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Production And CharacTeristics of A Bioflocculant Produced By Pseudomonas SP. STRAIN 38A

Soha Farag[1], Sahar Zaki[1], M.F. Elkady[2], and Desouky Abd-El-Haleem[1]

[1]Environmental Biotechnology Department, Genetic Engineering and Biotechnology Research Institute, City for Scientific Research and Technology Applications, Alexandria, Egypt.

Email: sohafarag88@yahoo.com

[2]Fabrication Technology Department, Advanced Technology and New Materials Research Institute (ATNMRI), City for Scientific Research and Technology Applications, Alexandria, Egypt.

Email: maroelkady@yahoo.com

ABSTRACT

Screening of microorganisms producing flocculating substances was achieved. A strain secreting a large amount of bioflocculant was isolated from wastewater samples collected from the inlet from Borg El-Arab industrial wastewater treatment plant. Based on the morphological properties and 16S rDNA sequence analysis, the isolate (designated 38A) was classified as pseudomonas sp. exhibiting the highest flocculating activity and a crude polymer yield of 3 g/L after 1 day cultivation. A bioflocculant produced by 38A was heat-stable and had strong flocculating activity in a wide range of pH with relatively low dosage requirement. By studying the flocculating activity of 38A, factors such as bioflocculant dosage, temperature and pH of the reaction solution were tested. The optimal conditions for the flocculating activity were 15 mg/l bioflocculant dosage and pH 7.0, at a temperature of 25˚C. Under these conditions, the flocculating rate of kaolin clay was 99.89%. Different cations were investigated, the best was the $CaCl_2$. Also the optimal concentrations for the flocculating activity was 1ml $CaCl_2$ 3%(w/v) which played the synergistic effect on kaolin flocculation. The bioflocculant of 38A was identified as a polysaccharide. Infrared spectra showed the presence of hydroxyl, carboxyl and methoxyl group in its molecules.

Keywords: pseudomounas sp. Bioflocculant; Bioflocculant production media; Time course; Bioflocculant characterization.

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INTRODUCTION

Flocculants have been widely used in a variety of industrial processes, such as wastewater treatment, the food and fermentation industries, drinking water purification, and industrial downstream processes [1, 2]. In general, flocculants are divided into chemically synthesized flocculants (organic and inorganic flocculants) and natural flocculants (chitosan, algin, and microbial flocculants) [3]. Although chemical high-polymer flocculants, such as polyacrylamide (PAM), are frequently used because of their low cost and marked effectiveness, most of them are difficult to degrade, and many of the monomers derived from synthetic polymers are harmful to humans [4, 5, 6]. Presently, several countries have banned or limited the use of such flocculants [7].

In recent years, bioflocculants have attracted wide attention, because they are biodegradable, harmless, and free of secondary pollution risk. Bioflocculants could be used as potential replacements for organic synthetic flocculants which possess inherent drawbacks of being a source of carcinogenic monomers, and being less biodegradable. Bioflocculants have been widely used in wastewater treatment, and in food-processing and fermentation industries [8]. However, high production costs associated with relatively expensive substrates, such as glucose, fructose and glutamate [9, 10] limit their application. However, bioflocculation is a dynamic process resulting from the synthesis of extracellular polymer by living cells. Bioflocculants are essentially polymers produced by microorganism during their growth, with their flocculating activity being dependent on the characteristics of the flocculants [11].

Many bacteria, fungi and actinomyces have been reported to produce extracellular biopolymers, such as polysaccharides, functional proteins and glycoproteins with considerable flocculating activity [12, 13]. Bioflocculants have still not been practically applied due to their low productivity, which means high cost [14]. Screening new microorganisms that could produce flocculants with excellent flocculating activity and less consumption [15, 16, 17], therefore, seems to be a feasible approach [18]. This paper aims to look for bacterial isolate(s) producing bioflocculants with high flocculation activities, and to characterize the flocculation properties of these bioflocculants for further application. To achieve this target, several bioflocculant-producing bacterial strains were isolated from activated sludge collected from the inlet of Burg El-Arab industrial wastewater treatment plant. A novel bioflocculant-producing strain 38A that attain highest flocculation efficiency was selected to be identified as Pseudomonas by 16S rDNA sequence analysis. The major components and functional groups of bioflocculant produced by strain 38A were examined by chemical analysis, HPLC, amino acids analysis and Fourier-transform infrared (FTIR) spectrometry. The bioflocculant of strain 38A showed high flocculation rate in Kaolin suspension. The optimal conditions for flocculation efficiency were then investigated, such as the ranges of bioflocculant dosage, pH, and temperature.

