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Research Article

CHARACTERIZATION AND PRODUCTION OF EXOPOLYSACCHARIDES PRODUCED BY MICROORGANISMS FROM SALINE SOIL

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ABSTRACT

In the present study, isolation and characterization of microorganisms was performed by collection of saline sample obtained from Kilakarai a coastal town, Ramnad Dt. Serial dilution was carried out to isolate strains by spread plate and pure culture in Nutrient agar medium. The isolates were screened for exopolysaccharide production which was confirmed by formation of clear zone around the colonies. The dominant strains were characterized morphologically and biochemically. It was confirmed as *Bacillus cereus.* The production of exopolysaccharide was performed by selective media and it was confirmed by treatment of extracts in ice cold isopropanol. The study was further extended for optimization with different pH, temperature, different carbon and nitrogen sources. The optimum pH was found to be 7.0 and the optimum temperature was found to be 37^0c. Among the carbon sources tested sucrose was found to exhibit enhanced production, whereas peptone was found to yield an increased production among nitrogen sources. The functional group changes after production of exopolysaccharide was analyzed by FTIR. The total quantity of protein estimated was found to be 1.66g/lit. The High performance liquid chromatography (HPLC) analysis was performed to identify the metabolites which exhibited a prominent peak of 1.933 indicated fraction of exopolysaccharide metabolite present presumed to be deoxyhexoses and hexoses produced by *Bacilluscereus* for exopolysaccharide production. The other HPLC chromatogram with a peak value of 2.013 represent fraction of exopolysaccharides corresponds to alditol acetates of monosaccharaides.

INTRODUCTION

Exopolysaccharides producing bacteria which was found as indigenous soil (Bhaharuddin, etal., 2015).Some exopolysaccharides producing bacteria have been reported include *pseudomonas aeruginosa, erwinia, ralstonia and azatobactervinelandii,* exopolysaccharide protects the bacteria from a variety of environmental stresses (Iqbal, etal., 2002), protects cells from antimicrobial compounds, antibodies and bacteriophages or for stricking to other bacterial, animal and plant tissue (Wingender, etal., 1999).

Microbial exopolysaccharides are polymers that consist principally of carbohydrates and are excreted by some bacteria and fungi onto the outside of their cell walls. Their composition and structure is varied. The may be either homo or hetero polysaccharide and may also contain a number of different organic and inorganic consitutents (Sutherland, 1990).Increased attention was being paid to these molecules because of their bioactive role and their extensive range of potential applications in pharamaceuticals as antiangiogenic (Matou, etal., 2005) or antiviral agents (Arena, etal., 2009) and in agriculture and various other industrial areas (Sutherland et al 2002).

Exopolysaccharides shows different properties like, thickening, gelling, emulsifying, etc., (Nowdo, etal., 2012). In the present investigation much focus of attention was given to produce exopolysaccharides from marine saline samples and *Bacilluscereus* have been isolated which is known to produce extracellular polysaccharides.

Besides an attempt to address to study the functional groups associated with exopolysaccharides Fourier Transform Infrared (FTIR) spectroscopy was studied. The exopolysaccharides production depends on several factors such as pH, temperature, carbon source and nitrogen source and cultivation conditions were performed. Furthermore exopolysaccharide produced were quantified for protein content and carbohydrate. The study were further extended to study the antimicrobial activity

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of exopolysaccharides treated selected pathogens. The fractions of exopolysaccharides and its metabolites were analyzed for High Performance Liquid Chromatography (HPLC) analysis.

MATERIAL AND METHODS

Isolation and Screening of Exopolysaccharide Producing Microorganism

The soil samples were collected from Kilakarai, Ramnad dist. The samples were serially diluted and plated on nutrient agar plates to obtain isolates. It was screened to obtain pure culture isolates by following procedure by Emtiazi, et al., 2004.

Identification and Characterization of the Efficient Exopolysaccharide Producing Microorganisms Morphological Characterization

Morphological characteristics such as abundance of growth, pigmentation, optical characteristics, size and shape were studied on nutrient agar plates.

Biochemical Characterization

The biochemical characterization test were performed by following tests Indole, MR-VP test, citrate test, TSI test, starch hydrolysis test, nitrate reduction test, gelatin test and carbohydrate fermentation test was carried out.

