MEDIA OPTIMIZATION OF EXOPOLYSACCHARIDE PRODUCING BACTERIUM FROM MARINE SEDIMENT

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ABSTRACT: Exopolysacharides (EPS) are polymeric substances of microorganisms of high molecular weight and long chain composed of sugar residues secreted by them into the surrounding environment. The aim of this study exopolysaccharides producing strain was isolated from marine sediment in Parangipettai, South east coast of India. The strain shows mucoid growth on Zobell marine agar plate. It was identified as Pseudomonas putida by cultural, biochemical and 16S rRNA gene sequencing. Maximum EPS production was observed at pH 9 and the temperature is 30°C. The maximum EPS production was observed in Adenitol as a carbon source and nitrogen source is Ammonium bicarbonate. Lysine as a soul source of aminoacid leads to the maximum EPS production. Among the tested metal ions, the maximum amount of EPS production was observed in Ferric chloride.

Keywords: Exopolysaccharide, marine sediment, Pseudomonas putida, Optimization and ferric chloride.

INTRODUCTION:

Now a day, the marine environment is used for the production of exopolysaccharides. Marine microorganisms (bacteria, algae &viruses) including their biodiversity, ecology and biogeochemistry. Marine bacteria and marine algae are traditionally good substitutes of gums in microbial exopolysaccharides. The physical and chemical characteristics of variations are climatic, cultivation, production and population conditions. Marine environment are extremely changes in pH, pressure, temperature, salinity, nutrients levels, etc. The skeletons of marine organisms are responsible for biogenic sediments are very smaller in size. In the fact, marine microbes are the canary in the coal mine for the marine environment. Marine bacteria are used to isolate the bacterial strains, and its production and extraction of biofilm and Exopolysaccharides (EPS). EPS is used in medicinal and pharmaceutical industry. Extracellular biopolymers are classifying the polysaccharides, inorganic poly anhydrides, polyesters, polyamides and microcellular polysaccharides are also termed as the Extracellular polymer substances. EPS yields are produce different bacterial strains depends on the substrate composition and environmental conditions (Rabha et al., 2012).

Exopolysaccharides are high molecular weight of carbohydrate polymers that make up of a substantial component of the extracellular polymers surrounding most microbial cells in the marine environment. Exopolysaccharides are generally consists of mono saccharides and some non-carbohydrate substituents such as acetate, pyruvate, succinate and phosphate. Cell wall is made up of cellulose is one of the most studied exopolysaccharides in biofilm formation. Exopolysaccharides categorization are complex and in some instances characterization of factors are distinct in the groups and this is seen in the homo polysaccharides have been further

classified into groups of α-D-glycan, β-D-glycan, fructose and poly galantine; this groups are based on the linkage bonds and nature of monomeric units. The productions by bacteria have been shown to a several multiple functions including the promotion of the initial attachment of cells to solid surfaces. The major components of EPS are carbohydrate and proteins. Bacterial exopolysaccharides are known to identify the quantification of carbohydrate estimation. Naturally EPS are wide spread in the large variety of the sea, ocean and sediment soil particles. In the harse environment are desiccation bacterial cells are protects and the production of Exopolysaccharides (Passow 2000).

MATERIALS AND METHODS:

Source of the sample:

The pure marine sediment soil was taken from the Chidambaram Sea. The soil was serially diluted and poured it into the Zobell marine agar medium. The appearance of mucous and colloidal colonies is spread on the Zobell marine agar petriplate.

Conformation of the marine sample:

Gram staining:

Gram staining was first developed by Christian gram in 1884 to separate different bacteria into gran positive or gram negative. The clean class slide was taken and the marine bacterial culture was smeared on the center of the slides. Air dried and heat fixed at 1 minutes. Gram's reagents were added, first crystal violet were flooded on the slide and are kept for 1 minute. Wash dis.H_2O then gram's iodine was added it was washed and decolorized with ethyl alcohol. The ethyl alcohol was washed out and the counter stain Safranin was added. Examine under microscope to determine the type of bacteria present.

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Biochemical characterization:

Isolated Marine bacteria are conformed by the biochemical characterization method.

Indole test:

Tryptophan is an essential amino acid that can undergo oxidation by the enzymatic activities of some bacteria. It is acted upon by the enzyme tryptophanase. Ability to hydrolyze tryptophan with the production of Indole is not a characteristic feature of all microorganisms and therefore serves as a biochemical marker. The marine bacterial culture was inoculated on the sterilized tryptone water, incubate overnight at 24 hrs for 37°C, added few drops of kovaċ s reagent to deep tube cultures and agitate the culture were agitated gently.

