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Isolation and characterization of extracellular polysaccharides (EPS) producing bacteria and biofilm formation

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ABSTRACT
Extracellular polysaccharides (EPS) are essential microbial metabolites that are secreted by certain microbes, generally bacteria and fungi, which consist of mixture of macromolecules i.e., lipid, nucleic acid, protein, lipopolysaccharides, minerals and certain humic like compounds. This study primarily denotes about isolation, characterisation, application of EPS producing bacteria. Sampling of EPS carried out from latitude 22° 40' north and a longitude of 81° 45' east. Primary screening of bacterial isolates was carried out using string formation assay and total 5 isolates were selected for further production and characterisation assay. Bacterial EPS were evaluated by viscosity, density and dry weight. Dry weight of extracted EPS was found to be 0.120 gm, 0.091 gm, 0.086 gm, 0.097 gm and 0.117 gm for 5C, 2V, FKH30, FKH12 and FKH13 bacterial strains respectively. Viscosity is determined to the 1.1 mPa.s, 1.02 mPa.s, 1.03 mPa.s, 1.06 mPa.s and 1.04 mPa.s for 5C, 2V, FKH30, FKH12 and FKH13. As EPS provides the matrix for the biofilm formation, the capability of isolates to form biofilm were also determined by two methods which includes the formation on the glass slide and in the flask. Both methods give positive results. The efficacy of desired EPS producing strains was carried out with lab scale application of dye decolorization. Dye decolorization rate was found to be 98%, 23.07%, 43.63%, 26.98% and 20.42% for 5C, 2V, FKH30, FKH12 and FKH13 bacterial strains respectively. In addition, the lab scale application of degradation of agricultural waste was also checked which shows the positive result by partially degrading the waste. The most dominant bacterial strain was further carried out for its identification by morphological, cultural and molecular assays. Results from 16srRNA partial sequencing revealed isolate 5C as Bacillus subtilis 5C which can be used as promising bio-sorbent in the biodeterioration process.

Keywords*: Extracellular polysaccharide (EPS), Characterization of EPS, Viscosity, EPS precipitation, Biofilm formation, Dye decolorization .*

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INTRODUCTION

Extracellular polysaccharides (EPS) are essential microbial metabolite that are secreted by certain bacteria which consist of macromolecules i.e., lipid, nucleic acid proteins, lipopolysaccharides, minerals and certain humic like compounds. EPS are mostly made up of carbohydrate polymers with high molecular weight which is either secreted on the outer surface of the cell comprising capsular polysaccharide which forms capsule attached to cell surface or forms slime like layer (matrix) loosely attached to cell wall or either completely secreted into surrounding medium/environment.[1] EPS can be synthesized and secreted by bacteria, cyanobacteria [2], microalgae [3] , fungi [4]and yeast [5]. Certain microorganisms known to produce various types of EPS includes *Xanthomonas campestris* (Xanthan), *Streptococcus mutans* (Mutan). *Leuconostoc mesenteroides* (Dextran) *Bacillus subtilis* (Levan) *Pseudomonas elodea* (Gellan), *Alcaligenes faecais* (Curdlan) etc. [6] There are many variations in EPS composition and physicochemical characteristics The composition of EPS depends upon the various factors like culture medium composition, carbon and nitrogen sources, mineral salts, trace elements, type of strain, pH, temperature, oxygen concentration etc. [7,8] On the basis of composition: of EPS can be classified into Homopolysaccharides and

Heteropolysaccharides. The homopolysaccharides represents the group of polysaccharide having the long chain of similar type of monosaccharide subunits, whereas the heteropolysaccharides have the different monosaccharide units which are linked together at different position to form the oligounits forming long chain (branched or unbranched/chain).[8] The example of homopolysaccharide includes the dextran, levan, pullulan etc and of heteropolysaccharide includes the xanthan, hyaluronan, heteroglycans etc [9]. The chemical and physical properties of different kinds provides the many valuable application in the various industries such as food, pharmaceutical, cosmetic, and paint industry. Because of their physical and chemical properties of various types. EPS are used as flocculant, gelling agent, emulsifier and stabilizer, moisturizing agents, coating agent, thickeners.[10] The polysaccharides secreted by bacteria in their extracellular environment is integral in the matrix formation which is body constituent of biofilm, because it acts as adhesive and cohesive agent [11]. In the biofilm EPS provides the physical stability from harsh environments (i.e., High temperature and high pH and, host immunity. In addition to this, EPS also gives protection from drug and antimicrobial agents [12]. EPS acts as a reservoir of various biomolecules providing nutrients to organisms in the Biofilm [12].

