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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; $MgSO_4 \cdot 7H_2O$ 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.

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INTRODUCTION

Bioflocculant is a polymeric substance secreted by a large group of microorganism 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 the three components such as (i) inorganic flocculants (ii) organic flocculants (iii) synthetic flocculants. However, these flocculant causes 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 air-tight 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 52 -AGAGTTTGATC (C/A) TGGCTCAG-32 (forward) and 52 TACGG(C/T) TACC TTGTTACGACTT-32 (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

Table 1. PB design for the screening of critical media components involved in bioflocculant production

No.	Sucrose	$MgSO_4$	KH_2PO_4	K_2HPO_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_2PO_4, K_2HPO_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 s*train 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

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)x100;

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_2HPO_4 0.5 g; KH_2PO_4 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; NaCl, 0.02g; (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 $MgSO_4 \cdot 7H_2O$ 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. Form the obtained value, adequacy of the model was represented by high regression

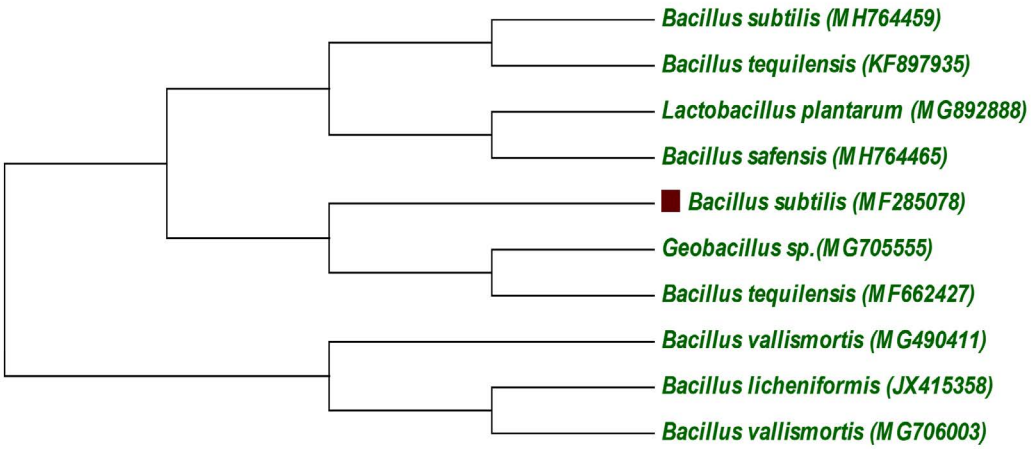

Fig. 1. Neighbor-joining phylogenetic tree based on 16S rDNA gene sequences of the strain *bacillus subtilis*

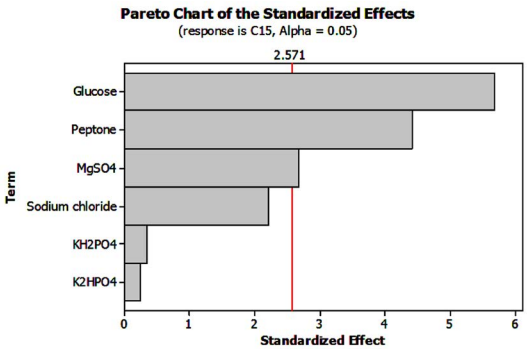

Fig. 2. Pareto chart responsible for selecting main components with respect to increase the yield of bioflocculant production for the strain *Bacillus subtilis*

coefficient value R^2=0.9354 obtained, with respect to enhance the bioflocculant production. 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* $MgSO_4$; $MgSO_4$* $MgSO_4$) 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 obtained[27].

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 strains[14,28].

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 inappropriate[16].

The effect of p^H on the flocculating activity was studied (Fig. 5(c)). The reaction was carried out with varying p^H (3-10). The maximum of 90 % flocculating activity was attained at the range of 7. Increasing and decreasing the p^H 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 min[9,29].

Structural and functional features of the bioflocculant

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 polysaccharide[30]. The weak absorption peak identified at 1048 cm^{-1} was related to all sugar

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	0.300000	3.80	2.54587
2.	14.0000	1.25000	0.400000	2.20	1.45692
3.	16.0000	2.00000	0.500000	3.60	4.58213
4.	14.0000	1.50000	0.400000	3.00	2.74568
5.	12.0000	1.25000	0.400000	2.50	3.28796
6.	14.0000	1.00000	0.400000	2.23	3.30164
7.	14.0000	1.25000	0.568179	1.00	0.99224
8.	16.0000	2.00000	0.300000	4.86	3.86979
9.	14.0000	1.75000	0.400000	2.00	2.14092
10.	17.3636	1.25000	0.400000	3.50	3.12291
11.	14.0000	1.25000	0.231821	3.20	3.17359
12.	12.0000	2.00000	0.500000	0.80	3.05435
13.	12.0000	0.50000	0.300000	3.80	1.86280
14.	16.0000	0.50000	0.500000	2.20	2.77654
15.	12.0000	0.50000	0.500000	2.80	3.27155
16.	10.6364	1.25000	0.400000	3.00	2.54587
17.	14.0000	2.25000	0.400000	2.90	1.45692
18.	16.0000	0.50000	0.300000	2.18	4.58213
19.	14.0000	-0.01134	0.400000	2.10	2.74568
20.	14.0000	2.51134	0.400000	3.00	3.28796