MATERIAL AND METHODS

Isolation and growth of bioflocculant-producing bacteria

Bioflocculant-producing bacteria were isolated from activated sludge sample collected from the inlet of Burg El-Arab industrial wastewater treatment plant. Isolation was carried out using an agar plate culture containing Trypticase soya medium (TSM) with composition per liter of 30 g Trypticase soya broth, 5 g potassium nitrate, 20 g L-glutamic acid and 20 g agar at pH 7.0. Bioflocculant-producing bacteria were originally screened based on colony morphology (mucoid and ropy). Then isolated strains were grown in 50 ml of TSM broth medium on a rotary shaker (200 r/min) at 30 °C for 48 h, and the resultant culture broths were examined for their flocculation activity. Flocculation activity was measured using a kaolin suspension as test material according to the method described by Dermlim [19]. Finally, one strain designated strain 38A with high flocculation activity for kaolin was selected for further study. Genomic DNA preparation of strain 38A, PCR amplification of its 16S rDNA, and sequencing of the PCR products were carried out. The 16S rDNA sequence data were compared with currently available microorganism sequences in GenBank. The GenBank accession numbers of strain 38A was JF715058**.** The composition of bioflocculant production medium was as follows: 20 g L-glutamic acid, 7 g NH_4Cl, 0.5 g K_2HPO_4, 0.5 g Mg SO_4, 40 mg $FeCl_3$, 150 mg $CaCl_2$ and 140 mg $MnSO_4$ per liter of deionized water at pH 7. To determine the time course, strain 38A was pre-cultured for 24 h in TSM medium and inoculated into production medium with the addition of 2% starter culture on a rotary shaker (200 r/min) at 30˚C for 5 days. Samples were taken every 12 h to measure cell growth and flocculation activity.

16S rDNA sequence determination and phylogenetic analysis

Genomic DNA preparation, PCR amplification of 16S rDNA, and sequencing of the PCR products were carried out as described previously [11] .The 16S rDNA sequence data were compared with currently available microorganism sequences in GenBank. The GenBank accession numbers of strain 38A was JF715058**.**

Bioflocculant production media and time course determination

Three conventional and four unconventional carbon sources; Glucose, Glutamic acid, Yeast extract, mollass, sunflower oil, wastewater collected from a paper factory at Burgelarab City and Whey wastewater collected from a milk factory at Burgelarab city were examined. The composition of the production media was as follows: carbon source (2% w/v), NH_4Cl 7g, K_2HPO_4 0.5g, Mg SO_4 0.5g, $FeCl_3$ 40 mg, $CaCl_2$ 150 mg and $MnSO_4$ 140 mg per liter of deionized water with initial of pH 7. To determine the time course of the strain 38A, the strain was pre-cultured and inoculated with the addition of 2% starter culture (24 h) on a rotary shaker (200 r/min) at 30˚C for 5 days. Samples were taken every 12 h to measure cell growth and flocculating activity as described previously.

Characterization of the bioflocculant

The protein content of the biopolymer was determined according to Bradford method [20]. The total sugar content of the bioflocculants was determined using the phenol-sulfuric acid method using glucose as the standard. Sugars and amino acids composition of the bioflocculant were determined by HPLC and amino acids analyzer as described previously by Abd-El-Haleem [21]. Infrared spectra of samples in the KBr pellet were recorded using a Fourier transform infrared (FTIR) spectrophotometer (AVATAR 360, USA). Solubility assay of the bioflocculant in distilled water and several solvents such as acetone, carbon tetrachloride, ethanol, isopropanol, hexane, methanol and nitrobenzene were performed [22].