MATERIALS AND METHOD

1 Sample collection and isolation

Soil sample were collected from the fields of Narmada District region, Gujarat (INDIA). 1g of soil was dissolved in 10 ml D/W. The sample was serially diluted till 10^{-6} and highest dilutions were then used for isolation of bacteria. Isolation was done on nutrient agar plate after the incubation of 24-48 hours at 37ºC.

2 Screening of eps producing bacteria

EPS producing bacteria gives a mucoid(gummy) type of colony on nutrient agar media which is main criteria for selection of EPS producing bacteria. Screening of EPS producing bacteria was done by string formation assay, gummy colonies of bacteria were extended by using nichrome wire loop which results in formation long filament and was re-streaked on another N-agar plate to maintain pure culture [13]

3 Eps production and Characterization

Screened bacteria was then inoculated in the nutrient broth and incubated in rotatory shaker for EPS production for 24-48hours at 37°C.

4 Dry Weight

Produced EPS was further proceeded for dry weigh estimation through alcohol/acetone precipitation method. In which, the media was centrifuged for about 10 min. at 5000 rpm. After which 10 ml of supernatant was taken and 20 ml of chilled acetone/alcohol was added and allowed to rest for 24 hours in the refrigerator which leads to formation of precipitates. At last weight the precipitates.[14]

5 Viscosity

Viscosity was one of the major criteria to check EPS production. Viscosity is the resistance of fluid to a change in shape or movement of neighbouring portions relative to one another. Viscosity measurement was performed by using Ostwald's Viscometer only after 3-4 days of inoculation [15].

6 Biofilm formation:

EPS provides the matrix (that is body constituents) for the biofilm formation. Therefore, the isolated EPS producing bacteria are further checked for the biofilm formation. The biofilm formation was determined by two methods. First includes the determination of biofilm in the flask containing nutrient broth, in which EPS producing bacterial culture was inoculated and was allowed for the incubation for about 3-4 days. Then by using the dye methylene blue, it was detected and optical density was checked using spectrophotometer. In the second method, biofilm was allowed to be formed on the clean glass slide. The glass slide was placed in slanting position in the petri plate containing inoculated nutrient medium and was allowed for the biofilm formation for about 3-4 days at 37°C.After incubation biofilm was detected using gentian violet.

7 Application

7.1 Dye degradation.

Due to great increase in urbanization a most common problem also increases which is release of dyes and untreated effluent in the natural water bodies. EPS produced by bacteria act as biosorbent hence help in dye removal [16]. EPS producing bacteria was inoculated in the nutrient broth containing Bromothymol blue and incubated at room temperature, allowed to degrade the dye. The amount of dye degradation was estimated by measuring the optical density at interval of every 24 hours.

7.2 Degradation of agricultural waste

The lab scale application of biodegradation of agricultural waste was done. The flask containing the 25ml of nutrient broth was inoculated with screened EPS producing bacteria and incubated for 24hours at 37°C.After incubation the small pieces of agricultural waste was added and was allowed for degradation,

was incubated in rotatory shaker at 250rpm at 37°C. The culture suspension was added on interval of 3-4 days.

8 Bacterial Identification

8.1 Cultural characteristics

Isolated bacteria were grown on Nutrient agar plates and observed for their colony characteristics.

8.2 Morphological characteristics

Gram's staining was performed on isolated bacteria and observed under microscope in oil immersion lens.