Methyl red test:

The methyl red test is employed to detect the ability of microorganisms to oxidize glucose with the production and stabilization of higher concentrations of acid end products. The overnight culture marine bacteria were inoculated on test tubes in sterilized MRVP broth and incubate at 37°C for 24-48 hrs. Following incubation, 5-6 drops of methyl red solution was added. Colour change in medium was examined and recorded.

Voges-proskauer test:

The test is determined to the capacity of some microorganisms to ferment carbohydrates with the production of non-acidic or its reduction products. The marine bacterial culture was inoculated on sterilized MRVP broth; incubate 48 hrs. at 37° C. Then add the Baritt's reagent A and B. Observed the colour change with the positive or negative reaction.

Citrate utilization test:

Simmons citrate agar was prepared and sterilized then allowed to solidify the agar slants. The marine bacterial culture was streaked on the marine bacterial culture. Colour change was observed by positive test or negative test.

Urease test:

Urease is a hydrolytic enzyme that attacks the nitrogen and carbon bond in amide compounds such as urea and forms the alkaline end product ammonia. Urea agar slant were prepared and sterilized at 121˚C for 15 minutes under aseptic condition. Adapting streaking on the sterile agar slants incubated the test cultures and the tubes are incubated at 37˚C for 24 – 48 hours. After 24 hours incubation, the tubes were examined for the presence of deep pink colour. Colour change was observed by produce the enzyme increases their ability to hydrolyze urea.

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Physiological characterization and optimization method:

Effect of pH on bacterial growth:

On the production media growth of marine bacteria at different pH is measured by turbidity method. OD of overnight culture at various pH(2,3,4,5,6,7,8,9,10,11,12) is measured at 600 nm using calorimeter.

Effect of temperature on bacterial growth:

On the production media growth of bacteria at differential temperature is measured. Optical density of overnight incubated marine bacterial cultures at various temperature (0 °c, 10°c, 20°c, 30 °c, 40°c, 50°c, 60°c) is measured at 600 nm using calorimeter.

Effect of carbon sources on bacterial growth:

On the production media growth of bacteria at different carbon sources is measured. Optical density of overnight incubated marine bacterial cultures at different carbon sources at 1% concentration (dextrose, sucrose, maltose, adenitol, trehalose, raffinose, galactose, arabinose, lactose, and mannose) is measured at 600 nm using calorimeter.

Effect of nitrogen sources on bacterial growth:

On the production media growth of bacteria at different nitrogen sources is measured. Optical density of overnight incubated marine bacterial cultures at different nitrogen sources at 0.5% concentration (casein, tryptone, ammonium bicarbonate, ammonium chloride, ammonium

sulphate, urea, gelatin, glycine, beef extract, peptone, yeast extract) is measured at 600 nm using calorimeter.

Effect of metal ions on bacterial growth:

On the production media growth of bacteria at different metal ions is measured. Optical density of overnight incubated marine bacterial cultures at different metal ions at 0.02% concentration (Nickel sulphate, Cobaltous chloride, lead acetate, lithium chloride, manganese sulphate, calcium chloride, ferric chloride, ferrous sulphate, copper sulphate) is measured at 600 nm using calorimeter.

RESULT

Screening of EPS producing Bacteria

Isolation of EPS producing bacteria from Marine Bacteria

Marine sediment soil was collected from Parangipettai, South East coast of India. The samples were serially diluted and plated for screening of efficient EPS producing bacteria. The plates were kept at 37°C for 72 hours. Selected strain which exhibited EPS production on Zobell marine agar (Fig. 1).

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Fig.1.

Fig.2.

The isolating Marine bacteria were selected based on the morphology and maximum EPS production on basal medium. The pure culture was isolated on the Zobell marine agar (streak plate) (Fig.2).

Identification of EPS producing microorganisms

The selected strain was identified by various physical, biochemical and molecular characters. This strain exhibited Gram negative, rod shaped and motile bacteria. According to Bergey's manual of Determinative Bacteriology, the selected microbes were identified as *Pseudomonas putida.*

All the biochemical studies were performed and results were presented (Table. 1.).

Table. 1. Morphological and biochemical characteristics of *Pseudomonas putida*.

S.No.	Characterization methods	Result
A)	Colony morphology	Observation
1.	Gram staining	Gram negative, rod
2.	Motility	Motile
B)	**Biochemical characters**	**Result**
1.	Indole production test	Negative
2.	Methyl red test	Negative
3.	Vogesproskauer test	Negative
4.	Citrate utilization test	Positive
5.	Oxidase test	Positive
6.	Catalase test	Positive
7.	Triple sugar iron test	Negative
8.	Urease test	Negative

16S rRNA gene sequencing:

The 16S rRNA gene of the *Pseudomonas* sp. was amplified using Polymerase chain reaction (PCR) with the help of 16S rRNA Universal primers. The sequences were compared against 16S rRNA sequences available in the RDP database (http:11rdp (me. msu. edu/)). The sequence analysis revealed that the strains were phylogenetically closely related to the genus *putida*. Blast analysis of the 16S rRNA sequence of isolate revealed that the selected isolates showed maximum similarity of 98% with *Pseudomonas putida*.

