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SCREENING AND MOLECULAR IDENTIFICATION OF POTENTIAL EXOPOLY-SACCHARIDES (EPSs) PRODUCING MARINE BACTERIA FROM THE BHAVNAGAR COAST, GUJARAT

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Marine environment, Marine bacteria, Exopolysaccharides (EPSs) production, Screening approaches and quantification, Molecular identification

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ABSTRACT: Marine environment is a complex habitat defined by a wide fluctuation in conditions such as pH, temperature, nutrient availability, osmotic pressure and salinity. Because of these various extreme environmental conditions, it harbors varieties of biodiversity such as bacteria, algae, fungi, coral reefs and sea weeds, as compared to terrestrial environment. For adaptation in these conditions marine organisms developed new adaptative strategies like synthesis of secondary metabolites. Exopolysaccharides (EPSs) is one of the important secondary metabolites produced by marine organisms. The present study focuses on the isolation of marine bacteria from different sites of Bhavnagar coast Gujarat, India and screening for EPSs producing marine bacteria by various approaches *viz*., using fluorescent Calcofluor white dye, Congo-red and trypan-blue agar plate assay method. Quantification of EPSs was done by using phenol-sulphuric acid method for total carbohydrate content. In this work marine bacterial isolate *Terribacillus saccharophilus* strain PS - 47 gave copious amount of EPSs 1052 µg/ml. Isolate will be further selected for use in various biotechnological applications.

INTRODUCTION: Life on earth mainly depends on the oceans and about 71% of the surface covered by marine environment which is a complex habitat defined by a wide range of physical, chemical, and geological variations. It exhibits high biodiversity as bacteria, algae, fungi, coral reefs and sea weeds as compared to terrestrial environment. Marine deep sea environment contains majority of biodiversity but due to difficulty in deep sea study, these environments are less exploited.

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Marine organisms not only maintain the pristine nature of the environment, but also serve as biological mediators through their involvement in the biogeochemical processes. Marine bacteria are remarkably adapted to diverse environmental conditions because they encounter a far greater variety of habitats and environmental conditions.

These environments are extreme conditions where organisms have developed new adaptative strategies like synthesis of secondary metabolites for survival in their surrounding microenvironment. Many of these compounds are produced by marine bacteria, without an apparent function in their growth and development. They are potent sources of various industrially important secondary metabolites including exopolysaccharides (EPSs), siderophores, biosurfactants, antibacterial, antiviral and anticancer compounds.

Extracellular polymeric substances are one of the important secondary metabolites which are produced mainly by marine bacteria for survival in extreme marine habitat. These compounds mainly contain 40 - 95% of polysaccharides, proteins, nucleic acid, lipid and humic acid substances. Most bacterial polysaccharides are synthesized intracellularly by substrate uptake, central metabolic pathways and polysaccharide production. These polysaccharides are exported to the extra-cellular environment as exopolysaccharides (EPSs) in the form of macromolecules. Bacterial EPSs mainly occur in two forms; as capsular polysaccharides (CPSs) and slime polysaccharides. CPSs are covalently or rigidly bound to the cell surface whereas slime polysaccharides are loosely attached.

The term EPSs was first used by Sutherland (1972) who described EPSs as high molecular weight carbohydrate polymers, which exhibits wide range of chemical structures. EPSs are hetero-polysaccharides, containing three or four different monosaccharides arranged in groups with repeating units. Monosaccharides most commonly found in marine EPSs are pentoses (D-aerabinose, D-ribose, D- xylose), hexoses (D-glucose, D-galactose, D-mannose, D-allose, L-rhamnose, L-fucose), amino sugars (D- glucosamine, D- galactosamine), and uronic acid (D- glucuronic acid, D- galacturonic acid). EPSs have average molecular weight ranging from 1×10^5 to 3×10^5 Da. Some EPSs are neutral molecules but majority are polyanioinic in nature because of the presence of uronic acids, inorganic residues such as phosphate, sulphate or pyruvic acids. The linkages that have been most commonly found are 1,4-β- or 1,3-β- in the backbones characterized by strong rigidity between monosaccharides and 1, 2-α- or 1, 6-α- linkages in the more flexible or loose bond to cell surface.