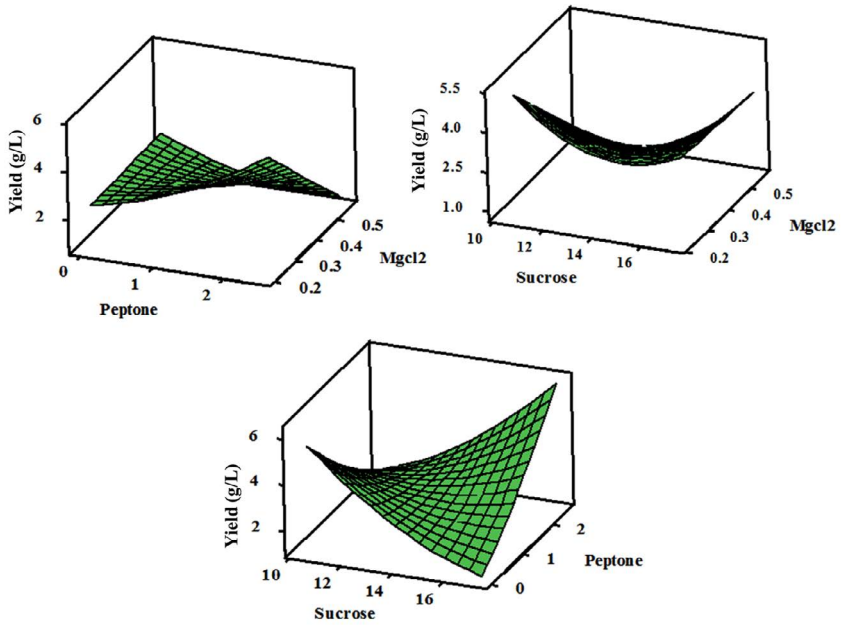

Fig. 3. 3-D response surface graphs derived from CCD showing the interactive efffects of different factors on the production of bioflocculant. (a) Interaction between Peptone and $MgCl_2$; (b) Interaction between Sucrose and $MgCl_2$; (c) Interaction between Sucrose and Peptone.

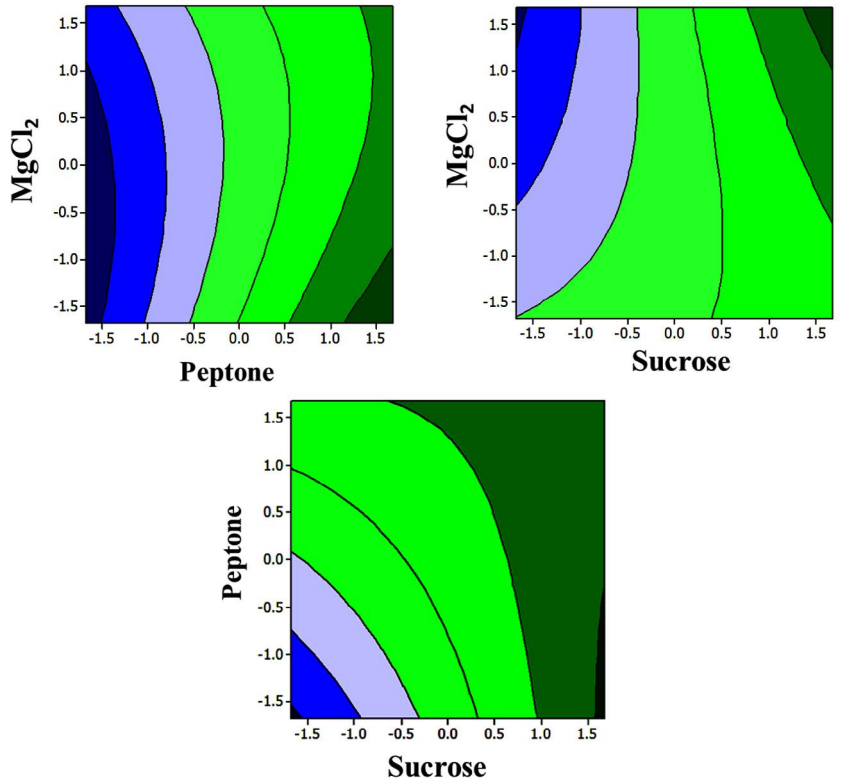

Fig. 4. Contour plots for the bioflocculant obtained from CCD showing interactions between three different factors.