The phylogenetic relationship was obtained using neighbor joining by pair wise comparison among the 16S rRNA gene sequence of selected isolates with species. The dendrogram was constructed for their Phylogenetic relationship and it revealed that the isolate *Pseudomonas putida* was distinctly placed under separate clusters. The 16S rRNA gene sequences of the isolates had been submitted to the NCBI Genbank.

BC4 F - ***Pseudomonas putida***

AACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAG
GTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCG
GTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCT
TACCAGGCCTTGACATGCAGAGAACTTTCCAGAGATGGAT
TGGTGCCTTCGGGAACTCTGACACAGGTGCTGCATGGCTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTA
ACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGG
TGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGA
AGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCT
GGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGC
CAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGT

BC4 R – ***Pseud***

TCCAACGGCTAGTTGACATCGTTTACGGCGTGGACTA
CCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGC
ACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCG
CCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCT
ACACAGGAAATTCCACCACCCTCTACCGTACTCTAGC
TTGCCAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGG
GGCTTTCACATCCAACTTAACAAACCACCTACGCGCG
CTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTC
TGTATTACCGCGGCTGCTGGCACAGAGTTAGCCGGTG

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Fig.3. Aligned sequence data of *Pseudomonas putida*

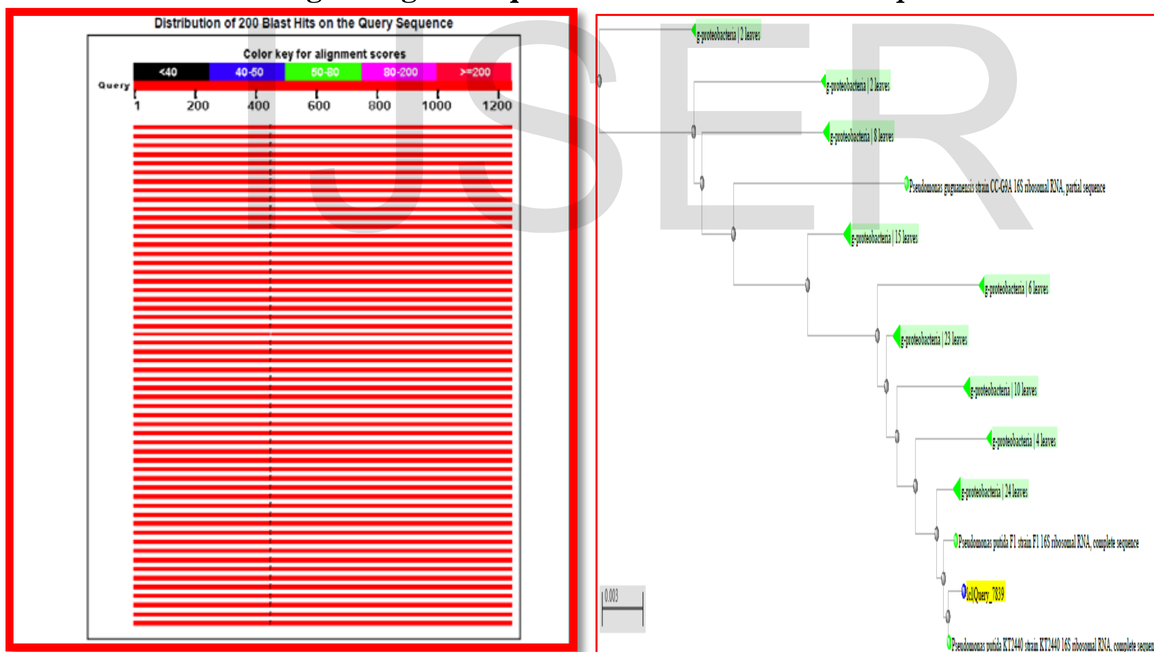

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Fig.4. Colour key alignment

Fig.5. Phylogenetic tree of *Pseudomonas putida*

EPS production

The bacterial EPS production recovered from basal medium was 108.54 ± 21.8mg/100ml of dry weight.

Optimization of cultural conditions for EPS production by *Pseudomonas putida*

EPS producting bacteria isolated from the Marine sediment was adjusted using various cultural conditions. The EPS production was assayed after 72 hours of incubation at 30°C under various parameters.

