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RSM for Accelerated Biofilm Formation that Facilitates Bioremediation and Characterization of Biofilm

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Abstract - Pseudomonas stutzeuri a sewage isolate has been reported to produce extracellular polymeric substances(EPS) invitro and forms high quality biofilm in natural environment .An attempt is made to increase the extracellular polymeric substances and its Biofilm forming ability of the isolate using Design Expert-8 a statistical software .Various factors like concentration of carbon source, Nitrogen source , pH, Temperature are known to influence the process of EPS synthesis. FTIR reports strongly infer the presence of anionic glycoprotein polymer with stretching- NH vibrations around 3100 - 3500nm and –CH vibration around 1637.99 indicating the presence of aldehyde group. HPLC further ensured the presence of glucose ,galactose ,rhamnose and verbacose at a concentration of 30%,22%,26%,25%and 5% respectively. Phenol sulphuric acid assay and Barfoards assay showed the presence of 60% and 30% of carbohydrate and protein components respectively. The polymer showed metal binding ability of 26% and 42% of lead and copper respectively complimented by its anionic nature. Extracted EPS showed noticeable emulsifying effect on diesel, engine oil, petrol and grease that can route this to successful tool for bioremediation.

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Abstract - Pseudomonas *stutzeuri* a sewage isolate has been reported to produce extracellular polymeric substances(EPS) invitro and forms high quality biofilm in natural environment .An attempt is made to increase the extracellular polymeric substances and its Biofilm forming ability of the isolate using Design Expert-8 a statistical software .Various factors like concentration of carbon source, Nitrogen source , pH, Temperature are known to influence the process of EPS synthesis. FTIR reports strongly infer the presence of anionic glycoprotein polymer with stretching- NH vibrations around 3100 - 3500nm and –CH vibration around 1637.99 indicating the presence of aldehyde group. HPLC further ensured the presence of glucose ,galactose ,rhamnose and verbacose at a concentration of 30%,22%,26%,25%and 5% respectively. Phenol sulphuric acid assay and Barfoards assay showed the presence of 60% and 30% of carbohydrate and protein components respectively. The polymer showed metal binding ability of 26% and 42% of lead and copper respectively complimented by its anionic nature. Extracted EPS showed noticeable emulsifying effect on diesel, engine oil, petrol and grease that can route this to successful tool for bioremediation.

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I. INTRODUCTION

A Biofilm is an aggregate of microorganisms in which cells adhere to each other on a surface. These adherent cells are frequently embedded with in a self produced matrix of extra cellular polymeric substances. (EPS) Biofilms have some major roles in Bioremediation .In brief bioremediation process uses microorganisms to remove detoxify or immobilize pollutants and does not require addition of harmful chemicals. Bioremediation is suitable for large area where contaminant concentration are relatively low and the hydrology of the soil does not support an aggressive chemical remediation statergy. In the last few years researchers have described the mechanisms of Bioremediation for numerous priority pollutants, including chlorinated hydrocarbons, polyaromatic hydrocarbons and heavy metals.

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II. BIOFILM FORMATION

Biofilm community are known to mobilize these accumulated heavy metals and involve in degrading the compound. Bacterial attachment is mediated by fimbriae and exo polysaccharide that act to form a bridge between bacteria and the conditioning film. These biofilms are used for the treatment of waste water and sewage .If the contaminated water pass through biofilm the microorganisms in the biofilm would eat and thus remove the harmful organic material from the water. They can be used for remediation of contaminated soil and ground water for cleaning up oil and gasoline spills. P.*aeruginosa* forms surface – associated communities called Biofilms. Compared with free swimming culture, Biofilms resist clearance by the host immune system and display increased resistance to antimicrobial agents[1] Psedomonas *species* forms bio emulsifier of peptide glycolipid type where the sugars are hydrophilic and create a good micelle that are semi soluble and involve them in pseudo solubilization [2].Microbial biofilm formed on abiotic surfaces is an important area of research because of the wide range of possible aspects and the disinfected resistance of the cells. The comparative and comprehensive analysis of all documented data concerning EPS production can enable the development and effective control strategies for Biofilms. The potential role of EPS has been documented by [3].Generally membrane biofilm have been believed to be minimized during the operation of membrane Biofilm Reactor for waste water treatment and reuse. The biofilm on the membrane surface was responsible for the removal of low molecular weight Organic matter by use of easily degradable organic matters. [4]The extracellular matrix produced by Bacillus *subtilis* B-1 an environmental strain forming robust floating biofilms was purified and investigated. [5]The structure of Biofilm was dramatically influenced by EPS production or capsule formation .This work is a study on the effect of various factors like pH, Temp and oraganic chemical components like carbon source and protein source using statistical software Design Expert-8 on EPS synthesis. The isolate being obtained from natural environment, forms EPS of high application value like

metal Binding ability and emulsifying property suitable for Bioremediation process.