Production and Extraction of Exopolysaccharide

Production was carried out with the mediumconsisting of following components peptone 10gm, Beef extract 3gm, Sodium chloride 5gm and sucrose 2% in 50ml of liquid medium. Media were sterilized and pH was adjusted to 6.5. The flasks were incubated a rotary shaker at room temperature for 3days (72hrs).At the end of incubation cells were harvested by centrifugation for 20mins at 10,000rpm. After centrifugation two volumes of ice cold isopropanol were added into it and stored overnight at 4^0c. Precipitated was formed. Precipitated material was collected by centrifugation (10,000rpm at 20mins) and the pellets were dried at 100^0c and weigh the pellets.

Optimization of Exopolysaccharide Producing Microorganism

To determine the effect of different parameters. Exopolysaccharide production was optimized under different environmental and nutritional conditions pH (4, 5, 6, 7, 8), Temperature (25^0c, 37^0c, 45^0c, 55^0c), Carbon Sources (Sucrose, Fructose, Dextrose, Mannitol) and Nitrogen Sources are (Peptone, Beef extract, Ammonium chloride, Ammonium sulphate, Potassium nitrate).

Estimation Carbohydrate and Protein Content of Crude Exopolysaccharides

The total carbohydrate content was estimated by Phenol Sulphuric Acid method. The amount of protein present in the exopolysaccharide was estimated by Lowry's et al.,1951, method.

Antimicrobial Activity of Exopolysaccharides Treated Against Bacterial Pathogens Using Well Diffusion Method

Antimicrobial activity of the extract of compounds was determined using well diffusion method. It was performed by sterilizing Muller Hinton agar media. After solidification, wells were cut on the Muller Hinton agar using cook borer. The test bacterial pathogens were swabbed onto the surface of Muller Hinton plates. Wells were impregnated with 25µl of the test samples. The plates were incubated for 30min to allow the extract to diffuse into the medium. The plates were incubated at 30^0c for 24 hours, and then the diameters f the zone of inhibition were measured in millimeters. Each antibacterial assay was performed in triplicate and mean values were reported.

Antifungal Activity of Exopolysaccharides against Fungal Pathogens Using Well Diffusion Method

Antifungal activity of the extract of compounds was determined using well diffusion method. It was performed by sterilizing Muller Hinton agar media. After solidification, wells were cut on the Muller Hinton agar using cork borer. The test fungal pathogens were swabbed onto the surface of Muller Hinton agar plates.

Determination of Functional Groups of Exopolysaccharides By Ftir Analysis

FTIR is perhaps the most powerful tool for identifying types of chemicals bonds (functional groups). The wavelength of light absorbed is characteristics of quantitative analysis.

HPLC Analysis of Exopolysaccharides

The isolated crude potential basal EPS were analyzed with a high performance liquid chromatography (HPLC) system (Agilent 1100) equipped with aqueous GPC startup Kit column and eluted with distilled water at a flow rate of 1.0 ml/min at 20c. The separated components were monitored by a refractive index detector. The EPS after hydrolysis dissolved in methanol and analyzed in HPLC. The column was calibrated with different molecular mass standard and a standard curve was then established.

Emulsification Assay

For determination of the emulsification index, 2 ml of supernatant or cell suspension and 3 ml of a selected hydrocarbon were mixed in a test tube and vortexed for 2 minutes. The test tubes were maintained at 25℃ and the height of emulsion layer was measured after 24 hours to determine the emulsification index (E_{24}) is as follows:

$$E_{24} = \frac{\text{height of emulsion layer} \times 100}{\text{Height of total solution}}$$

