Production and Flocculating Performance of Bioflocculant by Bacterial Strain and its Application for Municipal Wastewater Treatment

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Abstract

In the present research, bioflocculant was produced from Bacillus subtilis using molecular methods. The structural and functional features of the bioflocculant were determined that it consisted of 64% carbohydrate and 18% protein. Production medium for bioflocculant mainly comprised Sucrose 16 g; Peptone 2 g; MgSO\(_4\) · 7H\(_2\)O 0.3 g were statistically optimized to increase the bioflocculant production. They were further characterized by Field emission scanning electron microscopy (FE-SEM), and Fourier-transform infrared spectroscopy (FTIR). Therefore, they could be considered as a suitable method for treating municipal wastewater based on high flocculating rate.

Keywords: Bioflocculant, Wastewater, RSM, Kaolin, Treatment, COD, BOD.
INTRODUCTION

Bioflocculant is a polymeric substance secreted by a large group of microorganisms such as bacteria, fungi etc.\(^1\)\(^-\)\(^3\). They are the macromolecular polymer such as polysaccharide, nucleic acid, and glycoprotein\(^4\). The bioflocculant consists of monosaccharide units such as mannose, rhamnose, or glucose\(^5\). Generally, flocculants are divided into three components such as (i) inorganic flocculants (ii) organic flocculants (iii) synthetic flocculants. However, these flocculants cause environmental toxic issues, and health issues to animals and humans\(^6\). On comparing the bioflocculant secreted by the microorganism, with synthetic flocculants\(^7\)\(^,\)^8, they are non-toxic, harmless and biodegradable. Therefore, research has been focused recently on bioflocculant.

Many bioflocculant produced from various microorganisms has been described such as *Proteus mirabilis*\(^9\), *Bacillus firmus*\(^10\), *Azotobacter* and *Bradyrhizobium*\(^11\), *Streptomyces* and *Cellulomonas*\(^3\), and *Aspergillus*\(^12\). Bioflocculant have been extensively used to treat brewery wastewater, meat processing wastewater, river water, starch wastewater\(^13\)\(^,\)^14, molasses wastewater\(^15\), dye solutions\(^16\), and to purify drinking water at low temperature\(^17\).

In this study, a novel bioflocculant-producing strain was isolated from the sewage water and was identified as *bacillus subtilis* by 16S rDNA sequence analysis. Further, research has focused to optimize the medium composition to enhance bioflocculant production via Response surface methodology (RSM). The major components of the bioflocculant were studied. Various factors (dosage of the bioflocculant, time, pH, electrolytes) affecting the flocculation process were investigated to find out the flocculating activity in Kaolin suspension. Finally, bioflocculant developed from *bacillus subtilis* was used in the municipal wastewater to reduce BOD, COD and other physico-chemical parameters.

MATERIALS AND METHODS

Bioflocculant producing bacterial strain isolation

Sewage sample was collected from the drainage mixed into the Cauvery in an airtight bottle. Serial dilutions were carried out from processed water sample in the nutrient broth. Distinct colonies in their morphological characteristics were selected and inoculated into 50 ml fermentation medium and kept it for 48 hrs at 37°C with continuous shaking. After the incubation, culture broth were analyzed to observe the flocculating activity. Based on flocculating activity, the strain was selected, then stored at 4°C.

Identification of strain

The selected strain was inoculated into LB medium for 16 hrs at 37°C with continuous stirring at 150 rpm. The isolated genomic DNA from the selected strain was further subjected to PCR analysis. The primers used were 5'-AGAGTTTGATC(C/A) TGGCTCAG-3' (forward) and 5'-TACGG(C/T)TACC TTGTTACGACTT-3' (reverse). The PCR program consists of 30 cycles with 94°C (1 min), 55°C (30 s), and 72°C (1.5 min)\(^18\). The sequence

