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Optimized Bioflocculant production from Fungi using Response Surface Methodology

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Abstract: In the present investigation bioflocculant production from fungiwas investigated as microbial bioflocculant has advantages over chemical flocculant. They are found to be economical, potential flocculating effects, biodegradability and harmless to humans and the environment. Based on these properties it is proved to be advantageous in many ways and may potentially be applied in production industries for various purposes. In this study, a total of four fungal isolates were obtained and the isolates were screened using screening media for flocculant production, measured using the kaolin solution as a test material. The flocculant activity was measured using UV-Vis spectroscopy and studied for its bioflocculant activity. The functional groups were analysed by Fourier transfer infrared radiation (FTIR). Further, for better quantification of the flocculant production, Response surface methodology (RSM) was used to design an experimental set up which could be used to study the effect of different parameters on the bioflocculant production.
Keywords: Bioflocculant, screening media, flocculant activity, Response surface methodology.

Introduction

Majority of today's industries are opting to use flocculating agents in processes such as fermentation and waste water treatments. Flocculants help in the aggregation of the cellular and colloidal substances which aids in easy separation[1]. Flocculants can be classified as (1) inorganic flocculants such as aluminium sulphate or polyaluminum chloride, (2) organic synthetic polymers such as polyacrylamide derivatives or polyethylene imines, and (3) flocculants from biological resources, such as chitosan and microbial extracellular macromolecules[2]. Bioflocculants are produced by a diverse group of bacteria, fungi, actinomycetes, yeasts and algae. In contrast to the chemical flocculants, the bioflocculants are more widely used as it is a non-toxic and easily degradable substance.

The composition of these bioflocculants can be polysaccharide, proteins or even glycoprotein. The bioflocculants are most often extracellular[3]. Bioflocculants have certain advantages when compared to their chemical counterparts with respect to ecological safety and treatment[1]. Bioflocculants possess flocculation and adhesion properties, similar to some amphoteric polymeric flocculants. These materials are desired for their properties of conventional flocculants, biodegradability and eco-friendly nature[4], and can be uniformly and reliably produced by fermentation processes[5]. The mechanisms of flocculation are thought to be either by the bridging theory and the patch model[4]. Even though a number of bioflocculants have been discovered only a small number of these have been used as it has been hard to actualize industrialization of these bioflocculants

due to culturing costs and low production yield[6]. Thus, research is being conducted on bioflocculants in order to reduce bioflocculant production costs and increase flocculant yield and applicability[7].

A group of researchers reported that the media composition also plays a vital role in obtaining bioflocculants in high yields[1], therefore the present study was undertaken to involve the optimization of parameters using Response Surface Methodology (RSM) in order to get an optimal flocculant activity.

Materials and Methods

Sample collection and isolation of fungal strains The soil sample was collected from Ambur textile industry, Tamil Nadu, India.The fungi were isolated from the soil by performing serial dilution and plating them onto PDA plates. The plates were incubated at room temperature for 7 days. The fungal strains thought to have potential flocculation activity was sub-cultured and screened for bioflocculant activity.

Screening of the fungal isolates for bioflocculant production.

The fungal isolates were screened for the bioflocculant production using screening medium, bioflocculant production broth (BPB) which consists of: 10g glucose, 2g KH_2PO_4, 0.2g $MgSO_4.7H_2O$, 0.1NaCl, 0.5g $CaCO_3$, and 0.5g yeast extract for 1000ml and the pH was maintained at 7.0.the medium was sterilised and was inoculated with the pure fungal strain and was incubated on a rotary shaker at 120 rpm, 37 ° C and for 3 days.

Screening for optimum carbon and nitrogen source

To evaluate the required amount of carbon and nitrogen source for the bioflocculant production aliquots of 2 mL were inoculated into 200 mL of sterile basal salt media (fermentation media) composed of the following (g/L): glucose, 10; $CaCl_2$, 0.15; K2HPO4, 0.5; $FeCl_3$, 0.04 and MgSO4·7H2O, 0.5;$MnSO_4$, 0.14g. The fermentation medium was adjusted to pH 7 and incubated at a temperature of 30 °C with an agitation speed of 160 rpm for a period of 72 h. The broth, after the incubation period, was centrifuged at 3,000 rpm for 30 min at 15 °C and the cell-free supernatant was assessed for flocculation activity. Fructose, sucrose, lactose, maltose and starch respectively served as sole carbon sources, while the nitrogen sources evaluated included urea, ammonium sulphate, ammonium nitrate, ammonium chloride, peptone, monovalent salts (KCl and NaCl), divalent salts ($MgSO_4$,$CaSO_4·H_2O$, $MnCl·4H_2O$, and $FeSO_4$) and trivalent salts ($FeCl_3$), respectively.