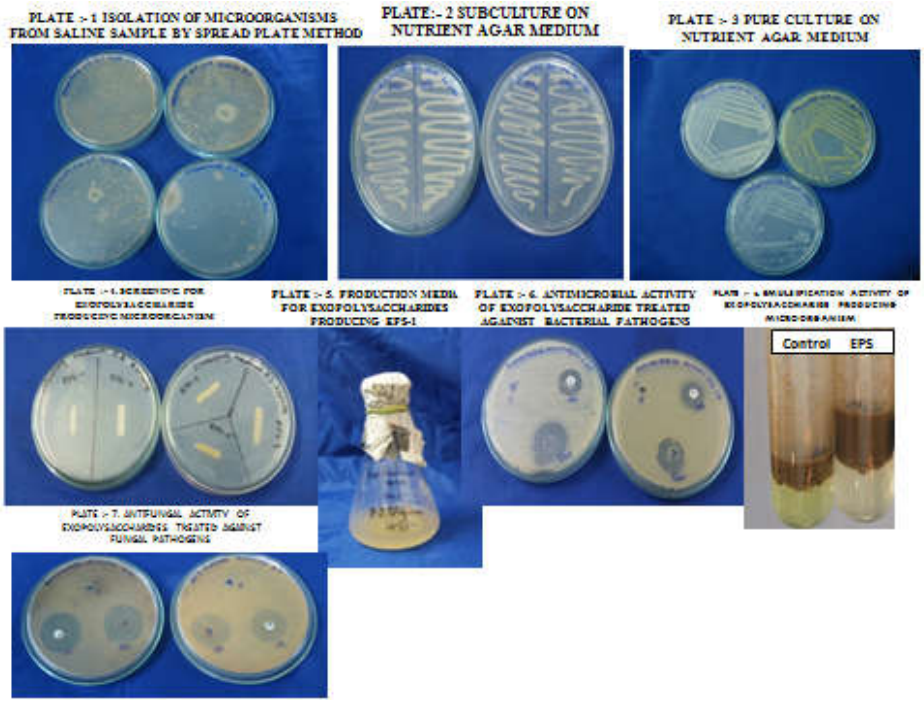
The results were compared with the positive controls such as SDS and Tween 80

RESULT

In the present study, different marine sediment samples from salt pans of Kilakarai, Ramnad (dist) were collected. The samples were serially diluted and spread plated incubated 37c for 24 hours. Five dominant morphologically distinct colonies were selected and pure cultured repeated streaking on nutrient agar plates. (Plate:-1, 2 and 3).

Out of five isolates one bacterial strain were screened for exopolysaccharide producing ability on basal medium. The zone formation around microorganism was identified as positive exopolysaccharide producing bacteria. The isolate EPS-1 showed maximum exopolysaccharide activity with zone

of about 2.1mm. Therefore these efficient exopolysaccharide producing strain EPS-1 were selected for further experimental study and biochemical study.(Plate:4).



Identification of Exopolysaccharide Producing Organism

Morphological and Biochemical Characterization

On nutrient agar medium the isolate EPS-1 was found to be gram positive was occurring simply. It produced endospore and motile in nature. On nutrient agar the colonies were non-pigmented. The biochemical test for the isolate EPS-1 reduced indole negative reaction. It fermented sugars, methyl red test, voges proskeur revealed positive. The isolate utilized sugars in triple sugar iron test. Nitrate was reduced to nitrite. Gelatin was liquefied, starch was hydrolysed. The isolate EPS-1 utilized sucrose and the result was found to be positive. From the results it was observed that the organism belong to genus is *Bacillus cereus*. The results were compared in accordance with Bergey's manual of determinative bacteriology. The results exhibit in Table:-1.

Table 1 Morphological and Biochemical Characterization of Exopolysaccharides

S.No	Tests	Results
	Morphological characterization	
1	Gram staining	Positive
2	Endospore staining	Positive
	Biochemical characterization	
1	Indole test	Negative
2	Methyl red test	Positive
3	Vogesproskeur test	Positive
4	Citrate utilization test	Positive
5	Triple iron agar test	Alkaline sland
6	Starch hydrolysis test	Positive
7	Gelatine hydrolysis test	Positive
8	Nitrate reduction test	Positive
	Carbohydrate fermentation test	
9	Sucrose	Positive
	Dextrose	Negative

Production of Exopolysaccharides

The isolate EPS-1 is used for production of exopolysaccharides. Exopolysaccharide have been reported to play a significant role in providing protection to the cell and also it contributes to soil aggregation due to its gluing properties. The isolates were used for exopolysaccharide production in specific media and the cells were harvested to obtain exopolysaccharides. The results showed on Table:- 2 and Plate:-5.

Table 2 Production of Exopolysacharide Producing Microorganism

S.No	O.D at 580nm	Crude EPS production activity (g/l)
1	1.72	17.20

Optimization of Exopolysaccharide Producing Microorganism

Effect of ph on Exopolysaccharide Production

EPS strain 1 was inoculated into nutrient broth and peptone and incubated at different pH 4, 5, 6, 7 and 8 in a rotary shaker in incubator at 30°c. The optimum pH was found to be pH-7 for exopolysaccharide production (Fig:1).

Effect of Temperature on Exopolysaccharide Production

EPS strain 1 was inoculated into nutrient broth and peptone at different temperature from 25°c, 37°c, 45°c and 55°c in rotary shaker was incubator and temperate. 37°c was found to be optimum for exopolysaccharide production. (Table: 4 and Fig:2).