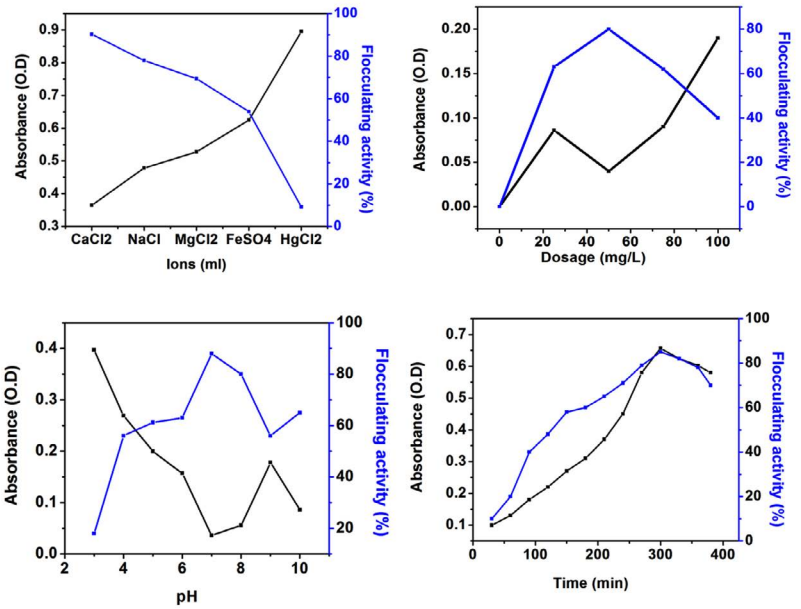

Fig. 5. Effect on the conditions of bioflocculant production. a) Effect of ions on bioflocculant production; b) Effect of dosage on bioflocculant production; c) Effect of pH on bioflocculant production; d) Effect of time on bioflocculant production.

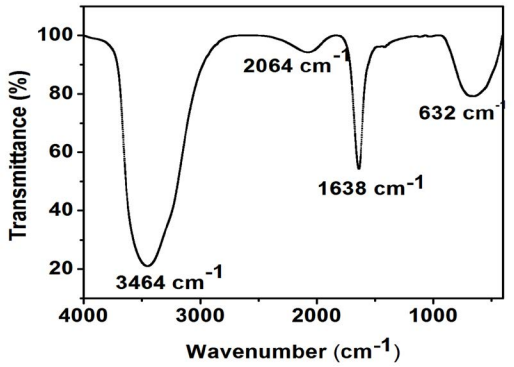

Fig. 6. FTIR spectra of the bioflocculant produced by *bacillus subtilis*

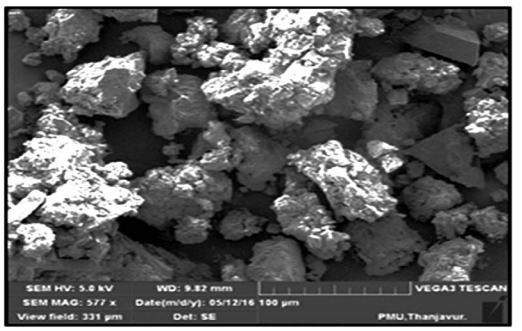

Fig. 7. SEM image of the bioflocculant produced by *bacillus subtilis*

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
$MgSO_4$	-0.4025	-2.68	0.044
KH_2PO_4	-0.0525	-0.35	0.741
K_2HPO_4	0.0358	0.24	0.821
Peptone	-0.6642	-4.42	0.007
Sodium chloride	-0.3308	-2.20	0. 079

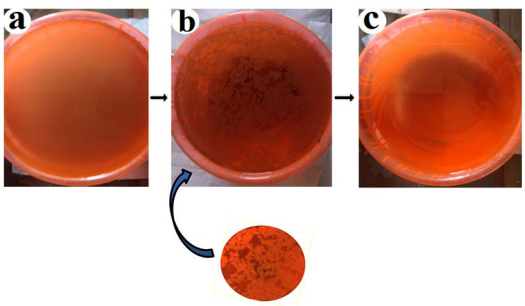

Fig. 8. a) Municipal wastewater added with the bioflocculant; b) After the incubation, flocculated particles flocculated on the surface of the wastewater; c) Treated wastewater after the treatment.

derivatives[31]. 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_2 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 wastewater[6,32]. 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 *Bacillus 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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