EPSs production requires about 70% energy, representing a significant carbon investment for marine bacteria. However, the usefulness concerning EPSs are significantly higher than the cost of its synthesis. The benefit of EPSs includes increased growth and survival under adverse conditions. Most marine bacteria produce maximum EPSs either in stationary or exponential phase, under nutrient (*e.g.* nitrogen, phosphate, sulphate, potassium *etc*.) depletion conditions or in response to other environmental stresses. These stresses are characterized by osmotic pressure, temperature, salinity, against possible predators as well as under high levels of heavy metals. EPSs have several functions including protection from desiccation, cryoprotection, stabilization of enzymes by buffering pH, salinity fluctuation, nutrient storage and in temperature changes. Because of their diverse structural and physico-chemical properties, they are employed in several industries, having a variety of biotechnological applications ranging from pharmaceutical industries, immunomodulatory, antiviral effects, bone regeneration, food-processing industries for their gelling and thickening properties. Some EPSs are employed as bioflocculant in waste water treatment and as biosurfactant in bioremediation processes [1 - 4].

The present study focuses on the isolation of marine bacteria from different sites of Bhavnagar coast Gujarat - India and screening the isolates for EPSs production using fast and reliable screening approaches and identification of most potent EPSs producer PS - 47. EPSs have also been quantified for the selection of the potential isolate that could be used for various biotechnological applications.

MATERIAL AND METHODS:

Study Site and Sample Collection: Water and sediment samples were collected from Alang, Koliyak, Ghogha and New-Port around Bhavnagar coast, Gujarat India (21°46'N - 72°11'E). The samples were collected at quarterly intervals during Sep - 2014 to June - 2015. Water samples were collected in sterile glass bottles and sediment samples were collected in polythene zip bags. The samples were transferred to the laboratory within 6 hours and stored at 4 °C until further analysis.

Isolation of Marine-Bacteria: Water and sediment samples were serially diluted up to 10^{-4} with sterile distilled water and subsequently. 0.1 ml from all dilutions were spread on different media *viz.* Basal salt supplement (BSS), Natural seawater medium (NSWM), Yeast malt glucose agar medium (YMG) and Zobell marine agar medium (ZMA). The plates were incubated at 30 °C for 24 hours [5 - 8]. YMG medium supported maximum number of isolates hence was used for further studies.

Screening for EPSs Producing Marine Bacteria: Morphologically distinct bacterial isolates obtained on above selective media. These isolates were further screened for the production EPSs using various approaches as below:

Calcofluor White Dye: Calcofluor white dye was used to detect EPSs production by the ability of the bacterial strains to developed fluorescent colonies as determined by emission of blue-green fluorescence under UV light. 0.02% solution was sterilized separately and 20 µg/L concentration was added in YMG medium after autoclave. After 24 hour, grown colonies were suspended in 10% KOH solution and observed under UV illuminator. Calcofluor White dye known to combine with polysaccharides and gives intense blue-green fluorescence under UV illuminator [9, 10].

Congo-Red and Trypan-Blue Agar Plate Assay Method: Screening for EPSs production was further carried out by using other dyes *viz*., Congo-red and trypan-blue agar plate assays. Isolates were cultivated on YMG at 30 ºC for 24 h having Congo red and trypan blue (with 250 µg/L dye concentration). Dye solution was sterilized separately and added to the medium after autoclaving. These dyes are more sensitive than calcofluor white dye as Calcofluor white dye binds only with externally secreted EPSs in the medium whereas Congo-red and trypan-blue dye increases the mucoidness of the colonies [11 - 15].

Quantification of EPSs Production: The isolates obtained as above were quantified for EPSs production using methods as mentioned below:

Precipitation: 50 ml of YMG medium was dispensed in 250 ml Erlenmeyer flask and sterilized at 121 ºC for 15 minutes. After sterilization, the medium was inoculated with 1 ml of inoculum (OD-1.0 at A600 SHIMADZU UV-Spectro-photometer) and incubated on an environmental shaker (Thermo-Scientific MaxQ) at 30 ºC for 24 hours. After incubation, the broth culture was inactivated at 100 ºC for 20 min. The cells were harvested by centrifugation at 10,000 rpm for 20 min and the supernatant was subjected for deproteination using Sevag method. The polysaccharides were mixed with Sevag reagent, 5:1 (v: v) $CHCl_3$: n-BuOH and stirred for 30 min. Then, the mixture was centrifuged and the upper polysaccharide solution was collected. The polysaccharide solution was further deproteinized with Sevag reagent for 5 times until the absence of white layer between polysaccharide solutions, ensuring that the polysaccharide was free from proteins. After removal of the Sevag reagent, the resulting polysaccharide solution was precipitated by adding double volume of chilled ethanol and the final mixture was kept at 4 °C overnight. After incubation, the precipitates were collected by centrifugation at 10,000 rpm for 20 min and the precipitated EPSs were quantified by phenol-sulfuric acid assay after drying for three days at 80°C [2, 16 - 18].

