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Production and characterization of a bioflocculant produced by two bacterial strains by using Response surface methodology

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Summary: Two very potentexopolysaccharidebioflocculantproducing strains were isolated from compost pit and named as JA1 and JA2. They were identified using 16S rRNA gene sequence method. FTIR analysis was done to confirm the presence of bioflocculant produced by the two bacterial strains. Response surface methodology (RSM) was employed to optimize the production medium for increasing biomass production for bioflocculant using a Plackett-Burman experimental design to aid in the first step of optimization. Edible glucose, magnesium sulphate and ammonium chloride were found to be significant factors affecting biomass production. Based on the response surface and canonical analysis, the optimum concentrations of the critical components were obtained as edible 9.7814 g/l glucose, 0.4887 g/l magnesium sulphate and 1.1704g/l ammonium chloride.

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Keywords: Bioflocculant, FTIR, Response surface methodology.

Introduction

Flocculation is a process whereby chemicals stimulate aggregation of colloids and other suspended particles in a suspension to form floc. Flocculation is an easy and effective method of removing suspended solids, colloids and cell debris etc. The flocculating agents are classified into three groups: (1) organic synthetic flocculants include polyacrylamide derivatives and polyethylene imine; (2) inorganic flocculants such as aluminum sulphate, ferric chloride and polyaluminum chloride; (3) naturally occurring flocculants are chitosan, sodium alginate and bioflocculants[1].Bioflocculant is a kind of biodegradable macromolecular flocculant secreted by various microorganisms. It is nontoxic and the degradation intermediates are not secondary pollutants [2]. Bioflocculants are mainly used for protein, glycoprotein, polysaccharide and nucleic acid [3]. Some of the chemically synthetic flocculating substances are harmful to human and environment, example the monomer of acrylamide is not only neuro-toxic and strong human carcinogen, but also non-degradable in nature. Several types of bioflocculant synthesized by some microorganisms are identified recently and a large number of them have been purified and reported to belong to functional proteins or functional polysaccharides [4]. The low yield and high cost are the major factors limitating development of bioflocculants for commercial use in wastewater treatment. The production limitations, mutational methods to obtain more efficient strain and a search for low-cost

feedstockhave been active areas of research [5].Abd-El-Haleem et al.[6]reported thepotent microorganism produces natural organic macromolecule substances that can flocculate suspended solids, cells, colloidal solids, etc. Generally, soil and activated sludge samples are the major sources for isolating biflocculant–producing microorganisms.The most reported bioflocculants are polysaccharides and proteins. Some of theproteinaceousbioflocculants are produced by *Bacillus subtillis*[7], *Bacillus licheniformis*[8], *Pacilomyces*sp. [9], *Nocardiaamarae* YK1 [10]. Few examples of polysaccharide bioflocculantsare *Alcaligeneslatus* KT201 [11],*Enterobacter*sp. [12], whileglycoproteinsbioflocculant*Arcuadendron*sp. TS-4 [13].Most of the researchers havefocused on the isolation of bioflocculant microorganisms such as bacteriabut not much work is reported using RSM for the optimization of maximum bioflocculant production from fungal sources.

Materials and methods

Sample collection

Bioflocculant-producing microorganisms were isolated from vermicompost pit, Palakkad, Kerala. Characterization of JA1 and JA2 bacterial strain was done on the basis of the colony morphology, biochemical characteristics following Bergey's manual of systematic bacteriology, Gram's staining and sequence analysis of 16S rRNA gene was performed.

16S rRNA gene sequence

The pure culture of the isolated was inoculated in nutrient broth for 24 h followed by DNA extraction using AmpurE bacterial gDNA Mini Spin kit (Amnion Biosciences Pvt. Ltd. Bangalore, India). These 16S rRNA gene fragments were amplified by polymerase chain reaction (PCR) using universal forward and reverse primers. PCR reaction mix of 50 µl final volume contained: 50 ng sample gDNA, 100 ng forward primer, 100 ng reverse primer, 2 µl dNTP's mixture (10 mM), 5 µl 10X *Taq* polymerase buffer, 3 U *Taq* polymerase enzyme and PCR grade water to make up the volume. Amplified PCR product was sequenced by using ABI3730xl genetic analyzer (Amnion Biosciences Pvt. Ltd. Bangalore, India). The sequencing result was submitted to the GenBank National Center for Biotechnology Information (NCBI) database.