III. Materials & Methods

a) Screening and Isolation

Number of sewage samples with high Biofilm were screened for EPS former. A Basal nutrient Broth was used for primary isolation. Nutrient Agar and Mac conkey Agar medium were used for isolation process using streak plate technique. Mucoid colonies were selected for further Biofilm forming ability. Selected colonies were then analyzed by Biochemical tests.

b) Invitro Biofilm Formation

Prepare Brain heart infusion broth and inoculate the isolates. Add 100ml of sterile BHI broth to micro titer plate in all the wells and 100μl of standardized inoculum 1x10^{8}CFU/ml (app) of various isolate into the well. Incubate for 24 - 48 hours time at preferred temp and remove the supernatant. Discard the non-adherent cells by washing with 100 μl of sterile phosphate buffer saline. Fix the biofilms by incubating them for 1 hour at 60°C and then stain with 100μl of Huckel crystal violet for 15 min was with water to remove the excess stain. Dry the plates for 30 min at 37 and resolublize the dye in 150ul of 95% v/v ethanol in the well, Measure the absorbance (A620) of the resolublized dye with the micro titer plate reader.

RSM To Enhance EPS Forming Ability. The method involves number of empirical techniques to evaluate the correlation of experimental factors and predict the critical concentration of dependent and independent variables. Based on prior experiments the factors like pH, Temp carbon source (Sugar),protein (yeast extract) were found to be major influencing factor for EPS production.

Thus these variables were selected to find the optimized condition for higher polymer production using CCD and RSM. The range & level of experimental variables investigated in this study are presented in Table – 1.

The central value zero chosen for experiment were glucose – 3gm/100ml, yeast extract – 0.3gm/100ml, pH -7 and Tem -33°C.For statistical calculations the variables X_i were coded to x_i according to equation 1.

$$X_i = x_i\,(x_i - x_i^2)/\Delta x \qquad (1)$$

After considering several experimental designs, a four variable experimental design proposed by Box Behnken was used to optimize the critical composition required for high yield of Exo polysaccharide. TABLE -2. A quadratic model was used to estimate the response of dependent variable .Where Y is predicted response and A, B, C, D are independent variables, b_0 is constant and b_1 ,b_2 ,b_3 ,b_4 are coefficients.

$$Y = b_o + b_1A + b_2B + b_3C + b_4D \qquad (2)$$

The production was optimized by using Box Behnken design when EPS production is related to independent variables by a response equation.3

$$Y = f(x_1 , x2_2 , x_3 , x_4 \ldots\ldots\ldots x_n)$$

The true relation between Y and X may be compli2cated; in most cases it is not known. A quadratic polynomial can be used to represent the function in the range interest (Annadurai & Sheeja)

$$Y = R_0 + \sum R_iX_i + \sum 2R_{ii}X_i2 + \sum\sum R_{ij}X_iX_j + E\text{--}3$$

Where X_1, X_2, are independent variables which affect the response Y. R_o , R_i , R_{ii} , R_{ij} (i=1-k and j=1-k) are known parameters, E is the random error.

IV. Characterization of Eps

a) FTIR analysis by KBR technique

The major functional groups of the EPS were identified using FTIR spectrum. 0.5 mg of dried sample was ground with 150mg of KBr crystals. The mixture was pressed using hydraulic press. The discs were subjected to FTIR analysis using Perkin Elmer IR spec.

b) NMR analysis

Bruker Advance 600 MHz sample was exchanged twice with D_2o with intermediate lyophilisation and then dissolved into 500μl of D_2O to a final concentration of 50 mg/ml, chemical shifts are reported in ppm, relative to sodium-d4-trimethylsilyl propionate for H- and $CPCl_3$ for ^{13}C – NMR spectra.

c) Emulsifying activity

EPS dissolved in 5ml distilled water (0.5 w/w) was mixed with 5 ml each of hydrophobic substances in test tube. The tubes were vortexed to homogeneity and left to stand for 24 hours at 4° C. Emulsifying activity was expressed as the percentage of total height occupied by the emulsion after 24 hours,petrol, kerosene, diesel, lubricant oil and grease were the hydrophobic substances used.

d) Heavy Metal Binding Ability

Metals salts like cobalt chloride and lead acetate were used 0.5% w/v of anionic polysaccharide were put into dialysis tubing in flask with 20ml of each metal salt solution and shaken at 1000 rpm for 24 hours at 30 ° C. The quantity of metal bound to polymer was calculated by measuring the ions in solutions at 0 hours and remaining after 24 hr by atomic absorption spectrometry. Controls were made with 5ml distilled water in dialysis tubing with various metal salt solutions.