Carbohydrate Screening Assay: The phenol-sulfuric-acid-method still represents the common procedure for the fast determination of total carbohydrate content of bacterial and plant polysaccharides. This method was first described by Dubois *et al.,* 1956 [19]. Orsod *et al*., 2012 [20] and Seedevi *et al*., 2013 [21]. Who also support these methodology for quantification of total carbohydrate content. Extracted dry EPSs were dissolved in distilled water and measured for total carbohydrate content by phenol-sulfuric acid assay using glucose as standard. Aliquots of dissolved EPSs, 1 ml of 5% (w/v) phenol and 5 ml of H_2SO_4 were taken in test-tubes. The mixture was incubated at 30 ºC for 20min and EPSs were quantified by its absorbance at 490 nm [22].

Identification of Potential EPSs Producing Isolate PS - 47: The most potent EPSs producing bacterial isolate PS - 47 was isolated from Bhavnagar coast, Gujarat-India and it identified based on their morphological and molecular characterization 16S rDNA analysis sequencing. Phylogenetic analyses of the aligned sequences were performed with the help of program MEGA.

RESULTS AND DISCUSSION: Present study aimed at Exopolysaccharides (EPSs) producing marine bacteria isolated from Bhavnagar coast, Gujarat, India. The EPSs producing bacterial isolates were essentially selected basis on the development of mucoidness of the colonies. This characteristic, as stated by Rühmann *et al*., (2015) [3] and Fang and Catchmark (2015) [23], who studied the production of EPSs, in unexplored diversity of

microbial exopolysaccharides. Evans (2015) [24] and Patil and Shirsath (2015) [25] have selected EPSs producing strains on the basis of the mucoidness of bacterial colonies cultured on solid medium. Screening of EPSs production using "mucoid" and "slimy" morphology has also been carried out by Subair (2015) [26]. Four different sites of Bhavnagar coast Gujarat *viz,* Alang, Ghogha, Koliyak and New-Port were selected for the present study. **Fig. 1B** indicated the count of morphologically distinct 99 marine bacterial isolates obtained from various sites of Bhavnagar Coast. The results indicated that maximum 35 EPSs producing isolates were obtained from New-Port, 26 isolates from Alang, 21 from ghogha and least *i.e.* found 17 from Koliyak. **Fig. 2** represents the count of morphologically distinct bacterial isolates obtained during various seasonal variations Sep - 2014 to Jun - 2015. The results indicated that maximum EPSs producing isolates were obtained in Mar-2015, *i.e.* 29 from sea-water samples and 12 from sediment samples, followed by Dec - 2014, 17 were from sea-water samples and 17 from sediment and least EPSs producers were found in Jun-2015 and September - 2014.

As shown in **Fig. 2** sea water samples showed more number of isolates (60) then sediment samples (39), Thus, The overall results indicated that sea-water harbored more bacterial diversity than sediment samples. Highest bacterial count was found in sea water than sediments samples in the season of March - 2015. For the isolation of EPSs producing marine bacteria various media *viz.*, BSS, NSW, YMG and ZMA were used and 99 morphologically distinct isolates were designated as PS 1 to PS 99. The isolates were selected on the basis of their slimy and mucoid appearance on the above media. Maximum numbers of isolates 49 were obtained on YMG medium followed by 31 on ZMA medium, 11 on NSW medium and least *i.e.,* 8 were on BSS medium. Thus, maximum numbers of marine isolates were obtained on YMG medium as shown in **Fig. 1A**.