8.3 Biochemical Characteristics

Different biochemical tests were performed to identify the bacteria

8.4 Phylogenetic analysis of 16s rDNA sequencing.

The 16srRNA sequencing is considered to be the standard method to identify and for taxonomic classification of bacterial species. The 16s rRNA sequencing of isolate FKH13 was done at Gene Explore lab, Ahmedabad. After sequencing, obtained sequence was compared with the NCBI database using BlastN. Then alignment and construction of phylogenetic tree using maximum likelihood was done with the help of ClustalW and MEGA 11, respectively.

RESULT AND DISCUSSION

Isolation and Characterization:

Figure 1 shows the soil sample collection site. Total 12 bacterial strains were isolated from the soil sample collected from the field region of Narmada district, Gujarat (INDIA) on the nutrient agar plate.

Screening of EPS producing bacteria

Out of 12 bacteria isolated, 5 EPS producing bacteria screened on the basis of mucoid(gummy) colony characteristic and string formation. The screened bacteria were 5C, 2V, FKH30, FKH12, FKH13 which shows positive result. In **figure 2** we can observe the string formation which was formed because of viscous EPS production.

EPS Production and Characterization

After the incubation period the inoculated nutrient agar broth becomes viscous and shows sticky/ropy broth with foam formation.

Dry weight.

The EPS produced was characterized using its dry weight estimation. Dry weight shows the yield of EPS in the broth [17]. The produced EPS was precipitated using the alcohol/acetone precipitation method and dried at 50-55^{o}C in hot air oven overnight and weighed. **Figure 3** shows the results of dry weight estimation of EPS. From the results we can observe that the bacterial isolate 5C and FKH13 gives the greatest amount of EPS which is obtained 0.120g and 0.117g respectively, than the other isolates 2V, FKH12 and FKH30.

Viscosity

Viscosity is the key characteristic to identify EPS production in the broth. Viscosity is estimated using the Ostwald's viscometer. The results of viscosity of EPS produced are as shown in **table 1**. From the results we have observed that the viscosity of EPS produced by isolate 5C is 1.1mPa.s, which is much higher than the viscosity of EPS produced by the other isolates 2V,FKH30,FKH12 and FKH13 which is obtained 1.02, 1.03, 1.06 and 1.04mPa.s, respectively. **Figure 4** shows the graphic study of results of viscosity, from which we can easily observe the results by comparative study that the bacterial isolate gives the highest result.

Biofilm formation

The biofilm formation was done by two methods which includes the flask method and on the slide. The flask method was done for the quantification of biofilm formation capability of isolates. The optical density was measured after the addition of dye and application of acetone in the broth at 490nm. Table 2 shows the result of optical density measured at 490nm in the flask method. The results shows that the 5C gives the highest results than the 2V, FKH30, FKH12 and FKH13, which shows the higher capability of 5C to form denser biofilm.

In the second method the biofilm formation was done on the slide by placing the slide in the slanting position with partially submerged in to the inoculated broth in the petri plate. The detection of biofilm formed on the slide is done using the gentian violet dye as the application of dye will stain all the organisms (**Figure 5** shows the biofilm formed on slide and **figure 6** shows the detection of biofilm formed on slide using the gentian violet). All the isolates give the positive results for the biofilm formation. This method shows only ability of isolates to form biofilm.

Application

1)Dye decolorization

Table 3 shows the rate of dye decolorization obtained. **Figure 7** shows the comparative graphic study of dye decolorization rate, from which we can observe that 5C give the highest decolorization rate, 98%, by decolorizing the dye bromothymol blue.

2) Degradation of Agricultural waste

The lab scale application of degradation of agricultural waste was checked. As the 5C gives the maximum yield of EPS than the other isolates, it also shows the degradation potential. The degradation of agricultural waste was proceeded for the 21 days. By comparing the test flask with the control flask, it was observed that the agricultural waste was partially degraded after 21days.

Bacterial Identification

After the whole study, we observed that the isolate 5C is most promising EPS producing bacteria by giving the higher results in the estimation of viscosity, dry weight and biofilm formation capability. Therefore, we have further done the identification of 5C on the basis of its cultural and morphological characteristic, biochemical tests and 16s rRNA sequencing.

1) Cultural characteristics

Table 4 show the cultural characteristics of isolate 5C. Its cultural characteristics shows that it has smooth and mucoid colony which is the one of the main characteristics for the EPS producing colonies.