Bioflocculant purification

The method described previously by Li [23] was used to purify the bioflocculant. Strain 38A was cultivated for an optimal period, subsequently the viscous culture broth was centrifuged to remove cell pellets (5000×g, 30 min), thereafter, the supernatant was concentrated to 0.2 volume with a rotary evaporator and dialyzed overnight at 4˚C in deionized water. Three volumes of cold anhydrous ethanol (4˚C) were added to the dialyzed broth. The precipitate obtained was redissolved in deionized water followed by the addition of 10% cetylpyridinium chloride (CPC) with stirring. After several hours, the resultant precipitate was collected by centrifugation (5000×g, 15 min) and dissolved in 0.5 M NaCl. Three volumes of cold anhydrous ethanol (4˚C) were then added to obtain the precipitate, which was then washed with 75% ethanol three times and lyophilized to obtain purified bioflocculant.

Flocculation properties of the purified bioflocculant

Flocculating activity was measured using a clay suspension as test material according to the method described previously by Kurane [14] and Dermlim [19] with minor modifications. The mixture containing 200 ml kaolin clay suspension (5 g/l) and the pH value was adjusted to 7 using 1 M NaOH or HCl, 0.5 ml sample (supernatant centrifuged at 5000g for 30 min) and 10 ml 3% (w/v) CaCl2 solution was used in a modified jar test procedure. The suspension (200 mL in 0.5 L jar) from one beaker was circulated at 240 rpm for 3min, followed by slow mixing at 80 rpm for 2 min and left standing for 3 min. The supernatant was measured for absorbance at 550 nm. A control was prepared using the same method but the sample was replaced by distilled water. The flocculating activity was calculated according to the following equation:

Flocculating activity % = (A - B)/A *100

Where A and B are the optical densities of the control and the sample, respectively. The strains possessing the culture broth with the highest flocculating activity against a kaolin suspension were selected and further identified.

To determine the best dosage of the biopolymer, purified bioflocculant was dissolved into a suitable volume of distilled water to yield a flocculant solution (0.1g/L). Then the effect of its concentration was examined by measuring the flocculating activity using different dosage of polymer ranged from 1 to 50 mg/L. The effect of pH (1, 3, 5, 7, 9, 10 and 11) and temperature (5, 25, 35, 50, 65, 75, 85 and 100˚C) of the kaolin suspension on the flocculating activity of the purified biopolymer was studied by measuring the flocculating activity of the reaction mixture containing the optimum concentration of biopolymer at the specified ranges of pH and temperatures [24, 25]. The pH stability of the biopolymers was determined [25, 26] by measuring the flocculating activity after 24 h pre-incubation at various pH (1, 3, 5, 7, 9, 10 and 11) and compared with that of normal polymer solution at pH 7. Thermal stability of the biopolymer was determined [24, 25] by measuring the flocculating activity after 30-min incubation at various temperatures (5, 25, 40, 60, 80 and 100 ˚C) and compared with that of 30˚C. As well as the cation types (NaCl, KCl, $CaCl_2$, $MgCl_2$, $AlCl_3$, $ZnCl_3$, and $FeCl_3$) and the effect of cation concentrations (0.5-200 ml/L reaction mixture) were also studied.

RESULT AND DISCUSSION

Isolation, selection and identification of bioflocculant-producing bacteria

Total of four slime-forming or mucoid colonies of bioflocculant-producing bacteria (9B, 10A, 38A and 38C) were selected on the basis of their flocculation activities over 75%. As shown in Table 1, strain 38A was the most effective bacterium with flocculating activity exceeding 97%. The colony of 38A was orange yellow, circular and smooth; its 16S rDNA gene was sequenced (~ 800 bp) after PCR amplification and compared with sequences deposited in GeneBank database. The highest level of 16S rDNA sequence similarity to *pseudomonas* was 99%.