Batteries of 99 different morphologically distinct isolates were obtained on various media as shown in **Fig. 1A**. YMG is the most suitable media for isolation of EPSs producing marine bacteria. Merma *et al*., (2017) [27], have examined the production of EPSs producing isolates *Rhodococcus opacus* on YMG media, however, work on *Grifola frondosa* on YMG medium display highest polysaccharide production which was done by Latha and Baskar (2014) [28] and Yeon-Ran Kim. (2003) [29], Sung-Hwan Ko *et al*., (2000) [30] also used YMG as a suitable medium for EPSs production. This trend was followed by the present finding which suggested maximum EPSs production on YMG medium most suitable medium as per Mehta *et al*., (2014) [31]. NSW and BSS media were not well reported for in as EPSs production media but as supporting reference of Iwabuchi N (2002) [5], used NSW medium and S. Bragadeeswaran *et al.,* (2011) [32], used BSS as EPSs producing medium for their research. On the basis of results found as shown in **Fig. 1B** New-Port exhibits as maximum numbers of EPSs producing bacterial isolates site because as it being hyper saline environment with extreme salinity and pH As stated by Kumar *et al*., (2007) [33] which support this result that there is no single set of culture conditions that guarantees for high EPSs production, as bacteria differ in the critical factors: carbon and nitrogen source utilization, mineral requirements, temperature and optimal pH, salinity for their growth and EPSs production.

Alang is ship breaking yard, previously reported for the ship-breaking activities and making this site as heavy polluted site. Second highest EPSs producing bacterial count were found in alang, being polluted and coastal marine site as supported by Iwabuchi N *et al*., (2002) [5] examined on extracellular polysaccharides produced by marine *R. rhodochrous* S-2 (S-2 EPS), the rough strains of rhodococci acquired resistance to the hydrocarbons. EPSs production is affected mainly by the seasonal variations, stress conditions in which bacteria harbor and nutrient stress conditions.

In this present study high EPSs producing diversity was found in March - 2015 in sea water samples which is a summer season as shown in **Fig. 2**. In December - 2014 also found noticeable isolation of EPSs producing isolates followed by September-2014 and least were found in July - 2015. By Overall study from sea-water samples obtained more distinct Isolates than sediment. These results are supported by Passarelli *et al*., (2015) [34] who stated both bound and colloidal carbohydrates displayed seasonal variations in their

monosaccharide composition. Glucose was mainly responsible for seasonal differences; it accumulated in summer and was recorded in lower proportions in other seasons.

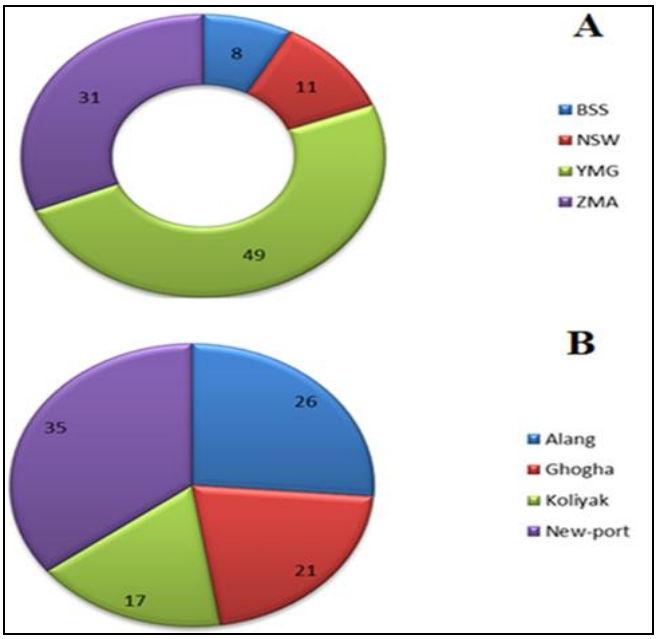

FIG. 1A AND 1B: A - MORPHOLOGICAL DISTINCT MARINE BACTERIA OBTAINED ON VARIOUS MEDIA; B - MORPHOLOGICAL DISTINCT MARINE BACTERIA OBTAINED FROM VARIOUS SITES OF BHAVNAGAR COAST GUJARAT, INDIA