2) Morphological characteristics

Table 5 shows the result of gram staining. The Gram staining results shows that 5C is Gram positive bacteria, rod in shape.

3) Biochemical Identification

The various biochemical tests were performed, in which carbohydrate utilization, catalase, amylase, protease, lipase production and gelatine liquefaction test were positive. **Table 6** show the result of various biochemical tests that were performed.

4) Phylogenetic analysis of 16s rDNA sequencing

The bacteria were identified by 16s rRNA sequencing. Then analysed by BlastN, which shows the highest similarity with the *Bacillus subtilis.* Then phylogenetic tree has been constructed using MEGA11. Phylogenetic analysis also placed the strain among the *Bacillus subtilis* **(Figure 11)**.

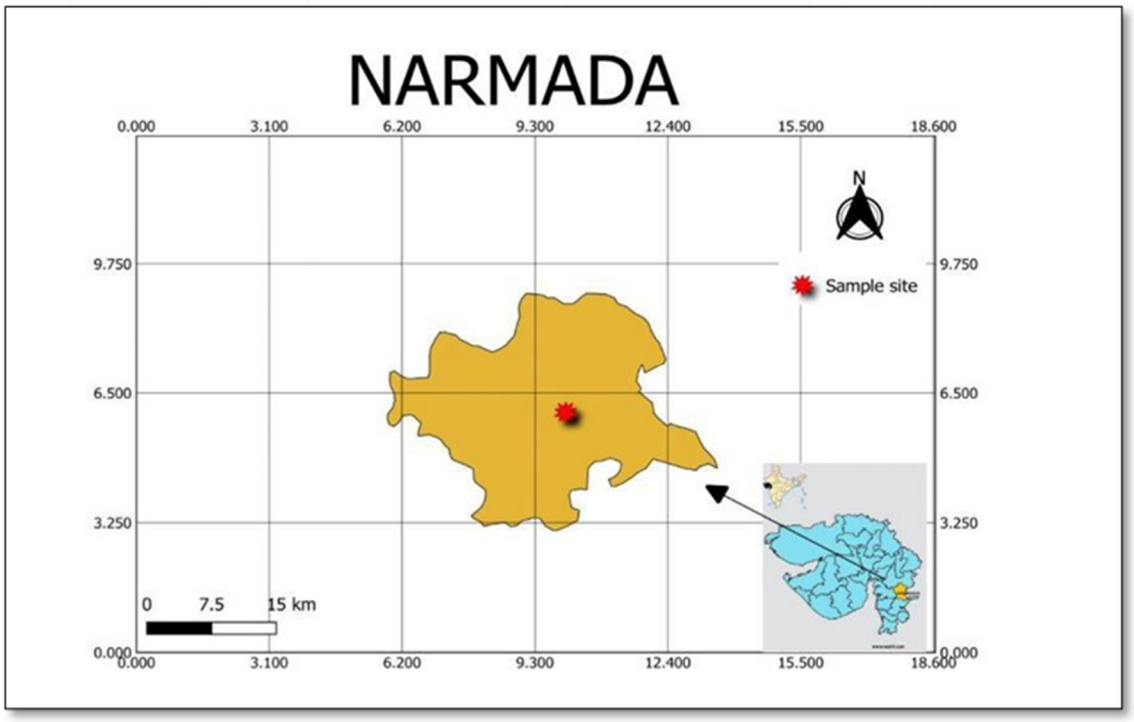
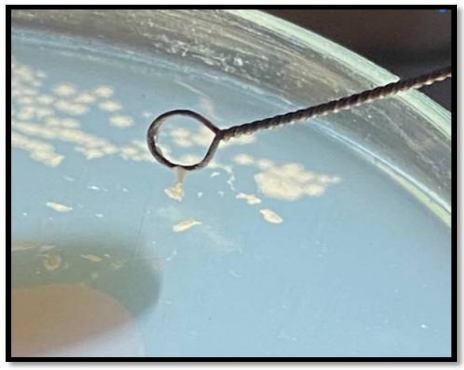
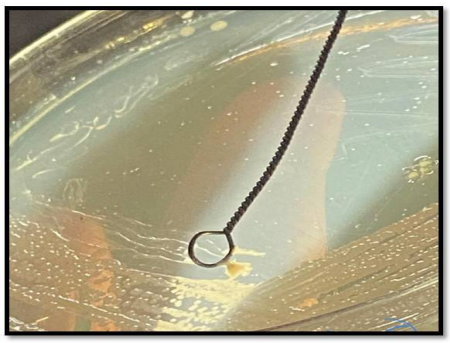