V. Results

a) Identification

The isolate that formed high amount of Extra cellular polymeric substance was identified as P.stutzeuri. The organism formed large mucoid colonies on nutrient Agar and no colonies on Mac-conkey medium. To our surprise, the organism could also grow on high salt medium – Mannitol salt Agar proven as selective medium for staphylococcus aureus. P.Stutzeuri formed pink colonies on Mannitol salt Agar. The organism was germ negative and non-motile, non capsulated. FITR – Reports revealed the presence of amino groups and aldehyde groups specific for protein – polysacharide nature of extracellular polymeric substance. The stretching C-H vibrations around 1637.99 clearly indicates the presence of anionic carbohydrate. Bonds around 3100 to 3500 clearly ensures the presence of N-H groups which indicates presence of carbohydrates with proteins in the Exoploymeric substance.

HPLC – Reports further ensures presence of glucose, fructose, galactose, Rhamanose, verbose at a concentration of 30%,22%,26%,25% and 5% respectively. The extracted Extracellular polymeric substance could bind 26% of lead and 42% of copper as detected by Atomic Absorbtion spectrophotometer, as they were identified as anionic carbohydrates. The extracted Exopolymeric substance showed good Emulsification index on diesel, Engine oil, petrol and kerosene. (Table-3)

Statistical approach towards increased production of extracellular substances is summarized here in form of tables and equations. (Data not given fully)

Statistical testing of the model was done by partial sum squares – Type III ANOVA and the results are tabulated (Table – 4). The calculation of regression analysis gives the value of the determination coefficient (R^2 = 0.9469) which indicates that only .06%of the total variations are not explained by the model and the P value Prob F less than 0.0500 indicate model terms are significant. This proves all the factors are significant EPS forming ability. The statistical analysis of the design shows a high precision of the quadratic model that reflects the high degree of fitting between the predicted and the experimental data.(Table-1). This great similarity between the predicted and the observed results reflects the accuracy and the applicability of the Box Behnken model in the optimization of this Biofilm forming process.

VI. Discussion

Increasing population accumulates heavy pollution directly & indirectly. We indeed are in need of eco friendly chemicals and process that help us to make this planet a comfortable place for life. This thought evolved in Bio medication processes where living organisms and there products are used in removal of contaminants. In nature bacteria and fungi frequently inhabit distinct environmental riches at the interface between two phases such as air and water or water and a substratum. In these locations cells are anchored together by means of multivariate combination of biomolecules which form a barrier surrounding the cells and acts to protect against adverse conditions such as temperature or from chemical attack, such as chlorine in potable water. [6]Response of biofilms to toxic compounds has been modeled using mono-type inhibition kinetics [7] Bacterial biofilm formation is thought to enhance survival in natural environment and during interaction with hosts. [8]The Plc R mutant of B.*cereus* strain ATCC 14579 developed significantly more biofilm than the wild type and produced increased amounts of bio surfactant. Bio surfactant production is needed for Biofilm formation. [9] Bacterial extracellular polysaccharides are a key constituent of the extracellular matrix material of biofilms. [10] The extracellular polymeric substance extracted in this study is known to constitute 66% of extracellular polysaccharides and 10% of protein. Bio stabilizers are known to disperse one liquid to other. They are proteins with hydrophobic moiety and initially bind to hydrocarbon in a reversible manner. Polysaccharides then attaches to protein and stabilizes the oil in water emulsion. The EPS extracted in this study is more efficient in this process as they are reported to have high emulsifying index. The actual and potential applications of phototrophic biofilms in waste water treatment, bioremediation , fish feed production, bio hydrogen production and soil improvement. [11]