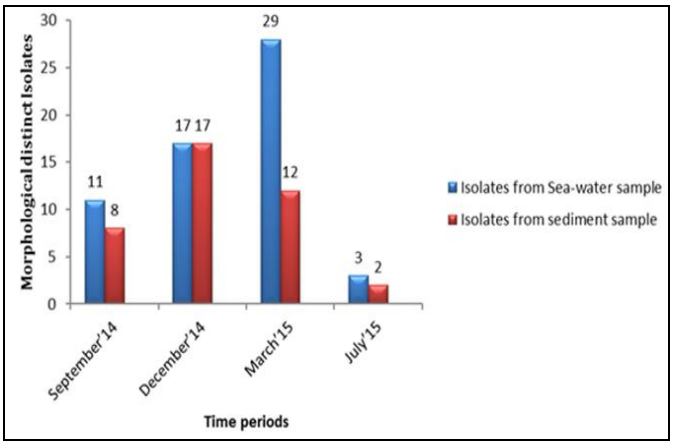

FIG. 2: MORPHOLOGICAL DISTINCT MARINE BACTERIA OBTAINED DURING SEP - 2014 TO JUL - 2015 FROM VARIOUS SAMPLES

EPSs producing isolates were primarily screened by Calcofluor white dye method in which dye directly binds with EPSs which is externally secreted in the medium. Amongst 99 isolates 57 gave positive results by green-blue fluorescence under UV light. These 57 isolates were further screened by more accurate and responsive dyes *viz*., congo red and trypan blue dye. Out of 57 isolates 35 showed positive results on above three dyes **Fig. 3** and **4**. Total 35 EPSs producing isolates were subjected for secondary screening in which precipitation of EPSs were done using ethanol. Most EPSs are decidedly soluble in aqueous solutions, whereas the solubility can be acutely dropped off by using water miscible solvents. For the precipitation of EPSs, a solvent like ethanol was used in this work. The efficiency of precipitation of polymers depends on their chemical structure, molecular weight, the final concentration of polymer and alcohol used for the precipitation.

Quantification of total carbohydrate content presence in precipitated EPSs was done by phenol-sulphuric acid assay. This method detects virtually all classes of carbohydrates. High EPSs production was achieved by isolate PS-47 *i.e.* 1.052 mg/ml **Table 1**.

TABLE 1: QUANTIFICATION OF EPSs BY 35 MARINE BACTERIA

S. no.	Isolates	Quantification (µg/ml)
1	PS 1	877
2	PS 3	714
3	PS 4	266
4	PS 6	406
5	PS 7	406
6	PS 8	112
7	PS 11	644
8	PS 12	755
9	PS 14	294
10	PS 15	77
11	PS 16	56
12	PS 17	742
13	PS 20	896
14	PS 22	126
15	PS 26	224
16	PS 27	555
17	PS 28	663
18	PS 31	238
19	PS 33	91
20	PS 34	270
21	PS 37	728
22	PS 38	602
23	PS 40	881
24	PS 41	595
25	PS 42	161
26	PS 43	960
27	PS 45	49
28	PS 47	1052
29	PS 48	238
30	PS 49	602
31	PS 50	951
32	PS 52	126
33	PS 53	168
34	PS 56	910
35	PS 57	633