Media and cultivation conditions

The agar slant consisted of (g/l): beef extract 5; peptone 10; sodium chloride 20; agar, 20. The seed medium contained (g/l): glucose 10; NH_4Cl 1; $MgSO_4$. $7H_2O$ 0.5; yeast extract 0.6; NaCl 20; KH_2PO_4 2 and K_2HPO_4 5. The primary production medium consisted of (g/l) : edible glucose 10; $MgSO_4.7H_2O$ 0.5; NH_4Cl 1; yeast extract 0.6; NaCl 24; KH_2PO_4 2 and K_2HPO_4 5. The initial pH of all media was adjusted to 7.5 – 8.0 with HCl and NaOH. Seed preparation, a colony of cells was picked from an agar plate culture, placed into 50 ml of sterile seed medium in a 100 ml Erlenmeyer flask, and incubated at 28°C with shaking at 160 rpm for 22h. 50 ml of the production medium in a 250 ml flask was inoculated with 3% (v/v) of the seed culture, which contained approximately 2.7×10^7 cells/ml, and incubated at 28C and 160 rpm for 48 h.

Bioflocculant purification

To quantify JA1 and JA2 production, the fermentative cultures were centrifuged at 9800× g for 30 min at 4 °C. The supernatant was precipitated by the addition of 2.5 volumes of chilled waterless ethanol, incubated at 4° C for 24 h, and then centrifuged at 9800 × g for 15 min. The precipitate was collected, washed twice using 70% (v/v) ethanol, and then dissolved in distilled water. This procedure was repeated thrice, and final precipitate was dried at 80° C until a constant weight was achieved using a analytical balance [14].

Determination of flocculating rate

Flocculation activity was measured according to the method described by Kurane et al.[11]. Briefly, 5.0 ml of a 1% (w/v) $CaCl_2$ solution and 0.2 ml of a centrifuged fermentation culture supernatant were added in turn to 95 ml of kaolin suspension (5.0 g/l, pH 8.0). The mixture was stirred at 200 rpm for 1min, slowly stirred for 80 rpm for 5 min, and allowed to stand for 5 min. The optical density (OD) of the supernatant was measured with a spectrophotometer at 550nm. In the control experiment, 1 ml of culture broth was replaced with 1ml of fresh culture medium. The flocculating activity was calculated according to the following equation [15]:

Flocculation rate = (B-A)/ B× 100 %

where A and Bwere the OD 550 (optical density at 550 nm) of control and sample supernatant, respectively.

FT-IR spectroscopy analysis of purified bioflocculant

The functional groups of the bioflocculant were determined using Fourier transform infrared (FTIR) the frequency range of 4,000-500 cm^{-1} with a Fourier transform infrared (FTIR) spectrophotometer (8400 Shimadzu, Japan, with Hyper IR-1.7 software for Windows) with a helium-neon laser lamp as a source of IR radiation. Precipitate were prepared by grinding the extracted samples with potassium bromide in a mortar with 1:100 ratio and immediately analyzed in the region of 4,000-400 cm^{-1} at a resolution of 4 cm^{-1}.

Response surface methodology (RSM)

The response surface methodology is an empirical statistical design that conducts multiple regression analysis of the input data to solve variable equations simultaneously [16].The experiment was conducted to optimize the medium composition for the growth of bacterial strain JA1 and JA2 using Plackett-Burman (PB) design with the Minitab16 software package. Analysis of Variance (ANOVA) was used for the data analysis to obtain the interaction between the process variables and the responses.

Before RSM was applied, appropriate culture conditions for JA1 and JA2 were determined by varying one factor at atime and keeping others constant. These preliminary studies revealed the best carbon and nitrogen source as glucose and ammonium chloride for JA1 as well as JA2. $MgSO_4$ was also supposed to have positive effects on bioflocculant production.

Fractional factorial design (FFD)

FFD are the initial optimization steps to identify which component(s) of the medium is most important. Its purpose is to check for the important nutrients and interactions between them with a set of few experiments rather than one by one. It helps in reducing the number of experiments with all the variables taken into consideration. The variable identification was done using 2 - level FFD. The variables considered for the design are listed in table 1. The FFD analysis was conducted with Minitab 16^{TM} software taking each

Table 1.Coded values of the variables of FFD.