Determination of flocculating activity

The flocculating activity was determined by using a suspension of kaolin clay as a test material for flocculating activity determination. Using a suspension of kaolin clay as test material, flocculating activity was determined according to Kurane et al.[8] as modified by Gao et al.[6] The kaolin clay was suspended in distilled water at a concentration of 5 g/L at pH 7 and was used as stock solution. The kaolin clay suspension (9 mL), culture supernatant (0.1 mL) and 1% CaCl2 (0.25 mL) were mixed in a test tube. A reference tube in which the culture supernatant (control) was replaced with distilled water was also included and measured under the same conditions. The final volume of the mixture was made up to 10mL with distilled water. After mixing gently, the solutions were allowed to settle for 5 min. at room temperature. The optical density (OD) of the clarifying upper phase solution was measured at 550nm with a UV spectrophotometer and the flocculating activity determined as follows:

$$\text{Percent flocculating rate} = [(B - A) / B] \times 100\%$$

where, A and B are optical densities at 550 nm of the sample and control respectively.

FTIR analysis

The supernatant containing the bioflocculant were tested for the functional groups present which was determined using a Fourier transform infrared (FT-IR) spectrophotometer (2000 FTIRS Spectrometer; Perkin Elmer Systems, Waltham, MA, USA) over a wave number range of 4000 to 500 cm^{-1}. The FT-IR spectrum was obtained from the potassium iodide with the sample. The sample was later mixed with KBr powder to form pellet and analyzed using the diffuse reflectance accessory.

Response Surface Methodology

Critical media components for the production of bioflocculant by the mixed culture were assessed using the Plackett-Burman (PB) design in an "n" variable screening of n + 1 experiments. The carbon, nitrogen and cation sources yielding optimal flocculation activity were evaluated with other media components. The "n" variables were glucose (10-12.5g), $CaCl_2$ (0.3-0.5), NH_4Cl_2 (1.0-1.5g), K_2HPO_4 (5-6.5g) and were investigated at two levels (concentrations) of each variable, ''high" and ''low" which were used and was designated as +1 and −1 respectively. All experimental trials were carried out in triplicate and the average flocculation activity was used as the response variable.

Results and Discussion

Isolation and screening of fungal strains

Three isolates were obtained from the soil sample upon serial dilution on PDA medium. All the three isolates were subjected to screening for bioflocculant activity. Out of the three isolates, one fungal strain showed 75% flocculant activity while the other two showed less than 50% of flocculant activity. Thus, the most potent fungal strain was taken into consideration for further experimental purposes.

Determination of flocculant activity

The flocculant activity was detrmined based on the UV-Vis spectroscopic analysis. After centrifugation the optical density of the sample was measured at 550 nm and further the flocculant activity was calculated to be 75% for PA.

IR spectra of the bioflocculant produced indicate the presence of two peaks at 3329.14 cm^{-1} which is strong and broad and 1613.78 cm^{-1} having medium narrow peak (Figure 1). The first peak corresponds to the presence of (O-H) stretch in the compound which is H-bonded. This peak points towards the existence of alcohols or phenols as the functional groups in the compound isolated. Similarly, N-H bend was present at 1613.78 cm^{-1} showing the presence of amines in the structure of the bioflocculant.