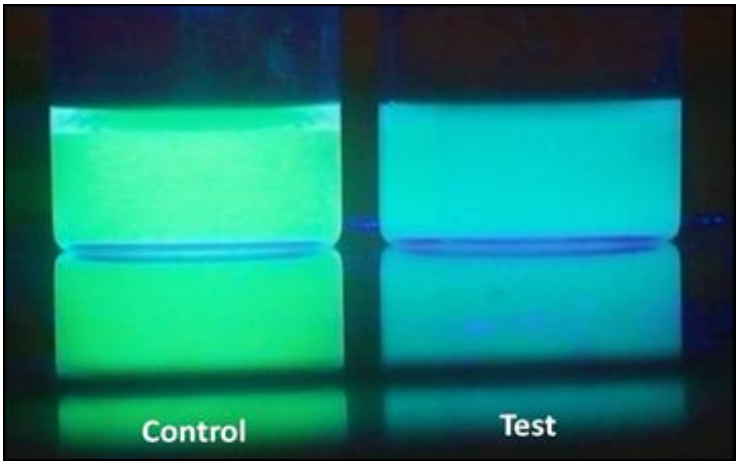

FIG. 3: EXOPOLYSACHHARIDES PRODUCTION BY *T. SACCHAROPHILUS* STRAIN PS - 47 USING CALCO-FLOUR DYE

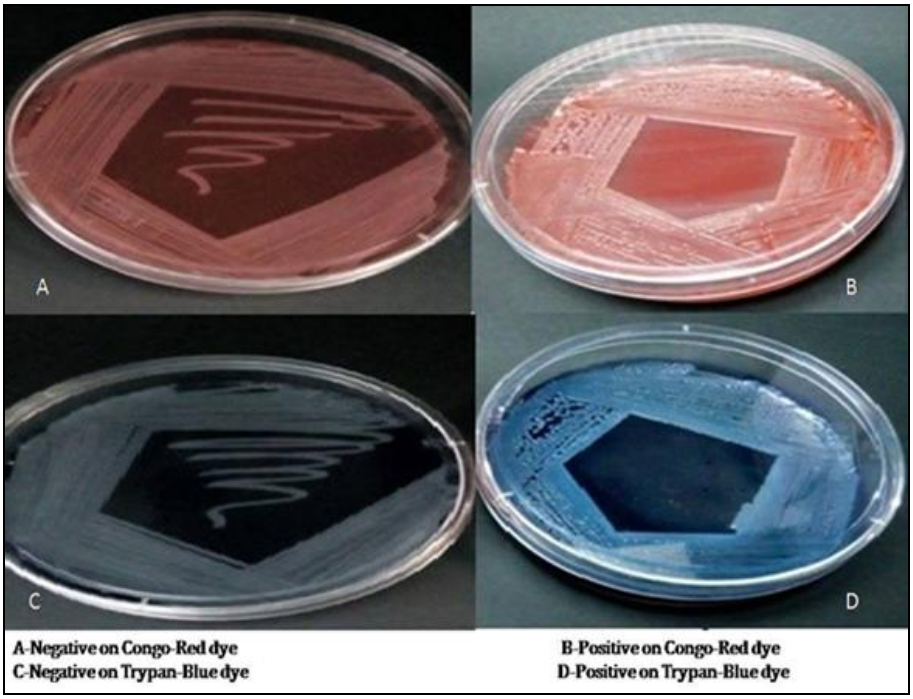

FIG. 4: EXOPOLYSACHHARIDES PRODUCTION BY USING CONGO RED AND TRYPAN BLUE DYES BY *T. SACCHAROPHILUS* STRAIN PS 47

EPSs producing isolates were screened by Calcofluor white dye which is fast EPSs detecting method. As reported by Kaufusi *et al*., (2004) [35], who studied the detection of EPSs using this dye, change fluorescence negative colonies can easily be identified. Further confirmatory screening assays were done by other dyes *viz*., Congo- red and Trypan-blue, is known to interact with β-(1-3) - and β-(1-4) - glucans and was successfully used to identify biofilm formation by EPSs from different Staphylococci strains. Some alternative method of screening EPSs formation by Congo-Red Dye, Castellane *et al*., (2014) [36] and Liu *et al*., (2013) [37] who studied the EPSs production by these dye.

Quantification was done by measurement of total carbohydrate content by phenol-sulphuric acid assay. This result supported by Vijaybhaskar P *et al*., (2011) [2] who obtained around 0.23 mg/ml and Saravanan P *et al*., (2007) [38] obtained 1.8 mg/ml of EPSs.

In present study from a battery of 99 isolates screening were undertaken by using various approaches for obtained EPSs producing bacterial isolates. Results revealed that PS - 47 was highest EPSs producer followed by followed by PS 46 *i.e.* 0.960 mg/ml and PS 50 *i.e.* 0.951 mg/ml produced copious amounts of EPS in culture medium.

EPSs producing marine strain PS - 47 was selected on the basis of their purity of bacterium, uniform colony morphology and observed to be the most potent EPSs producer from the study site Bhavnagar coast and hence further studied for molecular identification. Marine bacterium strain PS-47 was found to be a gram-positive and rod-shaped and formed smooth, white gummy mucoid colony after 24 h of incubation at 30 ºC on YMG agar medium plates. Identification using 16S rDNA gene sequence analysis (The sequence has been deposited in NCBI GEN Bank with accession No. KX788920). Approximately 1.1 kb 16S rDNA partial fragments were blast against database. Blast result showed 99% similar to *Terribacillus saccharophilus* (T); 002 - 048; AB243845 with 1415 unique common oligomers. Phylogenetic analysis neighbor joining revealed that marine bacterium strain PS - 47 closely related to *Terribacillus saccharophilus* (T); 002 - 048; AB243845 and *Terribacillus saccharophilus*; RB589; AB243847 **Fig. 5**.