Effect of pH on bacterial growth and EPS production

The various pH tested for the maximum production of EPS was reecorded at pH 10 (0.835±0.004 OD) next to that maximum EPS production was observed at pH 9 (0.798 ± 0.00611 OD). Minimum EPS production was observed at pH 3 (0.03 ± 0.004 OD) (Fig. 6)

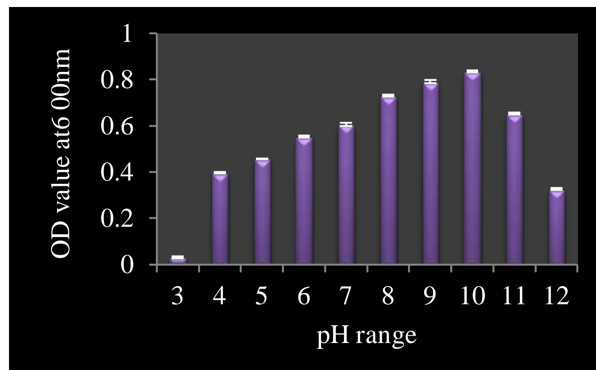

Fig. 6., Effect of pH on bacterial growth

Effect of temperature on bacterial growth

The various temperature tested the maximum EPS production was obtained at 30°C (0.871 ± 0.0036 OD) followed by this at 20°C (0.547 ± 0.005568 OD) was the second best temperature on EPS production. On the other hand, the minimum amount of EPS production was observed at 10°C (0.077 ± 0.004583 OD) (Fig.7).

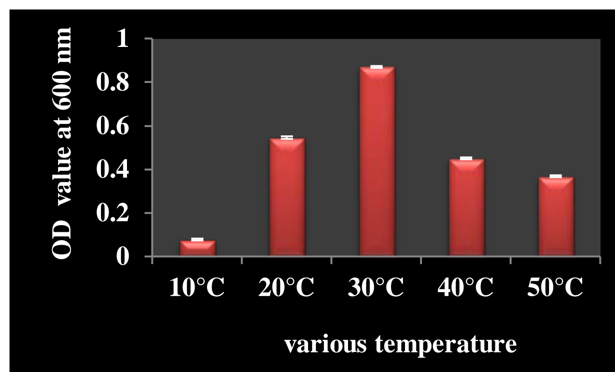

Fig.7. Effect of Temperature on bacterial growth

Effect of carbon source on bacterial growth

The effect of carbon source on EPS production by after 72 hours incubation at 30°C. Here the maximum EPS production was observed in Adenitol (0.822 ± 0.0110 OD) supplemented medium. The minimum EPS production was observed in Raffinose (0.03 ±0.004041 OD) provided medium (Fig.8).

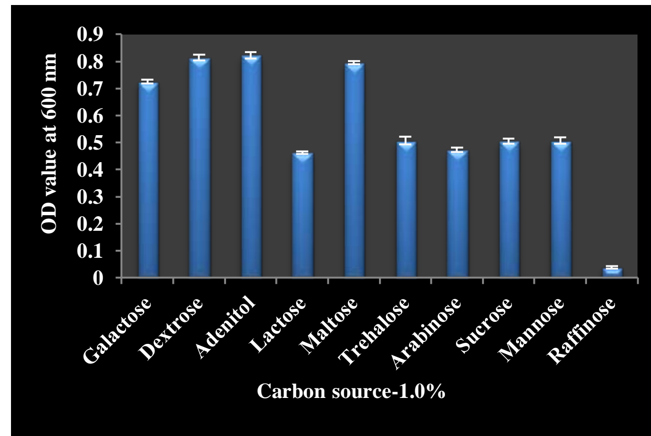

Fig.8. Effect of carbon source on bacterial growth

Effect of nitrogen source on bacterial growth

The effect of nitrogen source on EPS production by after 72 hours incubation at 30°C. Here the maximum EPS production was observed in Ammonium bicarbonate (1.176 ± 0.0088 OD) supplemented medium. The minimum EPS production was observed in Casein (0.03 ± 0.004583 OD) provided medium (Fig.9).

