elucidation of the produced biopolymer will be carried out in further experimentation.

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