| No. | Sucrose | MgSO\(_4\) | KH\(_2\)PO\(_4\) | K\(_2\)HPO\(_4\) | Peptone | Sodium chloride | Yield (g/L) observed value | Yield (g/L) predicted value |
|-----|-------|--------|-----------|----------|------|---------------|------------------------|--------------------------|
| 1.  | 10    | 0.3    | 2.5       | 5        | 1.5  | 0.02         | 3.80                   | 3.80000                  |
| 2.  | 12.5  | 0.3    | 2.5       | 5        | 0.5  | 0.2          | 2.20                   | 2.26667                  |
| 3.  | 10    | 0.5    | 0.5       | 3        | 0.5  | 0.2          | 3.60                   | 3.63333                  |
| 4.  | 10    | 0.5    | 2.5       | 5        | 0.5  | 0.2          | 3.00                   | 3.20000                  |
| 5.  | 10    | 0.5    | 2.5       | 3        | 1.5  | 0.02         | 2.50                   | 2.90000                  |
| 6.  | 10    | 0.3    | 0.5       | 5        | 1.5  | 0.2          | 3.23                   | 3.23333                  |
| 7.  | 12.5  | 0.3    | 0.5       | 3        | 1.5  | 0.2          | 1.00                   | 1.40000                  |
| 8.  | 12.5  | 0.3    | 0.5       | 3        | 0.5  | 0.02         | 4.00                   | 4.53333                  |
| 9.  | 12.5  | 0.5    | 2.5       | 3        | 1.5  | 0.2          | 0.50                   | 0.06667                  |
| 10. | 12.5  | 0.5    | 0.5       | 5        | 0.5  | 0.02         | 3.00                   | 2.90000                  |
| 11. | 12.5  | 0.3    | 2.5       | 3        | 0.5  | 0.02         | 3.20                   | 3.26667                  |
| 12. | 12.5  | 0.5    | 0.5       | 5        | 1.5  | 0.02         | 0.50                   | 1.60000                  |
obtained was compared with the NCBI database and was identified as *Bacillus subtilis*.

**Media component optimization with RSM**

Plackett–Burman (PB) strategy is the tool used to recognize significant components that affect the bioflocculant production. In the study, six medium components were studied such as Sucrose, Peptone, KH$_2$PO$_4$, K$_2$HPO$_4$, NaCl, and MgSO$_4$. All the variables were evaluated in 12 trials. Selected three components (Sucrose, Peptone, and MgSO$_4$) were optimized by RSM. The optimized result was subjected to analysis of variance (ANOVA).

**Production and purification of the bioflocculant**

*Bacillus subtilis* was inoculated into the medium designed and incubated at room temperature with continuous stirring for 72 hrs$^{19}$. The bioflocculant derived from *Bacillus subtilis* strain was partially purified based on the previously described method$^{20}$. The broth was centrifuged at 12000 rpm for 10 min. Chilled ethanol (1:3) was added with the supernatant followed by centrifugation at 12000 rpm for 10 min. The precipitate obtained was then dissolved in deionized water.

**Structural analysis of the bioflocculant**

Phenol sulfuric acid method were used to determine the sugar content of the bioflocculant$^{9}$. Similarly, protein content of bioflocculant was determined by the Lowry method$^{21}$. They were exposed to Fourier transform infrared spectroscopy analysis (FT-IR; Cary 630, Agilent, and USA). Further, it was subjected to SEM (SUPRA 55 SAPPHIRE; Germany)$^{22}$.

**Determination of the flocculating activity**

Kaolin suspension (4.0 g/L) was added with varying amounts of bioflocculant solution (2–100 mg/L) and 9 mM CaCl$_2$ solution (5 mL) at pH 7.0. The flocculating activity was then determined according to the literature$^{23}$. Effects of pH (3-9), effect of time (0-300 min), effect of dosage (0-100 mg/L) and effects of different cations on the flocculating activity were examined to determine the maximum flocculation. KCl, NaCl, MgCl$_2$, CaCl$_2$ were tested as cationic sources.