Bioflocculant production media and time course determination

Bioflocculants may be produced relatively inexpensively by means of some cost-effective fermentation medium [17]. Seven carbon sources were used as a substrate for the production of bioflocculant and the results were shown in Fig 1. L-Glutamic, yeast extract, glucose, Sunflower oil, mollass, paper wastewater and whey wastewater were each deemed as suitable substrates for bioflocculant production. Sunflower oil is not biodegradable and may suppress the microorganism. The highest flocculating activity of 98.74% was observed for the L-Glutamic medium as a carbon and energy source.

The growth curve of strain 38A and the flocculating activity of the culture broth are shown in Fig.2. During the fermentation, the flocculating activity increased with culture time at first, and after reaching maximum value of 97.3% at 24h, decreased slowly thereafter. The cell grew rapidly with increase of culture time in the first 110 h of cultivation, and then leveled off. The flocculating activity curve was disparate to the cell growth curve and the flocculating activity decreased with increasing culture time, indicating that the flocculant was produced by biosynthesis during the growth of

38A, not by cell autolysis. However, the slight decrease of flocculating activity may indicate that this strain has a bioflocculant-degrading enzyme [27]. The same result was presented with strain W31 which reach maximum value of 90% at 60 h, and decreased slowly thereafter [11].

Characterization of the bioflocculant

The purified bioflocculant was composed of 94.61% carbohydrates and 2.85% of protein. Therefore, the bioflocculant produced by 38A was classified as a polysaccharide containing some protein. The amino acids composition of bioflocculant produced by strains 38A was determined after acid hydrolysis. As stated in Table 2, the polymer is rich in glutamic acid, aspartic acid and Glycine, respectively. However, HPLC analysis revealed that monosugar glucose is the major sugar components of the biopolymer exhibiting 95.5% of the analyzed sample.

FT-IR spectroscopy was performed on the purified bioflocculant (Fig. 3). The spectrum showed a broad, intense absorption peak at 3,433 cm^{-1}, characteristic of a hydroxyl group, which could be caused by the vibration of -OH or -NH in the sugar ring. A weak peak at 2,198 cm^{-1}, known to be carbohydrates, indicated C=H asymmetrical stretching vibration. An asymmetrical stretching peak observed at 1,658 cm^{-1} was characteristic of C=O stretching vibration in -NHCOCH3. The weak peak at 1,083 cm^{-1} may be assigned to be C=O symmetrical and asymmetrical stretching of a carboxylate group in the bioflocculant [7, 16, 28]. The absorption peak of CH at 769 cm^{-1} showed that 38A bioflocculant was composed of sugar derivatives. The presence of hydroxyl groups verified by the IR spectra within the polymer favored the probability of hydrogen bonding with one or more water molecules, so 38A bioflocculant exhibited high solubility in aqueous solutions, following the solubility principle “like dissolves like” [29]. A major condition for flocculation is that the molecules of flocculants could adsorb onto the surface of particles. The surface charge of kaolin particles in aqueous solution is negative. When 38A bioflocculant is approaching particles in solution, an attractive force must exceed the electrostatic repulsion force. The calcium ion is necessary for the flocculating activity of 38A bioflocculant on kaolin. This can be explained in that Ca^{2+} stimulates flocculating activity by neutralizing and stabilizing the residual charge of functional groups as the binding distance is shortened [30]. Then OH, COOH or COO^- group of the bioflocculant and H^+, OH^- group on the surface of particles might form hydrogen bonds as the bioflocculant chains approach the surface of particles [11].

This polysaccharide was found to be soluble in water, slight acidic and basic solution but insoluble in all tested organic solvents. Consequently, it can be recovered from the cell-free supernatant by precipitation with organic solvents such as ethanol. In an aqueous system, polysaccharide particles can take up water, swell and usually undergo partial or complete dissolution [31]. The abundance of hydroxyl groups builds up strong forces of attraction between polysaccharide molecules, and may result in relatively hard crystalline solids – where hydrogen bonding can occur. These forces are too great to be broken by organic solvents, so the polysaccharide was insoluble in organic solvents [29].