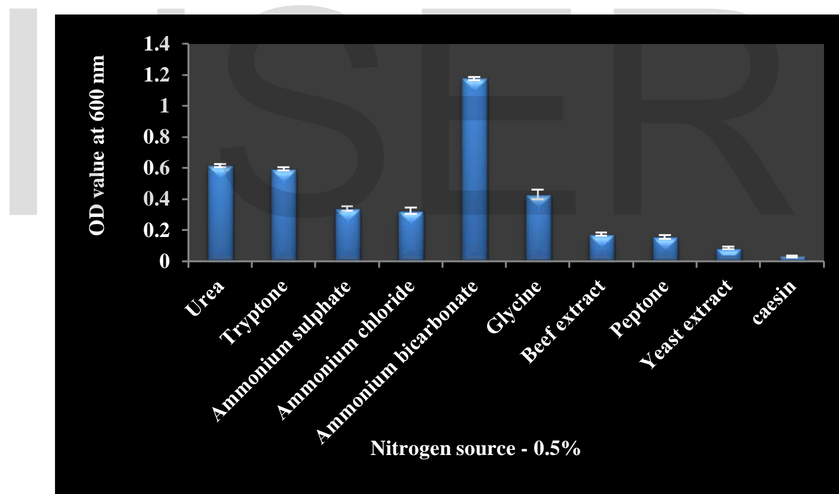

Fig.9. Effect of nitrogen source on bacterial growth

Effect of aminoacids on bacterial growth

The effect of various amino acids on EPS production by after 72 hours incubation at 30°C showed the maximum EPS production was observed in Lysine (0.778 ± 0.0060 OD) supplemented medium. Followed by this Cysteine (0.739 ± 0.0095 OD) was second best amino acids in EPS production, whereas, the minimum EPS production was observed in Glutamine (0.199 ± 0.0070 OD) (Fig.10).

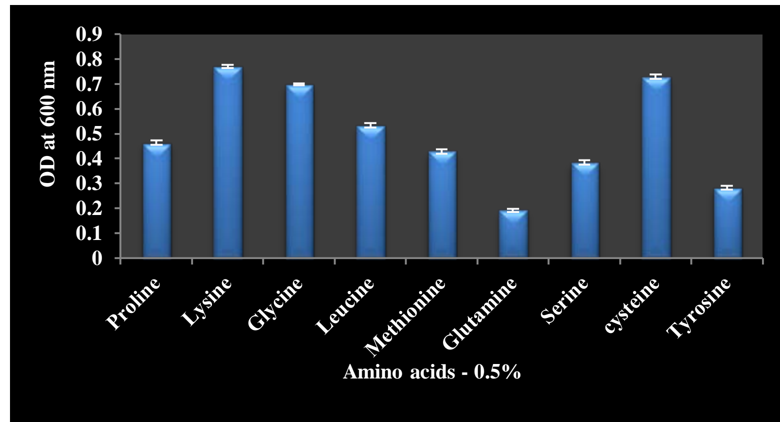

Fig.10. Effect of amino acids on bacterial growth

Effect of hydrocarbon on bacterial growth

The effect of different kinds of hydrocarbons was tested on EPS production after 72 hours of incubation at 30°C. Among the tested hydrocarbons, the maximum amount of EPS production was observed in Toluene (0.872 ± 0.005 OD) added medium. Olive oil (0.803 ± 0.0051 OD) comes second best hydrocarbons on EPS production. The minimum EPS production was observed in Xylene (0.078 ± 0.006028 OD) (Fig.11).

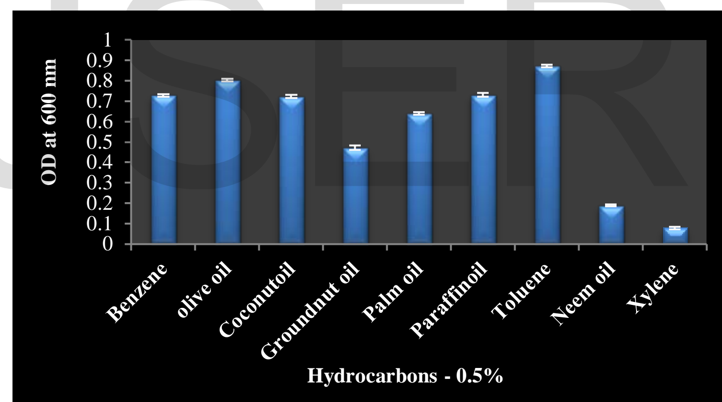

Fig.11. Effect of hydrocarbons on bacterial growth

Effect of surfactants on bacterial growth

The effect of different kinds of surfactants was tested on EPS production after 72 hours of incubation at 30°C. Among the tested surfactants, the maximum amount of EPS production was observed in Tween 20(0.724 ± 0.0065 OD) added medium. The minimum EPS production was observed in SDS (0.3266 ± 0.005 OD) (Fig.12).