**Application in Wastewater Treatment**

The experimental tanks were filled with 50 L of sewage water. 50 mg/L of bioflocculant and 10 mg/L CaCl$_2$ solution were added with the sewage to evaluate its effect on the wastewater. Untreated raw wastewater was considered as the control sample. Finally, parametric measurements such as chemical oxygen demand (COD), suspended solids (SS), and biological oxygen demand (BOD) were determined before and after the treatment. The experiment was repeated thrice and its

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**Table 2. Analysis of variance showing fitted quadratic polynomial model for optimization of bioflocculant production**

| Source          | DF | Seq SS   | Adj SS   | Adj MS  | F      | P       |
|-----------------|----|----------|----------|---------|--------|---------|
| Regression      | 9  | 16.3358  | 16.3358  | 1.81509 | 16.08  | 0.000   |
| Linear          | 3  | 7.1558   | 7.1558   | 2.44688 | 21.68  | 0.000   |
| Sucrose         | 1  | 0.5044   | 0.48428  | 0.48428 | 4.29   | 0.065   |
| Peptone         | 1  | 0.7992   | 0.9927   | 0.99269 | 8.80   | 0.014   |
| MgSO$_4$        | 1  | 5.8522   | 5.8522   | 5.85220 | 51.85  | 0.000   |
| Square          | 3  | 2.2622   | 2.2622   | 0.75408 | 6.68   | 0.009   |
| Sucrose * Sucrose | 1 | 1.9433   | 2.1435   | 2.14354 | 18.99  | 0.001   |
| Sucrose * Peptone | 1 | 0.3183   | 0.3051   | 0.30507 | 2.70   | 0.131   |
| MgSO$_4$ * MgSO$_4$ | 1 | 5.8522   | 5.8522   | 5.85220 | 51.85  | 0.000   |
| Interaction     | 3  | 6.9178   | 2.30593  | 2.30593 | 20.43  | 0.000   |
| Sucrose*Peptone | 1  | 4.6208   | 4.6208   | 4.62080 | 40.94  | 0.000   |
| Sucrose* MgSO$_4$ | 1 | 0.9522   | 0.9522   | 0.95220 | 8.44   | 0.016   |
| Peptone * MgSO$_4$ | 1 | 1.3448   | 1.3448   | 1.34480 | 11.92  | 0.006   |
| Residual Error  | 10 | 1.1286   | 1.1286   | 0.11286 |        |         |
| Lack-of-Fit     | 5  | 0.2387   | 0.2387   | 0.04773 | 2.56   | 0.163   |
| Pure Error      | 5  | 0.0933   | 0.0933   | 0.01867 |        |         |
| Total           | 19 | 17.4645  |          |         |        |         |

R-Sq = 93.54%; R-Sq (pred) = 74.58%; R-Sq (adj) = 87.72%
Flocculating activity was determined.
Flocculating activity (%) = \((A-B)/A\) \times 100;
A and B were the OD\(_{550}\) of the untreated and treated solution.

RESULTS AND DISCUSSION

Bioflocculant producing bacterial strain isolation
Total of 45 different morphological strains was screened from the sewage water. Strain showing best flocculating activity in Kaolin suspension was selected for further research. The strain had been subjected to the nucleotide sequence and confirmed to be as *Bacillus subtilis*. The nucleotide sequence had been submitted to GenBank (MF285078). A phylogenetic tree was constructed based on the comparison with similar sequences (Fig. 1). The strain isolated was Gram-positive, aerobic, rod-shaped bacteria\(^{24,25}\).

Media component optimization for bioflocculant production

In this study, Plackett–Burman strategy was used to find out the components that were mainly responsible for bioflocculant production. The components were Peptone 3 g; Sucrose 12.5 g; \(K\)\(_{2}\)\(H\)\(_{3}\)\(P\)\(_{2}\)O, 0.5 g; MgSO\(_4\)\(_{7}\)H\(_2\)O, 0.3 g; NaCl, 0.02 g; (Table 1). PB design had selected only three components based on Pareto chart (Fig. 2). The three components (Sucrose 16 g; Peptone 2 g; and MgSO\(_4\)\(_{7}\)H\(_2\)O 0.3 g) were further optimized by RSM. Central Composite Design (CCD) for RSM studies framed a total of 20 experiments with the highest yield of 4.86 (Table 3). The results obtained were further subjected to RSM. The second-order response surface model is depicted in Table 2. The model proved that coefficient of adjusted value \((R^2 = 0.8772)\) was also slightly similar\(^ {3,26}\). However, sucrose was an important factor since it had a statistical value of about 99% (Table 4). Three dimensional (3-D) response surface curves represented the interaction of substrates on the bioflocculant production (Fig. 3). Component interactions (Sucrose* Sucrose; Peptone* Peptone; MgSO\(_4\) * MgSO\(_4\); Sucrose* Peptone; Peptone* MgSO\(_4\); MgSO\(_4\); MgSO\(_4\) * MgSO\(_4\)) showed significant values for bioflocculant production (Table 2). Further, the contour plot suggested that the interaction
between the corresponding variables were significant (Table 2 and Fig. 4). Yield of obtained bioflocculant was 4.86 which was comparable with the predicted production obtained17.