Figure 1 : Soil sample collection site (Narmada district)

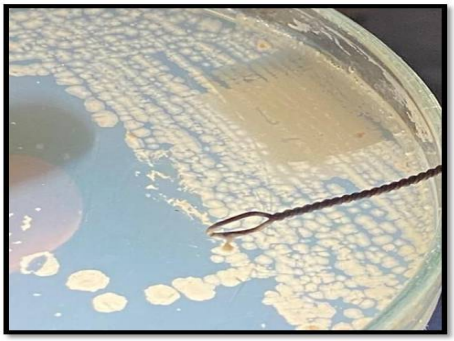
5C



2V



FKH30



FKH12

FKH12

Fig2: EPS Production

F

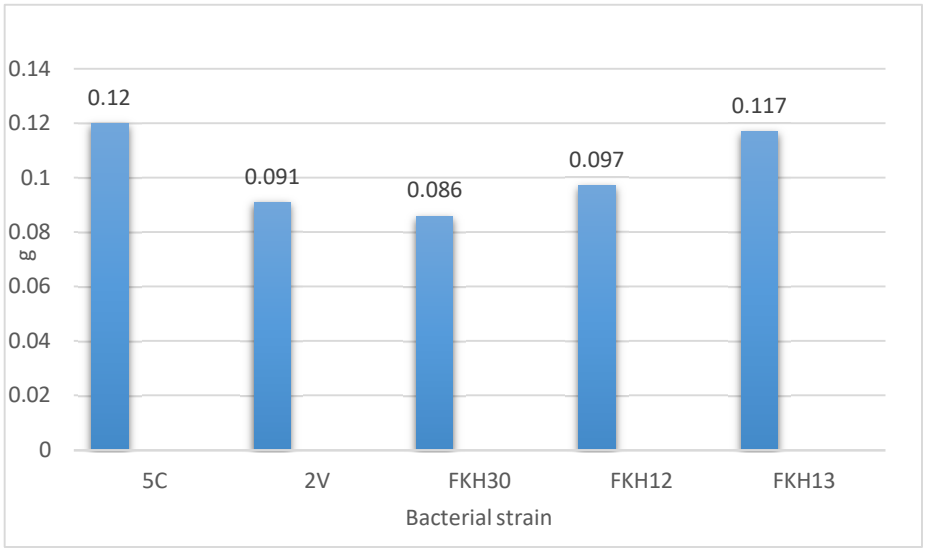


Figure 3: Graph showing the results of Dry weight of EPS precipitates

Table 1: Results of viscosity

Bacterial Name	Viscosity (mPs.s)
5C	1.1
2V	1.02
FKH30	1.03
FKH12	1.06
FKH13	1.04

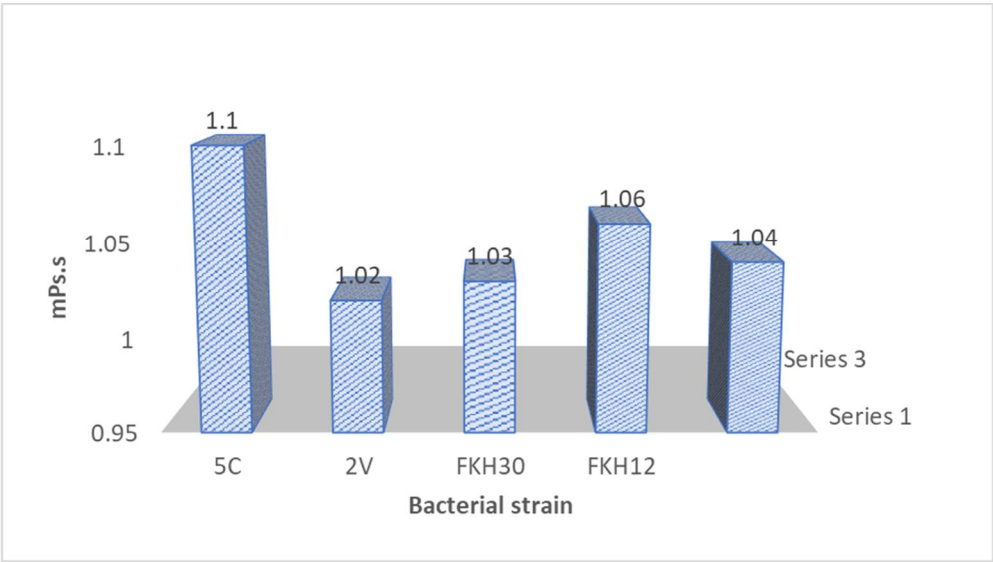


Figure 4: Graph showing the results of viscosity of EPS

Table 2: Result table of biofilm production in flask

Bacterial Name	Optical Density at 490 nm
Control	0.00
5C	0.44
2V	0.31
FKH30	0.34
FKH12	0.27
FKH13	0.40