Variables	Level of variables				
	-α	-1	0	+1	+α
Glucose	0	5	10	15	20
Magnesium sulphate	0.1	0.5	1.5	2.5	2.9
Ammonium chloride	0.1	0.5	1.5	2.5	2.9

variable at high (+1) and low (-1) level. The variables were coded according to the following equation:

$$xi = \frac{Xi - Xo}{\Delta Xi}$$

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where, xi is the coded value of an independent variable, Xi is the real value of an independent variable, Xo is the real value of an independent variable at the centre point, and ΔXi is the step change value[17]. If there is a significant difference between the means of the centre points and factorial points ($p<0.05$), the optimum range would lie within the design space.

Central composite design (CCD)

To obtain the optimum bioflocculant activity a 3-factor central composite design (CCD) was employed based on the identification of critical factors through screening. For the 3 significant factors, the design was made with full 2^3 factorial design. According to this design, the setup included 20 experiments with 6 replicates at the centre point. Further CCD was developed for the variables significantly affecting the bioflocculant production. The CCD of these three variables is given in figure 1. Analysis of Variance (ANOVA) was used for the analysis of the data to obtain the interaction between the process variables and the responses.

Fig 1. CCD experimental set up for JA1 and JA2.

Result and discussion

Bacterial Identification and 16S rRNS gene sequence

The bioflocculant producing bacterial strains (JA1 and JA2) were isolated from vermicompost soil and identified as Gram negative bacteria. The molecular characterization with 16S rRNA gene sequencing for both the strains JA1 and JA2 illustrated high degree of similarity with *Bacillus simplex* and *Pseudomonas moraviensis* respectively. Thus both the bacterial isolates were named as *Bacillus simplex* strain JAJDV1 and *Pseudomonas moraviensis* strain JAJDV2. The relationship between the isolate and the nearby phylogenetic relatives is given in the form of phylogenetic tree drawn using Mega 5 software. Figure 2 represents the tree for *Bacillus simplex* strain JAJVD1 and figure 3 related to *Pseudomonas moraviensis* strain JAJVD2.

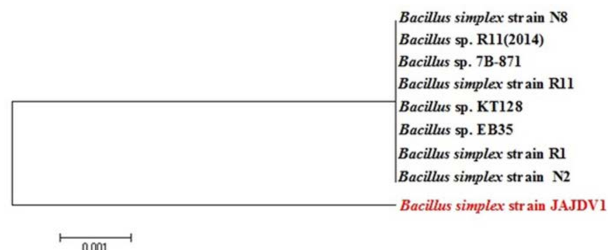

Fig 2.Phylogenetic tree of *Bacillus simplex* strain JAJVD1.

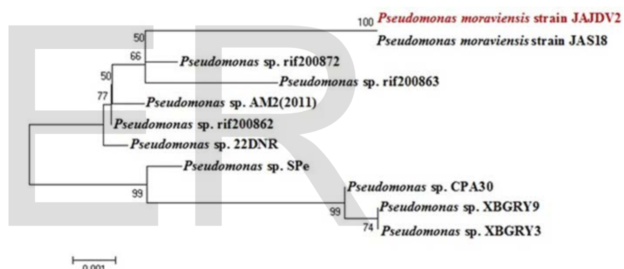

Fig 3. Phylogenetic tree of *Pseudomonas moraviensis* strain JAJVD2.

Effect of bioflocculant concentration

The relation between the concentration of JA1 and JA2 and its flocculating rate in kaolin suspension was investigated. The flocculating rates were higher than 90% when JA1 and JA2 the maximum range at 1.2 and 2.0 mg/l. It was reported that the flocculating activities of exopolysaccharidebioflocculants from *Sorangiumcellulosum*NUST06 and *Gyrodiniumimpudicum*KG03 were highest at 20 mg/l and 1.0 mg/l, respectively [18,19]. That suggested the efficiency of JA1 and JA2 in flocculating reaction at similar concentration to other polysaccharides.