**Factors influencing the flocculating activity of bioflocculant**

Effects of bioflocculant dosage, temperature, metal ions and pH on the flocculating rate were determined (Fig. 5). Bioflocculant activity could be achieved by altering the charges of the solution. Since bioflocculant was negatively charged nature in solution, positively charged ions were required to carry over the flocculating process (Fig. 5(a)). Therefore, different ions such as CaCl$_2$, NaCl, MgCl$_2$, FeSO$_4$, and HgCl$_2$ were examined to achieve higher flocculating activity. Similar kind of work has been reported with other strains14,28.

The flocculating rate was tested in the range of 0-100 mg/L, and the maximum flocculating rate was observed at an optimum bioflocculant dosage of 50 mg/L (Fig. 5(b)). It can also be observed that a higher or lower amount of bioflocculant caused poorer flocculating rate. Bioflocculant activity could not be reached maximum level when the bioflocculant dosage was inappropriate16.

The effect of pH on the flocculating activity was studied (Fig. 5(c)). The reaction was carried out with varying pH (3-10). The maximum of 90% flocculating activity was attained at the range of 7. Increasing and decreasing the pH would lead to the lower flocculating activity. Similarly, the effect of time on flocculating activity was also studied (Fig. 5(d)). The reaction was carried out by increasing the time from 0 to 400 min. The maximum 90% flocculating activity was obtained when it reached 300 min9,29.

**Table 3.** CCD matrix for critical media components showing observed and predicted values for the production of bioflocculant

| No. | Sucrose | Peptone | MgCl$_2$ | Yield (g/L) observed value | Yield (g/L) predicted value |
|-----|---------|---------|----------|---------------------------|---------------------------|
| 1.  | 12.0000 | 2.00000 | 0.30000  | 3.80                       | 2.54587                   |
| 2.  | 14.0000 | 1.25000 | 0.40000  | 2.20                       | 1.45692                   |
| 3.  | 16.0000 | 2.00000 | 0.50000  | 3.60                       | 4.58213                   |
| 4.  | 14.0000 | 1.50000 | 0.40000  | 3.00                       | 2.74568                   |
| 5.  | 12.0000 | 1.25000 | 0.40000  | 2.50                       | 3.28796                   |
| 6.  | 14.0000 | 1.00000 | 0.40000  | 2.23                       | 3.30164                   |
| 7.  | 14.0000 | 1.25000 | 0.568179 | 1.00                       | 0.99224                   |
| 8.  | 16.0000 | 2.00000 | 0.30000  | 4.86                       | 3.86979                   |
| 9.  | 14.0000 | 1.75000 | 0.40000  | 2.00                       | 2.14092                   |
| 10. | 14.0000 | 1.25000 | 0.40000  | 3.50                       | 3.12291                   |
| 11. | 14.0000 | 1.25000 | 0.231821 | 3.20                       | 3.17359                   |
| 12. | 12.0000 | 2.00000 | 0.50000  | 0.80                       | 3.05435                   |
| 13. | 12.0000 | 0.50000 | 0.30000  | 3.80                       | 1.86280                   |
| 14. | 16.0000 | 0.50000 | 0.50000  | 2.20                       | 2.77654                   |
| 15. | 12.0000 | 0.50000 | 0.50000  | 2.80                       | 3.28796                   |
| 16. | 10.6364 | 1.25000 | 0.40000  | 3.00                       | 3.12291                   |
| 17. | 14.0000 | 2.25000 | 0.40000  | 2.90                       | 1.45692                   |
| 18. | 16.0000 | 0.50000 | 0.30000  | 2.18                       | 4.58213                   |
| 19. | 14.0000 | -0.01134| 0.40000  | 2.10                       | 2.74568                   |
| 20. | 14.0000 | 2.51134 | 0.40000  | 3.00                       | 3.28796                   |