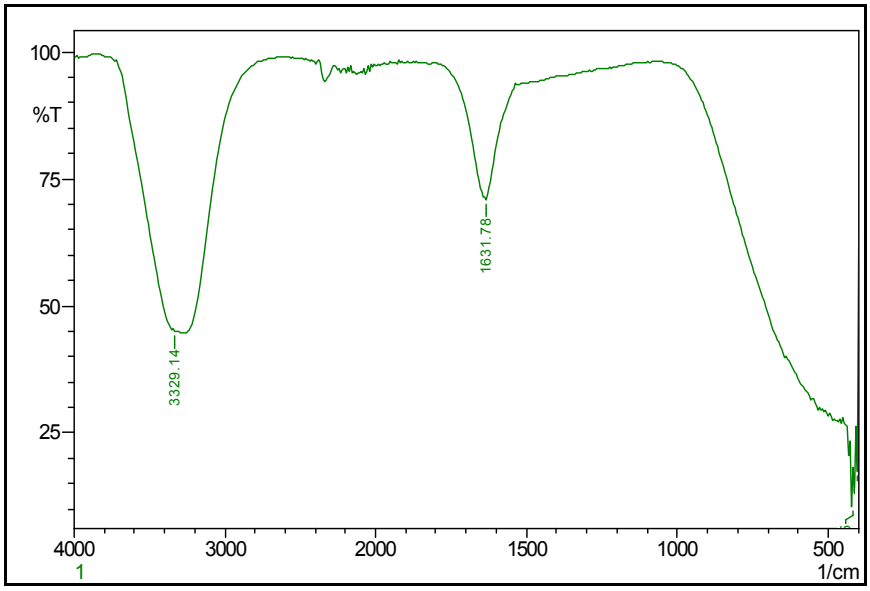

Figure 1. FTIR analysis of the bioflocculant produced.

Table 1. The OD obtained after incubation at 600nm is tabulated below

Run No	OD at 600nm	% activity
Control	1.206	
1	0.889	46.8852405
2	3.017	52.56584
3	1.219	19.5220564
4	0.488	80.1356551
5	0.691	63.3031509
6	0.534	78.0936013
7	0.971	40.0859038
8	1.012	36.6862355
9	0.817	52.8553897
10	0.968	40.33466
11	1.815	41.9930348
12	0.948	49.897512

Table1: Results of RSM indicating percentage bioflocculant activity.

Response Surface Methodology

The fungal strain showing maximum bioflocculant activity was taken up for further studies and RSM (response surface methodology) was carried out for this strain. The RSM was carried out using Plackett-Burman Design, which involved selection of critical parameters which might affect the growth of the organism and further affect the production of the bioflocculant. The parameters that were chosen for the Plackett-Burman Design were pH, temperature, percentage of glutamine/glutamic acid, carbon and nitrogen sources and inoculums size. The statistical software gave 12 experimental designs with different combination of the above mentioned parameters (Figure 2). The flocculation activity obtained upon incubation was extracted using the supernatant and kaolin clay and $CaCl_2$, measuring the OD at 550nm.

pH	Temperature	Glutamic acid (%)	Carbon source (%)	Nitrogen source (%)	Inoculum size (%)
4	35	1	0.2	0.2	3
7	28	5	0.2	0.2	1
7	35	5	0.2	2.0	3
4	35	5	2.0	0.2	3
7	28	1	0.2	2.0	3
4	28	1	2.0	2.0	3
4	35	5	0.2	2.0	1
7	28	5	2.0	0.2	3
4	28	1	0.2	0.2	1
7	35	1	2.0	0.2	1
4	28	5	2.0	2.0	1
7	35	1	2.0	2.0	1

Figure 2. FFD values of the experiment obtained using Minitab16 software.

The 3D response surface plots are an indicative of regression equations which tells us the relation between the variables and the response which in the present case is bioflocculant activity. These surface plots, therefore, allow for visualization of the optimum levels of each variable for the maximum production of microbial metabolites[9]. Figure 3 represents the response surface graphs for the bioflocculant production from PA. The ANOVA table give the R^2 value to be 98.46% indicating there is a statistical significance and also the $p<0.5$. The optimum hold values were obtained to be temperature 35 ˚C, pH 4, percentage glutamine at 5%, carbon source at 2%, nitrogen source at 0.2% and an inoculums size of 3% yielded the best results giving a flocculant activity of 80%.