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Analysis of the purified bioflocculant

Fourier- transform infrared (FTIR) spectrum of the pure bioflocculantJA1 showed broad absorption band at 3469 cm^{-1}, 3441 cm^{-1}, 2985 cm^{-1}, 2900 cm^{-1} O-H stretch carboxylic groups and 2013 cm^{-1} C≡N stretch, 1641 cm^{-1} C=O stretch and 1631 cm^{-1} N-H bend. The two other band at 1400 cm^{-1} N=O bend nitro group was present and 1078 cm^{-1}, 1045 cm^{-1} C-O stretch was presented. The JA2bioflocculant showed broad absorption band at 3439 cm^{-1}, 3417 cm^{-1}, 3242 cm^{-1} O-H stretch, 2927 cm^{-1} H-C-H asymmetric and symmetric stretch, 1631 cm^{-1}C-C=C symmetric stretch, 1400 cm^{-1} N=O bend was presented as given in figure 4.

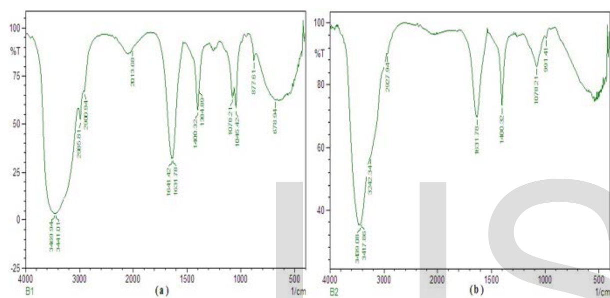

Fig 4. FTIR analysis of (a) *Bacillus simplex* strain JAJVD1 (b) *Pseudomonas moraviensis* strain JAJVD2.

Plackett-Burman (PB) design

The prominent effects of edible glucose, magnesium sulphate and ammonium chloride were likely due to the requirement of these medium components for substantial cell growth. Similarly, ammonium was reported to be important for the production of polysaccharide from a *Porphyridium* sp. [20]. Previous work also indicated that certain carbon to nitrogen (C/N) ratios performed an important function in microbial metabolism, including changing fatty acid composition from the heterotrophic *Chlorella sorokiniana* [21] and enhancing biological hydrogen production from *Clostridium pasteurianum*[22]. As an essential inorganic ion, magnesium was involved in many physiological functions, such as enzyme activity, cell growth, and cell division [23,24]. Therefore, it was likely that glucose is a played a key role in the metabolism of JA1 and JA2.

Three dimensional response surface plots graphically represent regression equations and are generally used to demonstrate relationships between the response and experimental levels of each variable. These surface plots, therefore, allow for visualization of the optimum levels of each variable for the maximum production of microbial metabolites [25]. Figure 5(JA1) and figure 6 (JA2) showed the response surface plots for the present study and illustrated the pair–wise interaction of the three variables. ANOVA table for both the samples is given in table 2 and table 3 for JA1 and JA2respectively. Accccording to the canonical analysis, the optimal concentrations of edible glucose, magnesium sulphate and Ammonium chloride were 14.1941, 9.7814 g/l, 1.1704, 0.4887 and 0.3296, 1.1704 g/l respectively. The maximum JA1 and JA2 biomass production corresponding to bioflocculant production was estimated to be glucose 14.1941, 9.7814 g/l and actual production obtained with the optimized medium was 14.1941 which is in close agreement to the model prediction.

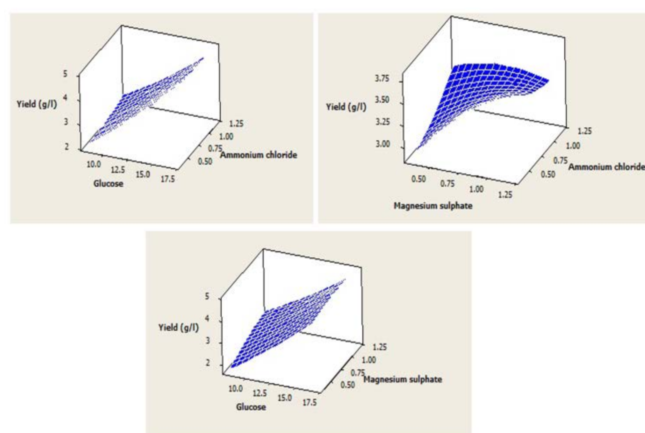

Fig 5. Response surface graphs of JA1.