Composition of the bioflocculant was determined as 64% total sugar and 18% protein. The functional group analysis of the bioflocculant derived from *bacillus subtilis* found out the spectrum at 3464 cm$^{-1}$ which was the characteristic of -OH, and -NH$_2$ groups. The spectrum displayed at 1638 cm$^{-1}$ was associated to the group of –COO$^-$ ion and thus, showing the presence of uronate in this polysaccharide30. The weak absorption peak identified at 1048 cm$^{-1}$ was related to all sugar...
Fig. 3. 3-D response surface graphs derived from CCD showing the interactive effects of different factors on the production of bioflocculant. (a) Interaction between Peptone and MgCl$_2$; (b) Interaction between Sucrose and MgCl$_2$; (c) Interaction between Sucrose and Peptone.

Fig. 4. Contour plots for the bioflocculant obtained from CCD showing interactions between three different factors.
Fig. 5. Effect on the conditions of bioflocculant production. a) Effect of ions on bioflocculant production; b) Effect of dosage on bioflocculant production; c) Effect of pH on bioflocculant production; d) Effect of time on bioflocculant production.

Table 4. Regression analysis showing critical media components in bioflocculant production

| Media        | Estimated Co-efficient | t-Value | p-Value |
|--------------|------------------------|---------|---------|
| Sucrose      | -0.8525                | -5.67   | 0.002   |
| MgSO₄        | -0.4025                | -2.68   | 0.044   |
| KH₂PO₄       | -0.0525                | -0.35   | 0.741   |
| K₂HPO₄       | 0.0358                 | 0.24    | 0.821   |
| Peptone      | -0.6642                | -4.42   | 0.007   |
| Sodium chloride | -0.3308            | -2.20   | 0.079   |

Fig. 6. FTIR spectra of the bioflocculant produced by *bacillus subtilis*

Fig. 7. SEM image of the bioflocculant produced by *bacillus subtilis*

Fig. 8. a) Municipal wastewater added with the bioflocculant; b) After the incubation, flocculated particles flocculated on the surface of the wastewater; c) Treated wastewater after the treatment.
The spectrum indicated the presence of a characteristic peak of 632 cm⁻¹ which showed the functional group of C-H bond. Therefore, -OH, -COO⁻, and NH groups were identified in the bioflocculant molecules (Fig. 6). SEM image of the bioflocculant appeared as an irregular shaped structure (Fig. 7).

**Treatment of municipal wastewater**

The trials were performed to find out the efficiency of bioflocculant for municipal wastewater⁶,³². The overall flocculating percentage of the bioflocculant was almost 90% with the dose of the bioflocculant (50 mg/L) (Fig. 8). The higher level of parametric studies indicated organic pollution in the samples. The treatment has shown the reduction of parameters such as BOD, COD, etc., that confirmed the removal efficiency of the bioflocculant.

**CONCLUSION**

Isolated bioflocculant-producing strain were identified as *B. subtilis*. It was composed of mostly polysaccharides and proteins. From the result, it was confirmed that amino, hydroxyl, and carboxylate groups identified in the bioflocculant molecules. They had a high flocculating ability at a minimal dosage requirement (50 mg/L), pH of 7 against Kaolin clay and municipal wastewater. Based on the outstanding features of the bioflocculant, it could be exploited in bioremediation. Further studies are required to carry out the gene responsible for flocculation.

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**DATA AVAILABILITY**

All data generated during the study are included in the manuscript.

**ETHICS STATEMENT**

This article does not contain any studies with human participants or animals performed by any of the authors.

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