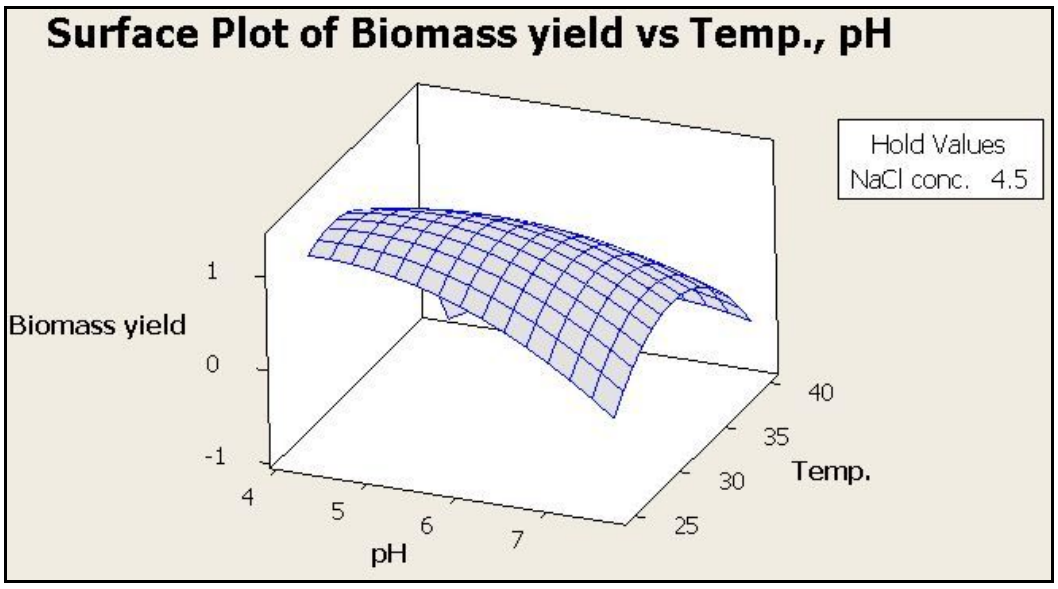

Figure 3. Surface plot of bioflocculant production against temp and pH.

There have been reports that carbon and nitrogen (C/N) ratios perform an important function in microbial metabolism, including changing fatty acid composition from the heterotrophic *Chlorella pasteurianum*[10]. Similarly, ammonium was found to be important for the production of polysaccharides from a *Porphuridium* sp.[11] Thus, it can be concluded that Response surface methodology was found to be an acceptable and proficient statistical tool in improving the bioflocculant production by optimization of the culture conditions. Figure 4 represents the contour plot of biomass against temperature and pH indicating the best fit for growth.

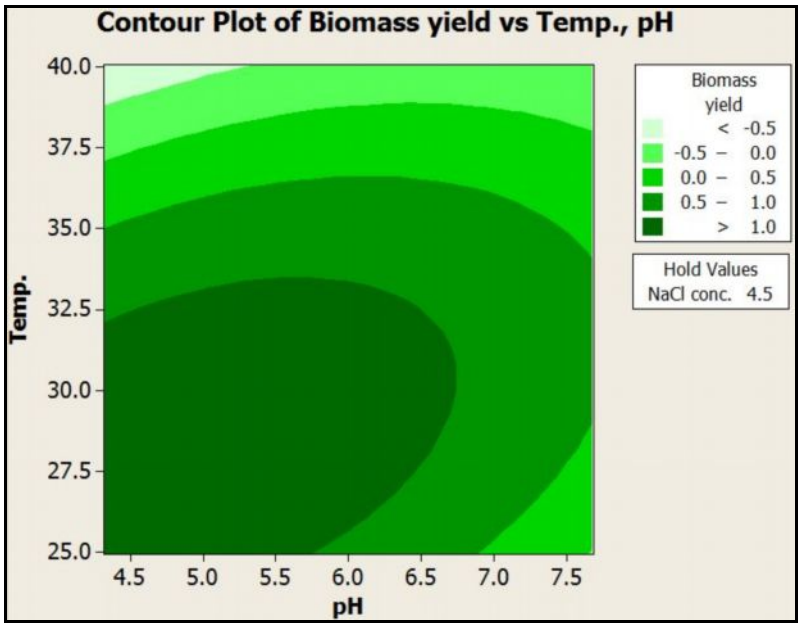

Figure 4. Contour plots for maximum biomass yield.

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