Effect of Carbon Source on Exopolysaccharide Production

The carbon sources include sucrose, fructose, dextrose and mannitol were supplemented in nutrient broth and

supplemented with 1% peptone. In order to enhance the exopolysaccharide production and among the different carbon sources tested sucrose was found to produce maximum yield for EPS-1 with 3.13g/l (Fig:3).

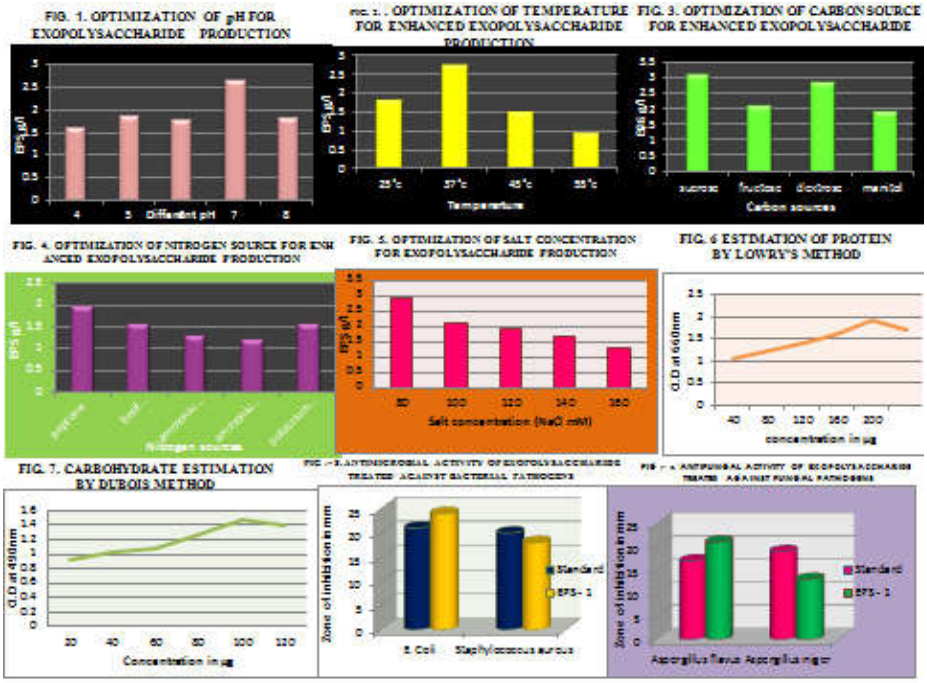
Effect of Nitrogen Source on Exopolysaccharide Production

In order to determine the effect of nitrogen source on exopolysaccharide production was studied. Different nitrogen sources are peptone, beef extract, potassium nitrate, ammonium sulphate and ammonim chloride. The results indicated peptone is the best nitrogen source for exopolysaccharide production with the yield of 1.60g/l (Fig:4).

minimum activity was observed against *E. Coli* (Plate: 6 and Fig: 8).

Determination of Antifungal Activity of Exopolysaccharide Producing Microorganisms

The susceptibility of various fungal pathogens to growth inhibition by the exopolysacharide produced microorganisms was presented in Table 11. The anti fungal activity of the fungal pathogen was detected by adding supernatant in to the well 72 hours zone formation was measured. It showed inhibitory activity against *Aspergillus niger and Aspergillus fslavus*.



Effect of Nitrogen Source on Exopolysaccharide Production

Figure:- 5 exhibit the results obtained with different concentration of sodium chloride tolerance to exopolysacharide producing microorganism. Among the different concentration of Nacl such as 50,100 and 150mg/l. The best results were obtained in 50mg/l of Nacl.

Estimation of Protein and Carbohydrate

The estimation of total protein content in crude exopolysaccharide was 1.66g/l (Fig:6).Estimation of carbohydrate content in exopolysaccharides the total carbohydrate content was estimated by phenol-sulphuric acid method. The among of carbohydrate present was found to be 1.35g/l (Fig:7).