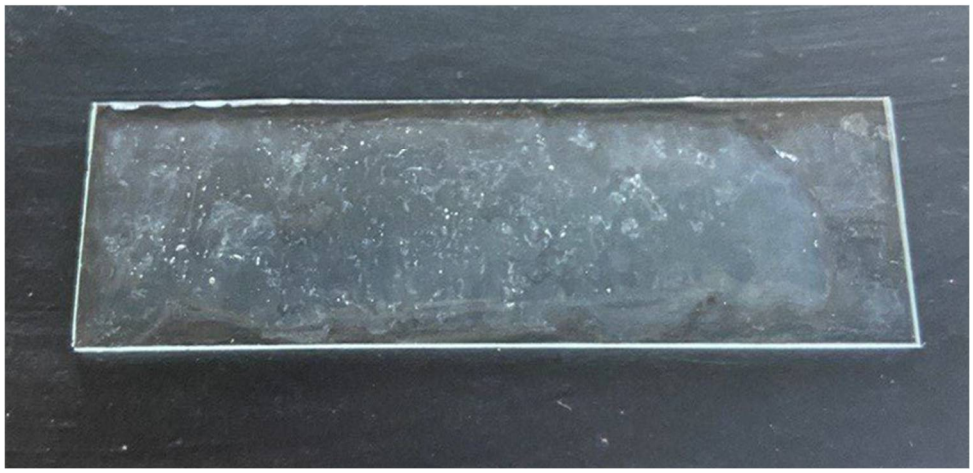


Figure 5 : Biofilm formation on the slide

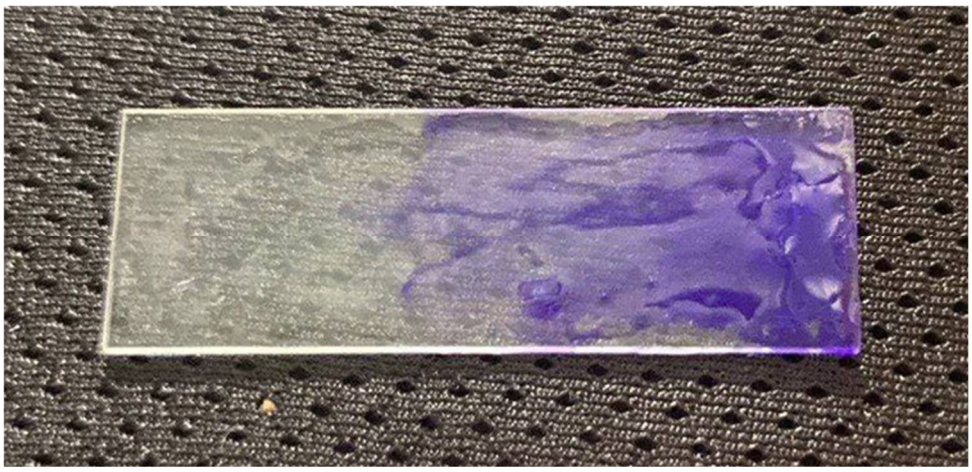


Figure 6 : Detection of biofilm on slide using dye gentian violet

Table 3: Results of dye decolorization

BACTERIAL NAME	DYE DECOLORIZATION (%)
Control	-
5C	98%
2V	23.07%
FKH30	43.63%
FKH12	26.98%
FKH13	20.42%

Figure 7 : Results showing the results of dye decolorization

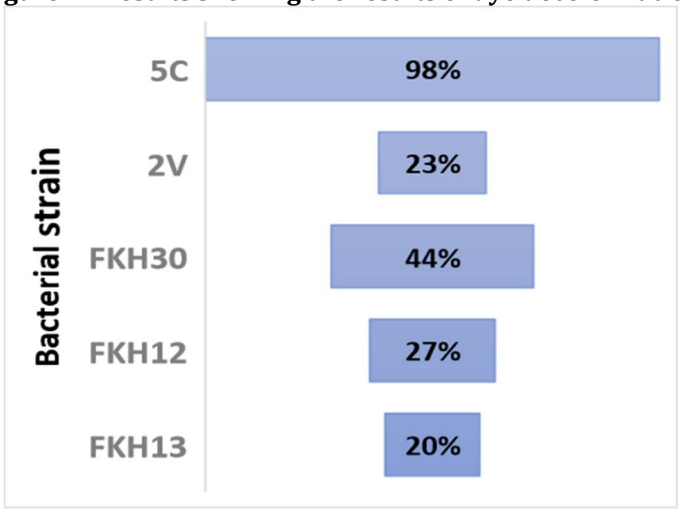


Figure 8 : 7th day result of biodegradation of agricultural waste

Figure 9 : 15th day result of biodegradation of agricultural waste

Figure 10 : 21st day result of biodegradation of agricultural waste

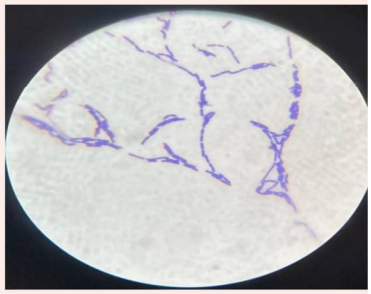
Size	Shape	Arrangement	Gram's Reaction	Microscopic Image
Big	Rod	Chain	Gram Positive	

Table 4 : Cultural characteristics of 5C

Table 5 : Morphological characteristics of 5C

Cellular morphology	Bacillus subtilis 5C	Cellular morphology	Bacillus subtilis 5C
Colony size	Medium	**Consistency**	Gummy
Colony shape	Round	**Opacity**	Opaque
Margin	Wavy	**Surface**	Smooth
Elevation	Slightly raised	**Colony colour**	Light pink

Table 6 : Biochemical test results of bacterial isolate 5C

Name of Test	Result	Name of Test	Result	Name of Test	Result
Indole production test	-ve	**Dehydrogenase test**	+ve	**Glucose utilization**	+ve (A, G)
Methyl red test	-ve	**Gelatinase**	+ve	**Fructose utilization**	+ve(A, G)
Voges-Proskauer test	-ve	**Lipase**	-ve	**Maltose utilization**	+ve(A, G)
Citrate utilization test	-ve	**Amylase**	+ve	**Sucrose utilization**	+ve(A, G)
H_2S production test	-ve	**Protease**	+ve	**Xylose utilization**	+ve(A, G)
Nitrate reduction test	-ve	**Cellulase**	+ve	**Mannitol utilization**	+ve(A, G)
Urea hydrolysis test	-ve	**Phenylalanine deaminase**	-ve	**Lactose utilization**	+ve(A, G)
Decarboxylase test	+ve	**Catalase test**	+ve		