The role of bacteria P.Putida in regulating the mobility of heavy metals in the soil environment was reported .[12] P. *stutzeri* is closely related to P.*putida* species of phytogenetic tree and also it could accumulate 26% lead and 42% copper. The anionic character of biofilm enables them to interact with metal cations and to form minerals. Biofilm mediated mineral formation on plant leaves that can enhance the leaf fossilization process has also been documented .[13]

FTIR analysis of EPS in this study reported they are anionic and thus implicate proven effects on metal binding property. Studies on Bio emulsifiers from marine Streptomyces Sp S1 was documented [14]. The fact is that these EPS may have marked by different functions based on its chemistry. [16] EPS with glycoprotein nature could be applied in the field of Bio and nano-technology such as lipid films and silicon wafers[17] Thus the EPS obtained from P.*stutzeuri* may have a promising application in such fields.[18] Presence of sugar and protein may help to render the protein more soluble.It is common to examine samples from waste water to screen bacteria with unusual metabolic properties such as degradation of anthropogenic compounds for Bioremediation.

Though the conventional method is simple and easy to apply for arriving at an optimal situation for biofilm formation, using statistical method to design an optimal medium is economical and often accurate with fewer residues as experienced by the author[19] use of statistical method for optimization process was proved to be cost effective. The use of Design Expert-8 model proves to be less time consuming though it needs expertise and prior experience with the product.

Table 1 : Observed and predicted value

Test No	Observed Value	Predicted Value	Residual Value
1.	.21	.21	-3.667
2.	.33	.33	6.333
3.	.22	.23	-9.667
4.	.23	.23	3.333
5.	.21	.22	-8.16
6.	.20	.21	-9.333
7.	.24	.23	6.000
8.	.21	.20	4.833
9.	.20	.20	8.125
10.	.27	.27	8.125
11.	.21	.21	-5.70
12.	.26	.26	-5.70
13.	.27	.27	-7.083
14.	.22	.22	4.792
15.	.23	.24	-2.37
16.	.21	.21	3.125
17.	.20	.20	4.042
18.	.26	.26	-5.958
19.	.19	.18	6.875
20.	.23	.24	-3.125
21.	.24	.24	3.75
22.	.25	.24	8.75
23.	.29	.29	4.167
24.	.21	.21	5.417
25.	.23	.22	8.200
26.	.23	.22	0.011
27.	.21	.22	-0.015
28.	.21	.22	-0.015
29	.23	.22	0.010

Table 2 : ANOVA table

Sample mg/ml	Diesel	Engine oil	Petrol	Kerosene	Tween 80	Copper binding	Lead binding
.5	20%	5%	negligible	10%	20%	3%	2%
1	50%	10%	negligible	30%	28%	8%	5%
1.5	80%	4%	10%	50%	40%	17%	12%
2	100%	51%	30%	60%	78%	26%	18%
2.5	20%	5%	50%	30%	80%	42%	26%

Table 3 : Emulsifying property and Metal binding property

Source of Variation	Sum of Squares	Degree of Freedom	Mean Square	F value	P value
A	.65	1	.057	17.83	<.0001
B	0.013	1	.65	101.44	.0036
C	0.023	1	.013	44.33	.0005
D	0.018	1	.023	10.34	.0011
AB1.600E-003	1	1.600E-003	.018	0.99	-
AC2 250E-004	1	2.250E-004	1.47	<0.0001	-
AD0.010	1	0.010	0.21	0.5637	-
BC1 .006E-003	1	1.600E-003	9.16	0.3411	-
BD0.010	1	0.010	1.47	0.2761	-
CD6.250E-004	1	6.250E-.004	9.16	.0013	-
$A^2$0.012	1	0.012	.57	.2966	-
B^2.602E-004	1	2.602E-004	10.78	.1156	-
$C^2$0.043	1	0.043	0.24	.0003	-
$D^2$9.081E-003	1	9.081E-003	39.16	.0123	-
Pure error	7.348E-003	4	8.32	0.3553	-
Correlation total	.82	28	_	_	-

VII. Conclusion

To conclude we say that Biofilm forming bacteria on the whole help as more in bioremediation and using statistical software like Design Expert help us in achieving a highly efficient process with less labor and is more economical. The cell free EPS of *P.stutzeri* binds heavy metals and also shows good emulsifying activity towards problem causing hydrocarbons and serve as better tool in bioremediation .As the prevalence of *P.stutzeri* or its growth is supported by a wide range of temperature and pH this serve as a promising tool in waste management. Further studies include other factors that influence this biofilm formation and tests for other supplemented activities of this EPS.

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