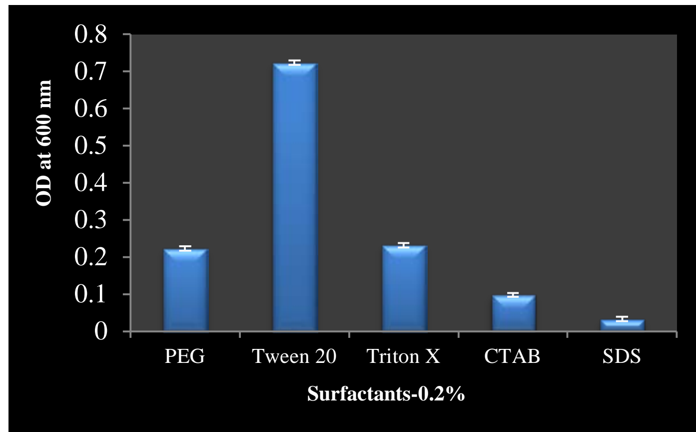

Fig.12. Effect of surfactants on bacterial growth

Effect of NaCl concentration on bacterial growth

The effect of different kinds of surfactants was tested on EPS production after 72 hours of incubation at 30°C. Among the tested surfactants, the maximum amount of EPS production was observed in 5.5% (0.77 ± 0.0055 OD) added medium. The minimum EPS production was observed in 7% (0.047 ± 0.0045 OD) (Fig.13).

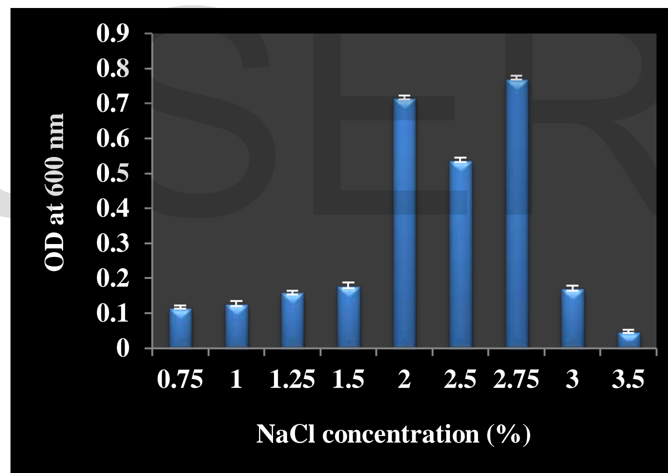

Fig.13. Effect of NaCl concentration on bacterial growth

Effect of metal ions on bacterial growth

The effect of different kinds of metal ions was tested on EPS production after 72 hours of incubation at 30°C. Among the tested metal ions, the maximum amount of EPS production was observed in Ferric chloride (0.961 ± 0.0199 OD) added medium. The minimum EPS production was observed in Zinc sulphate (0.101 ± 0.019 OD) (Fig.14).

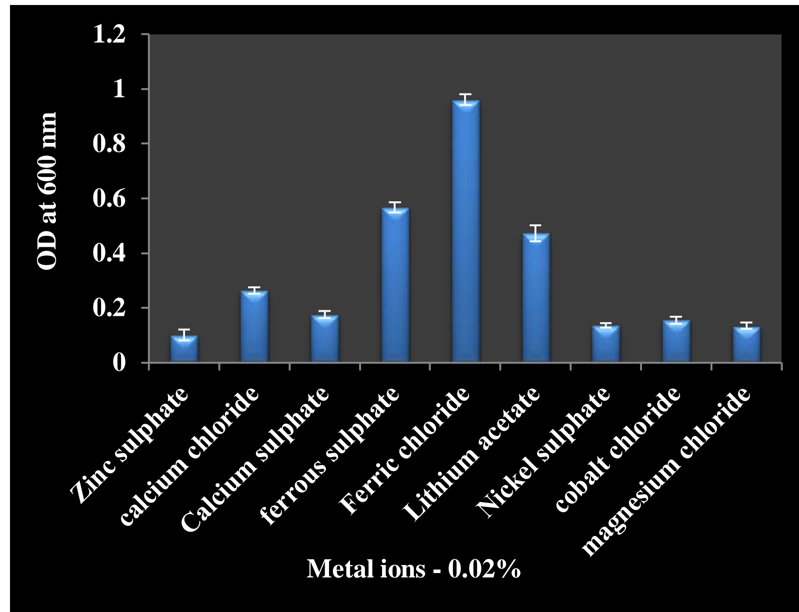

Fig.14. Effect of metal ions on bacterial growth

Effect of inoculum concentration in bacterial growth

The effect of different kinds of inoculum concentration was tested on EPS production after 72 hours of incubation at 30°C. Among the tested inoculum concentration, the maximum amount of EPS production was observed in 1.5% (0.799 ± 0.0095 OD) added medium. The minimum EPS production was observed in 3.0% (0.019 ± 0.0056 OD) (Fig.15).