Determination of Antimicrobial Activity of Exopolysaccharide Producing Microorganisms

The susceptibility of various clinical pathogens to growth inhibition by the exopolysaccharide producing microorganisms (EPS-1) was presented in Table and plate. The antimicrobial activity of the organism was detected by adding 50µlof supernatant into the well. After incubation, zone formation was measured. It showed inhibitory activity against *E. Coli,* and *Staphylococcus aureus,*. Among these, maximum activity observed against *E. coli* and *staphylococcus aureus* and

Among these Fungal pathogen maximum activity observed against *Aspergillus niger* and minimum activity was observed against *Aspergillus niger* (Plate:7and Fig: 9).

Emulsification Activity

Plate: -8 exhibit, the emulsification activity of exopolysaccharide producing microorganisms which revealed the best emulsification activity was obtained with crude oil.

FTIR Studies of Exopolysaccharide Producing Microorganisms

FTIR analysis which reveal functional group modification was detected by FTIR Spectrum from crude exopolysaccharide production.

The IR Spectrum of polymers has shown broad intense band at 4000cm^{-1}- 650cm^{-1}. Appearance of peak at a wave length of 2989.39cm^{-1} strongly suggest broad stretching of C-H bonds. Carboxyl group and its presence were confirmed in the previous studies also which is an indication of exopolysaccharide production. The IR spectrum of the crude polysaccharide sample showed band at peak value of 1070.49, 700.16 and 547.78 indicate characteristics of glucan. Polysaccharides C-O-C and C-O-P was found at 1070.49cm^{-1} was typical for glucose in pyranose form. Our results coincides with the work of Vijayabaskar et al., 2011. It was concluded

that exopolysaccharide extract was higher in sucrose as carbon source and peptone was found to yield more pronounced of exopolysaccharides. (Fig: 10).

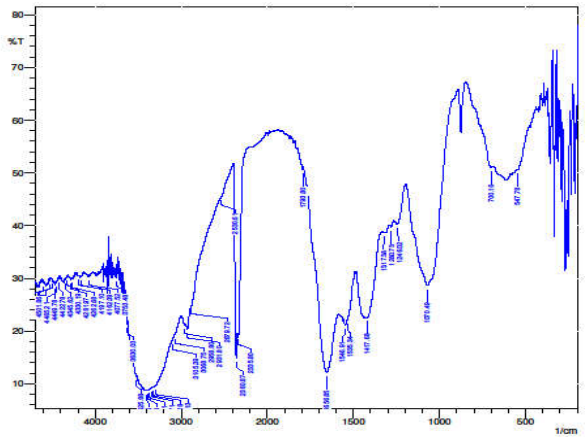


Fig 10 FTIR Analysis of Exopolysaccharide Producing Microorganism

Determination of Exopolysaccharide By HPLC

Determination of exopolysaccharide by High Performance Liquid Chromatography (HPLC) analysis using hexane as solvent, was analyzed. The exopolysaccharide production has been quantified by HPLC by *Bacilluscereus* from potential producing basal medium with independent peaks are identified in Retention Time. A prominent peak of 1.933 indicated the different fraction of metabolite was detected from exopolysaccharides. (Fig: 11). Besides the HPLC chromatogram with 2.013 and 2.433 represent fraction of exopolysaccharide which determine the derivative of alditol acetates which is used to determine the ratio's of monosaccharides. Besides, the other peak corresponds to hexose, deoxyhexose for bacterial exopolysaccharide production.

purpose and surviving in adverse traditions. In the present study, marine samplers were obtained from Kilakarai, Ramnad dist. The samples was serially diluted, spread plated and characterized.

In present investigation five different organisms were found to be distinct among which one strain EPS – 1 was found to be produce better yield of crude exopolysaccharide production. Screening of five different isolates was studied. Screening plays an important role in differentiating microorganisms according to their enzymatic activities (Mohamed, 1999). Two stages of enzymatic screening were performed was primary and secondary screening. The isolate EPS-1 were characterized to be gram positive rods, endospore forming and motile in nature and it was identified as *Bacillus cereus*. Biochemical characterization of the isolates revealed positive for methyl red, citrate utilization, starch, gelatin hydrolysis and nitrate reduction. The strain utilized glucose produced by bacteria which is indication of *Bacillus cereus*. Our study is in total agreement with work of (Lakshmi etal., 2013).

In the present study, exopolysaccharide production from marine bacterium *Bacillus cereus* were conducted use in basal medium containing peptone, beef extract, sucrose and sodium chloride for 72hours incubation. The maximum exopolysaccharide production was found to be 17.20g/l of exopolysaccharides at an pH of 6.5. Our results are in total accordance with (Kim etal., 2005). Similar findings were in (Kanieker, etal.,2009). The extraction of exopolysaccharides was carried out by centrifugation methods.