Keys: A=Acid production, G=Gas production

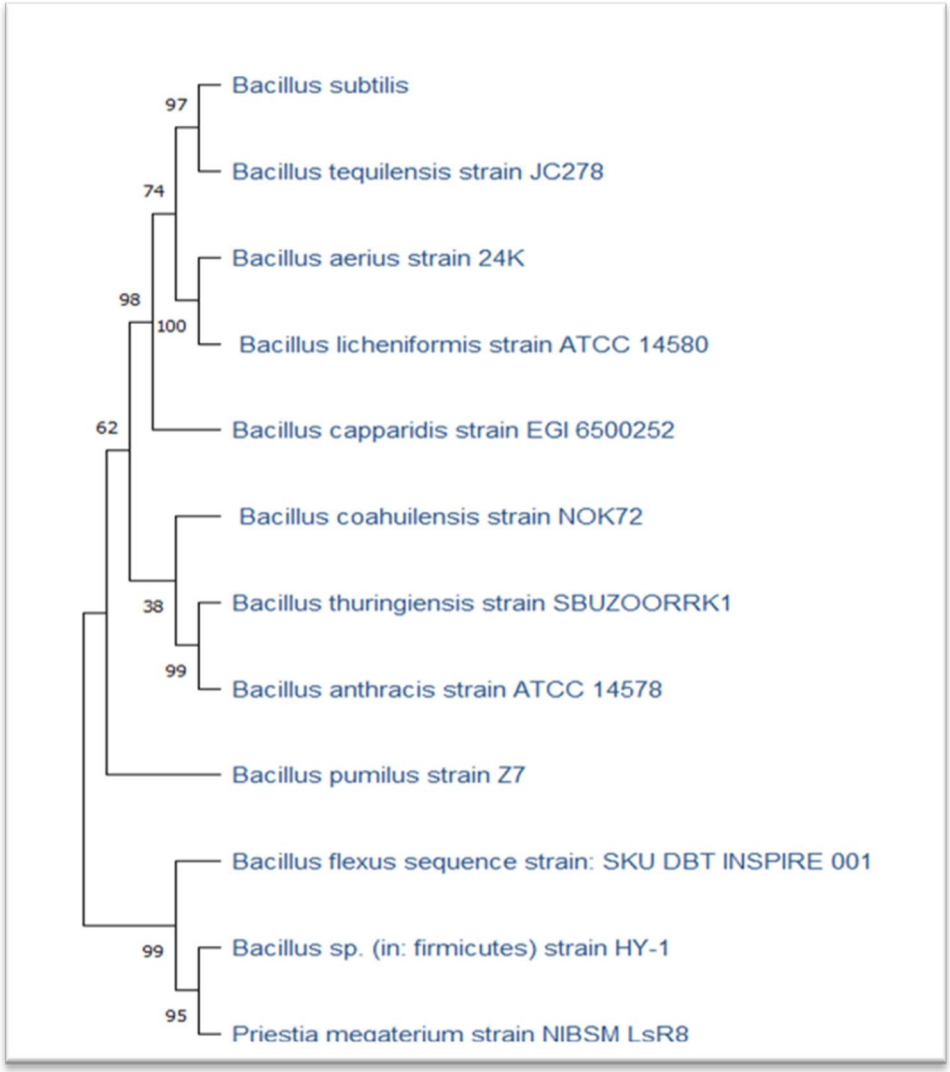


Figure 11 : Phylogenetic tree

CONCLUSION

Five EPS producing bacteria were isolated and screened from the soil sample in this study. These isolates were further characterized by its EPS's viscosity and dry weight estimation, with isolate 5C showing the best results. This study also involves the ability of isolates to form biofilm, which yields a positive result. furthermore, the lab scale application of dye decolorization and degradation of agricultural waste were checked which shows the positive result by decolorizing the dye and partially degrading the agricultural waste, respectively. Based on the results of the studies, isolate 5C which is identified as *Bacillus subtilis 5C* was determined to be a potential EPS producing bacteria due to its dye decolorizing and waste degrading capabilities with highly viscous EPS production.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE:

Not applicable

AVAILABILITY OF DATA AND MATERIALS:

WThe manuscript comprises all the applicable data in the text, tables and in the figures.

CONFLICT OF INTERESTS:

All the authors declare that there is no any conflict of interests for this publication.

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