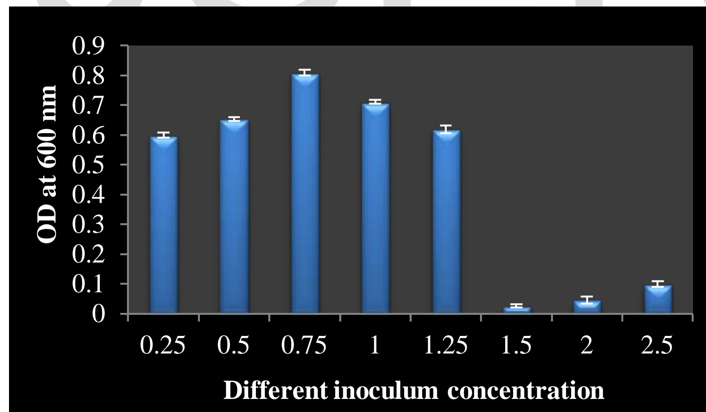

Fig.15. Effect of inoculum concentration on bacterial growth

Effect of incubation time on bacterial growth

The effect of different kinds of incubation time was tested on EPS production after 72 hours of incubation at 30°C. Among the tested incubation time, the maximum amount of EPS production was observed in (1.091 ± 0.0100 OD) added medium. The minimum EPS production was observed in 72 hours 120 hours (0.1 ± 0.003 OD) (Fig.16).

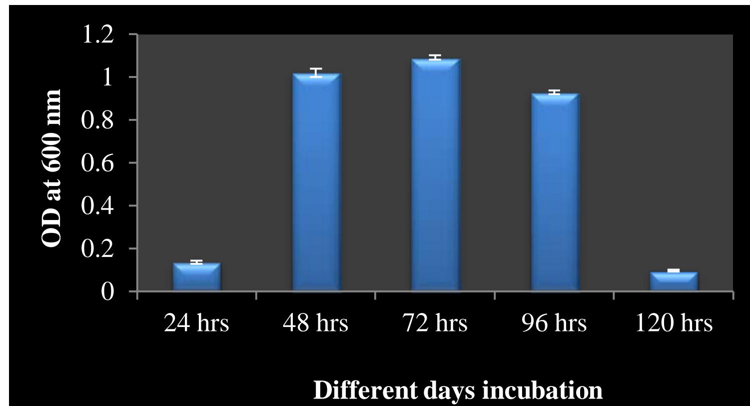

Fig.16. Effect of incubation time on bacterial growth

DISCUSSION

In the present study, we isolate EPS producing *Pseudomonas* sp. from marine sediment in and around south east coast, India. The serially diluted mucoid appearance was observed and identified the strains was taken based on the efficiency of EPS production observed by using carbohydrate estimation. Maximum production of EPS was observed from *Pseudomonas putida* basal medium was 108.54 ± 21.8mg/100ml of dry weight.Whereas Shankar *et* al., (2014) stated that elephant dung samples were taken from Shenbagathoppu, Srivilliputtur. The samples were serially diluted and plated for screening of efficient EPS producing bacteria, the selected strain which exhibited EPS production on Zobell marine agar medium. They recovered maximum production of EPS from *Frateuria aurentia* in basal medium 90.66 ± 16.8mg/100ml of dry weight.

In the present study maximum production of EPS was observed from *Pseudomonas putida* in Zobell marine agar medium 108.54 ± 21.8mg/100ml of dry weight. The optimal culture media determined as follows: After 72 hours incubation at pH 10, 30°C on optimized medium Adenitol- 1.0%, (carbon source), Ammonium bicarbonate- 0.5% (Nitrogen source), Ferric chloride-0.01% (Metal ions), Lysine-0.2% (Amino acid), Inoculum concentration-1.5%, Tween 20-0.2% (surfactant), NaCl-2.75%. *Pseudomonas putida* EPS content under the optimized conditions was better than that under the basic culture medium and initial conditions. Similarly Gao *et al*., (2012) observed optimal culture medium constituents. The optimized parameters for liquid fermentation were as Temperature-25°, Incubation time – 6 days, Initial pH 8.0, and volume of medium-150 ml. The optimized condition was 2.5 times better than under the basic culture medium and initial conditions.

In the present study the phylogenetic relationship was obtained using neighbour joining by pair wise comparison among the 16S rRNA gene sequence of selected isolates with species. The dendrogram was constructed for their Phylogenetic relationship and it revealed that the isolate *Pseudomonas putida* was distinctly placed under separate clusters. The 16S rRNA gene sequences of the isolates had been submitted to the NCBI Genbank. Whereas Berekaa,(2014) have investigate phylogenetic affiliation ofthis strain, the complete 16S rRNA gene was amplified, sequenced and deposited in the Genbank and given the accession number KC223618. Comparison of the obtained sequence with other sequences available at NCBI database revealed the greatest similarity to the corresponding sequences of many *Bacillus licheniformis*strains.