Optimization of exopolysaccharide producing microorganisms was carried out. pH as a strong influence on metabolic pathways and intermediate compounds generated by microorganisms. Each organism has an optimum pH for its growth and activity.

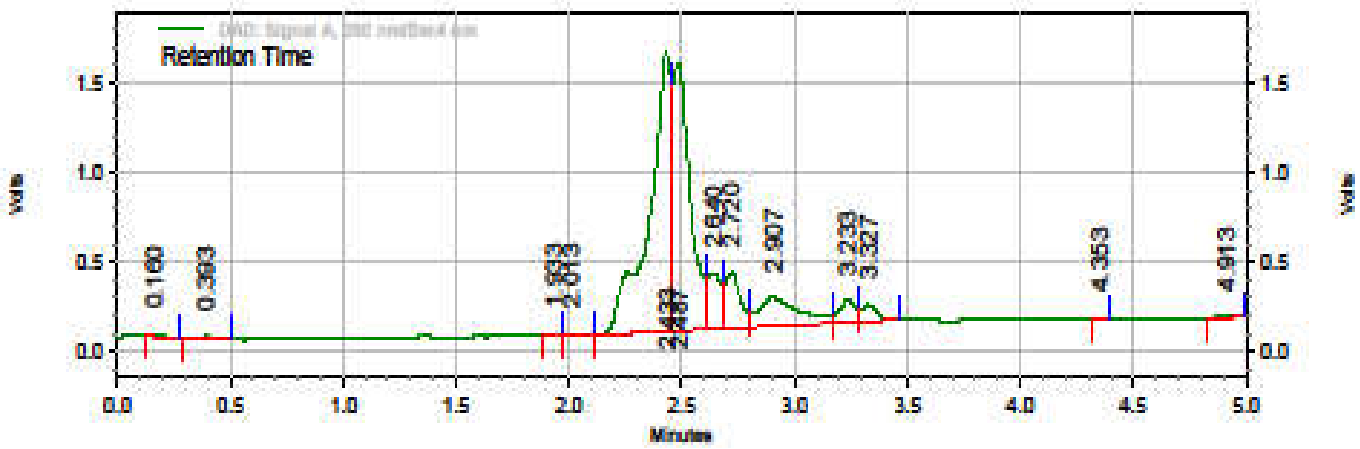


Fig 11 HPLC Analysis of Exopolysaccharide Producing Microorganism

DISCUSSION

Exopolysaccharides are high molecular weight polymers that consist of sugar residues widely differ in structure and function. Exopolysaccharides produced by Lactic acid bacteria possess the possibility of replacing stabilizers and thickners. Exopolysaccharides generally composed of monosaccharide's and some non-carbohydrate constituent like acetate and succinate. Marine bacteria are known to produce extracellular polysaccharides for their thriving fitness such as adhering

The pH dependent on factor such as temperature, substrate concentration, time of reaction. In which the reaction to be carried out and it is note worthy to indicate the source of enzyme (Pandy, 2000). In our study the optimum pH for exopolysaccharide production by *Bacillus cereus* revealed pH-7. Our results are in total agreement with (Mohamed etal., 2000).

Temperature is yet another parameter that has to be controlled from organism (Frankena etal., 1986) have shown a link

between enzyme synthesis and energy metabolism in *Bacillus* which was controlled by temperature and oxygen uptake. Temperature play a vital role on stability of the enzyme. The optimum temperature for exopolysaccharide production by *Bacilluscereus* exhibited 37°c. Similarly results was obtained by (Balaji etal., 2008). In order to determine the carbon source is different carbon sources were supplement to nutrient broth. Each organism has its own special conditions as carbon source for maximum enzyme production was reported by (Kumar Takgi, 1999). In our results sucrose was found to produce maximum yield for EPS-1 with 3.13g/l. The results were in total accordance with (Hoda etal., 2013).

The experiments was further extended to study the impact of nitrogen source for enhance exopolysaccharide production. Nitrogen source was the secondary energy source for the spoulating organism like Bacillus cereus which play a important role in growth of organism and enzyme production. In tune with above discussion Saber, etal., (2009) reported nitrogen source such as peptone, beef extract and casein as extra addition of nitrogen source to induce exopolysaccharide production. This was true in our study also, where in peptone was found to exhibit best nitrogen source.