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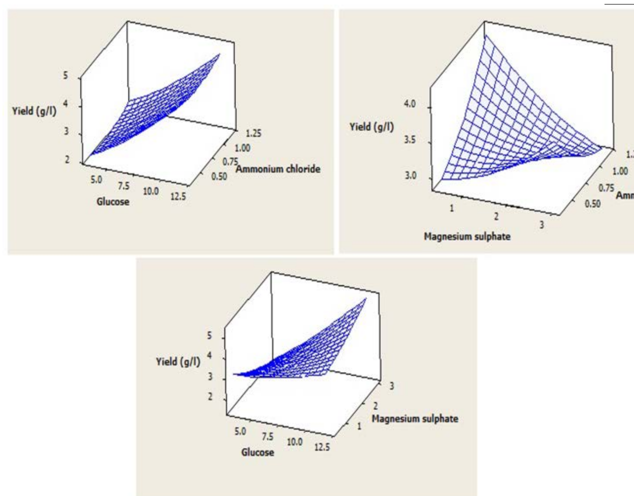

Fig 6. Response surface graphs of JA2.

Table 2.ANOVA table of JA1.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	7.80642	7.80642	0.86738	150.19	0.000
Linear	3	7.67161	7.67161	2.55720	442.80	0.000
Glucose	1	7.40100	7.40100	7.40100	1281.54	0.000
$MgSO_4$	1	0.26852	0.26852	0.26852	46.50	0.000
NH_4Cl	1	0.00209	0.00209	0.00209	0.36	0.561
Square Glu*Glu $MgSO_4$* $MgSO_4$ NH_4Cl* NH_4Cl	3 1 1 1	0.05018 0.01851 0.01966 0.01201	0.05018 0.01771 0.01655 0.01201	0.01673 0.01771 0.01655 0.01201	2.90 3.07 2.87 2.08	0.088 0.110 0.121 0.180
Interaction Glu* $MgSO_4$ Glu*NH_4 Cl,$MgSO_4$ *NH_4Cl	3 1 1 1	0.08464 0.02101 0.01711 0.04651	0.08464 0.02101 0.01711 0.04651	0.02821 0.02101 0.01711 0.04651	4.89 3.64 2.96 8.05	0.024 0.086 0.116 0.018
Residual error	10	0.05775	0.05775	0.00578		
Lack of Fit	5	0.00820	0.00820	0.00164	0.17	0.956
Pure error	5	0.04955	0.04955	0.00991		
Total	19	7.86417				

Table 3.ANOVA table of JA2.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	7.79379	7.79379	0.86598	344.89	0.000
near	3	6.86554	6.86554	2.28851	911.43	0.000
ucose	1	6.76832	6.76832	6.76832	2695.58	0.000
gSO_4	1	0.06098	0.06098	0.06098	24.28	0.001
H_4Cl	1	0.03624	0.03624	0.03624	14.43	0.003
uare u*Glu gSO_4* gSO_4 H_4Cl* H_4Cl	3 1 1 1	0.11614 0.03748 0.03571 0.04296	0.11614 0.05263 0.04352 0.04296	0.03871 0.05263 0.04352 0.04296	15.42 20.96 17.33 17.11	0.000 0.001 0.002 0.002
teraction u* gSO_4 lu*NH_4 ,$MgSO_4$ NH_4Cl	3 1 1 1	0.81211 0.52788 0.00035 0.28388	0.81211 0.52788 0.00035 0.28388	0.27070 0.52788 0.00035 0.28388	107.81 210.24 0.14 113.06	0.000 0.000 0.716 0.000
esidual ror	10	0.02511	0.02511	0.00251		
Lack of Fit	5	0.02202	0.02202	0.00440	7.12	0.025
Pure error	5	0.00309	0.00309	0.00062		
Total	19	7.81889				

Conclusion

This study showed that JA1 and JA2 is a bioflocculant-producing strain, it produced commendable bioflocullation. The JA1 and JA2 strain showed excellent flocculating rate of kaolin suspension .Response Surface Methodology (RSM) was employed to optimize the medium components for JA1 and JA2 biomass production from RSM was a reliable tool to optimize JA1 and JA2biomass production. Compared with the initial culture medium JA1 and JA2 was increased from 2.12 to 4.56 g/l after optimizing medium components.

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