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In the present study different carbon source were used in the optimization process. Among the carbon sources used Adenitol (0.822 ± 0.0110 OD) showed maximum amount of EPS production by *Pseudomonas putida.* Liu *et al*., (2011) find out that the effect of five different carbon sources on the EPS production and broth viscosity. The maximum EPS production was about 6.47 g/L and it was detected when lactose was served as carbon source.

In the present study different pH, temperature, NaCl concentrations were used in the optimization process. The optimal pH was 10 (0.835±0.004 OD), temperature 30°C (0.077±0.004583 OD) and the NaCl concentration of 5.5% (0.77 ± 0.0055 OD) showed maximum amount of EPS production by *Pseudomonas putida.*Likewise, the optimal pH was 7 (1.431 ± 0.0096 OD), temperature 30°C (1.661 ± 0.028 OD) and the NaCl concentrations 2.5% (1.138 ± 0.023 OD) showed maximum amount of EPS production by *Frateuria aurentia* (Sivakumar *et al*., 2012a).Whereas, Arun *et* al., (2014) find out the optimization of pH was (9.0-9.5), temperature (25-35°C) were analyzed in ZMA(Zobell Marine Agar) broth. The optimal pH was 9 (3.73 g/L), temperature 35°C (2.98 g/L) showed maximum amount of EPS production by *Halobacillus trueperi*.

Whereas Sung-Hwan Ko (2000) stated that the highest productivity of EPS-R was obtained at 20 to 25°C. At above 30°C, the productivity of EPS-R was reduced, contrast to an increase in the productivity of red pigments. The high productivity of EPS-R was yielded at pH 6 to 8. Especially, the highest productivity was acquired at pH 7 (9.19 g/L) with the yield ratio (YP/S) of 4.6. The yield of EPS-R was decreased under pH 5 and above pH 9.Similarly Kanmani*et al*. (2013) have find out the optimal pH, temperature and NaCl concentration for EPS production by *S. phocae*PI80, different pH (5-7.5), temperature (25-50°C) and NaCl concentrations (0-4%) were analyzed in MRS broth. The optimal temperature, pH and NaCl concentration for cell growth and EPS production were 35°C, 6.5 and 2-3%, respectively with the corresponding cell growth (OD-1.333 ± 0.02, 1.335 ± 0.05 and 1.358 ± 0.02) and EPS (g/L) production (7.8 ± 0.29, 7.9 ± 0.34 and 8.1 ±0.27).

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In the present study the carbon and nitrogen sources were used in the optimization process. Among the carbon sources used Adenitol (0.822±0.0110 OD) showed maximum amount of EPS production by *Pseudomonas putida.*Among the nitrogen sources used Ammonium bicarbonate (1.176±0.0088 OD) showed maximum amount of EPS production by *Pseudomonas putida.*Whereas the maximum amount of EPS was observed in jaggery (1.185 ± 0.003 OD) and in nitrogen source tryptone (1.248 ± 0.011 OD) was the best source for *Frateuria aurentia* (Sivakumar *et al.,* 2012). Whereas Wang et al. (2004) stated that a high level of EPS was obtained when maltose (carbon source) and glycerol (nitrogen source) were used as the carbon source. Among the carbon sources tested, maximum EPS (10.45 g/l) was obtained in the maltose medium. Among the nitrogen source tested, the maximum EPS production (10.53 g/l) was obtained in the peptone.

In the present study the effect of different kinds of metal ions was tested on EPS production after 72 hours of incubation at 30°C. Among the tested metal ions, the maximum amount of EPS production was observed in Ferric chloride (0.961 ± 0.0199 OD) added medium.Whereas, the adsorption capacity of EPS of *Azotobacter chroococcum* XU1 for lead and mercury at neutral pH showed that EPS had adsorbed 22.38 and 25.49 mg/g Lead and Mercury respectively (Rasulov *et al* 2013).

SUMMARY

Exopolysaccharides producing bacteria was isolated from marine sediment collected from Parangipettai, South East coast of India.

Biochemical characterization and 16S rRNA gene sequencing were done to identify the organism *Pseudomonas putida*.

Optimization was done to get maximum EPS production, for that parameters such as Adenitol- 1.0%, (carbon source), Ammonium bicarbonate- 0.5% (Nitrogen source), Ferric chloride-0.01% (Metal ions), Lysine- 0.2% (Amino acid), Inoculum concentration-1.5%, Toluene-0.5%(Hydrocarbon), Tween 20-0.2% (surfactant), NaCl-2.75%.

The physicochemical characterization was done for the EPS crude extract obtained from *Pseudomonas putida*.

The carbohydrate content was 0.834 ± 0.180 mg/ml in crude extract.

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