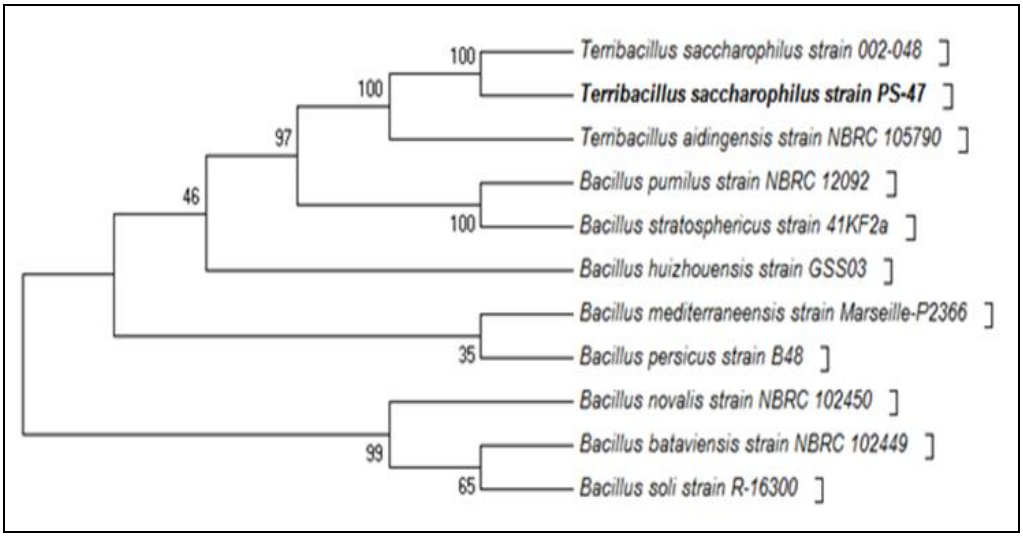

FIG. 5: PHYLOGENETIC TREE FOR THE *T. SACCHAROPHILUS* STRAIN PS 47

Characterization of various EPSs were separated on the basis of their functions *viz*., adsorptive, informative, surface-active, structural, nutritive, active, informative and redox- active. Four of functional molecules were present in *Bacillus* Sp. (Flemming 2007) [39]. An active type of EPSs is dominantly produced by *Bacillus* Sp. (Marvasi 2010) [40]. EPSs from *Bacillus amyloliquefaciens, Bacillus thuringiensis, Bacillus licheniformis, Bacillus subtilis* and *Paenibacillus favisporus* were mainly researched for plant growth promoting compounds, anti oxidant activities and effect of different stresses (Vardharajula 2011) [41].

In *Bacillus subtilis* have been demonstrated motility-to-biofilm transition. In *Bacillus subtillus* the reason of motility that a single protein EpsE present in EPS operon which is mainly work as flegger clutch. (Guttenplan 2010) [42]. *Bacillus subtilis* were also get attention for building of biofilm on substratum (Vlamakis 2013, Beauregard 2013) [43, 44], EPSs of *Bacillus licheniformis* were gave markable immunological activity (Liu 2010) [45], and recently various spp of Paenibacillus were identified for its high yield of EPSs and *Terribacillus saccharophilus* (T); 002-048 was isolated from field soil of Japan (An SY 2007) [46].

CONCLUSION: EPSs producing organisms are widely distributed in marine environment. EPSs function as an anchor between organisms and its environment for survival. The present study is a step towards understanding of isolation EPSs producing marine bacteria in the area around Bhavnagar coast, Gujarat, India and screening of these bacteria for EPSs production by various screening approaches and its quantification. Novel gram positive *T. saccharophilus* strain PS - 47 proved to be potential EPSs producer. Further study on optimization, chemistry and structure of bacterial EPSs will be carried out because most of marine bacteria and their metabolites are yet unexplored. This novel natural biological product is further utilized used in several industries like pharmaceutical, food industry and in environmental protection of natural habitats.

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