The estimation of total protein content for crude exopolysaccharide production was found to be 1.66g/l (Lowry etal., 1987). The amount of carbohydrate present in exopolysaccharide production was estimated by phenol-sulphuric acid method which showed a carbohydrate content of 1.32g/l of carbohydrates. Our results wherein total agreement with (DuBois etal., 1956). The present study susceptibility of various clinical pathogens to growth inhibition by the exopolysaccharide producing microorganisms was performed. The exopolysaccharide producing microorganism were determined by antimicrobial activity and antifungal activity for isolate EPS-1 maximum activity observed against *E. coli(21 and* 20*)* and *Staphylococcus aureus* (24 and 20) and minimum activity was observed against *staphylococcus aureus* (24 and 20) respectively.

Similarly the role of exopolysaccharide against fungal pathogen was studied. The zone of inhibition for exopolysaccharide *Aspergillus Niger* was found to be (17mm and 19mm) where the zone of inhibition for *Aspergillus Flavus* was found to be (21mm and 23mm*)*. Among this fungal pathogen maximum was activity observed against *Aspergillus flavus* and minimum activity was observed against *Aspergillus niger*. In order to ascertain the functional group present was analyzed by Fourier Transform Infrared spectroscopy. The functional group of parent molecules were changed into intermediates by oxidation and reduction reaction catalyzed by enzyme *Bacillus*. The IR spectrum indicated broad band at wavelength of $4000cm^{-1}$ – $650cm^{-1}$. the FTIR spectrum revealed a characteristic band of $2986.39cm^{-1}$ suggested stretching of C-H bands and carboxyl group was conformed in the present study. It was an indication of exopolysaccharide production.

Further on monitoring subsequent peaks revealed a band value of $1070.49cm^{-1}$ indicate characteristic of glucan. Hence, in the present study *Bacillus cereus* is a difficult organism to synthesize exopolysaccharides. The above results coinsides with the work of Vijayabaskar etal., 2011 in tune with above discussion exopolysaccharide extract was higher in sucrose as carbon source which was found to yield a pronounced exopolysaccharides.

Determination of exopolysaccharide by High Performance Liquid Chromatography (HPLC) analysis using hexane as solvent, was analyzed. The exopolysaccharide and it is metabolite production has been quantified by HPLC by *Bacillus cereus* with basal medium. A prominent peak of 1.933 indicated the different fraction of metabolite was detected from exopolysaccharides. Besides the HPLC chromatogram with 2.013 and 2.433 represent fraction of exopolysaccharide which determine the derivative of alditol acetates which is used to determine the ratio's of monosaccharides. Besides, the other peak corresponds to hexose, deoxyhexose for bacterial exopolysaccharide production.

CONCLUSION

The marine bacteria *Bacillus cereus* collected from salt pans of Kilakarai, Ramnad district. A total of 5 bacterial strains were isolated among which EPS-1 was screened for exopolysaccharide production. These strains were identified based on phenotypic, biochemical characteristics and screened for exopolysaccharide production. During screening, EPS-1 showed dominant zones. The strains were further studied for production and optimization for exopolysaccharide production. The optimum pH was found to be pH-7 and the optimum temperature was found to be 37°c.Enhancement of exopolysaccharide production by supplementation of carbon source, was studied and the best carbon source exhibited sucrose and the nitrogen sources revealed Peptone as best nitrogen source. The NaCl concentration were studied in different ratio, the high values were revealed in 80mm of salt concentration. The production of exopolysaccharide present in 17.20 g/l. The exopolysaccharide were extracted to determine antimicrobial and antifungal efficacy. The estimation of protein content in exopolysaccaride exhibited 170 in concentration µg/ml. The carbohydrate content present in exopolysaccharide producing showed 147g/l respectively. The studies were further extended to analyze functional group changes in exopolysaccharide production wit IR spectrum of polymers has shown broad intense band at $500cm^{-1}$ – $4000cm^{-1}$. The FTIR spectral studies revealed polysaccharides revealed C-O-C and C-O-P was found at $1070.49cm^{-1}$ was typical for glucose in pyranose form.The high performance liquid chromatography analysis was performed which exhibited a prominent peak of 1.933 indicated fraction of exopolysaccharide metabolite presumed to be deoxyhexoses and hexoses produced by *Bacillus cereus* for exopolysaccharide production. The other HPLC chromatogram with 2.013 represent fraction of exopolysaccharides correspond to alditol acetates of monosaccharides.

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