Flocculation properties of the bioflocculant

In order to coverage the flocculation properties of the purified bioflocculant, the effect of both the processing parameters such as the dosage, temperature and pH of the bioflocculant, in addition to the cation type and concentration and the operation conditions such as the temperature and pH of clay suspension on the flocculation activity were elucidated in details in this section.

Effect of dosage, temperature, and pH of the bioflocculant on flocculating activity

The effects of bioflocculant dosage, temperature and pH on flocculating activity were shown in Table 3. The effect of bioflocculant dosage showed that flocculating activity was constant 99.89 % in a wide range of bioflocculant dosages (15 - 45mg/L), Higher or lower dosages induced lower efficiency and so 15mg/L was an optimum bioflocculant dosage. The relationship between the dosage and flocculating activity of 38A was similar to that of bioflocculants produced by M-1 which has the maximum flocculating activity in an optimum dosage of 6.0 mg/l [10].

Thermal stability of the bioflocculant was also tested from 5–100˚C using 4mg/L bioflocculant dosage, the flocculating rate was above 75.79%, the highest flocculating rate of 82.68% was achieved at 25˚C, and the flocculating rate decreased slightly over 25˚C. The thermal stability was presumably because the main backbone of this bioflocculant was a polysaccharide [32]. Higher temperature induced higher flocculating activity. This may be explained by the release of extra and inner-cell polysubstances after heat at higher temperature. The same result was recorded by Gong [33] when they used bioflocculant SF-1 production from S. ficaria.

The pH stability of the bioflocculant was determined and compared with that of normal bioflocculant solution at pH 7. The purified bioflocculant had the optimum pH for flocculating activity at pH 7 and the flocculating activity decreased gradually in acidic and basic range as shown in Table 2. This suggested that the hydroxide ion (OH^-) may interfere with the complex formation of the polysaccharide and kaolin particles mediated by Ca^{2+}, consequently the kaolin particles were suspended in the mixture. The pH range for flocculation reaction of this polymer was wider than those of the polyglutamate from *Bacillus subtilis* PY-90 (pH range of 3–5) [34], and the cationic polysaccharide from *Paecilomyces* sp. I-1 (pH range of 4–8) [35].

Effect of cation types and concentrations on flocculating activity

Since the flocculating activity of the bioflocculant occurred in the presence of $CaCl_2$, the effect of cation alone and in combination with the bioflocculant was investigated. It was found that addition of a cation to the reaction mixture was necessary to induce the effective flocculation by forming complexes of the bioflocculant and kaolin clay mediated by a cation [36]. Different cation types (NaCl, KCl, $CaCl_2$, $MgCl_2$, $AlCl_3$, $ZnCl_3$, and $FeCl_3$) with the same anion Cl^- were tested at constant dosage 10 ml 3% (w/v) for 200 ml kaolin suspension. As shown in Table 2, flocculating activity of 38A bioflocculant was stimulated in the presence of Ca^{2+}, K^+, Na^+, but was inhibited by Al^{3+},Mg^{2+}, Fe^{3+}, Zn^{3+}. Among these cations, Ca^{2+} was the most favorable ion which produces the highest flocculating activity. So, $CaCl_2$ was selected for further experiments as shown in Table (3). This result was in the same direction of the result achieved by Liu [37]. They reported that the $MgCl_2$ cation has the highest flocculating activity of bioflocculant of MBF-W6 derived from *C. daeguense*.

Subsequently, the optimum concentration of $CaCl_2$ from 0.5 to 200ml/L was determined at 4mg/L bioflocculant dosage. As shown in Fig 4, it was observed that by increasing the concentration from 0.5 to 5ml/L the flocculation activity increased, after that increasing in $CaCl_2$ followed by decreasing in flocculating activity. The optimum flocculation activity (82.68156%) was achieved by using 5 ml/L $CaCl_2$. From this result we conclude that 15mg/L from the bioflocculant and 5 ml/L from $CaCl_2$ salt, the optimum dosages, at pH 7 achieving high flocculation activity 99.89%. The same result was reported previously Prasertsan [26]. Yokoi [38] emphasized that the cation could stimulate the flocculation by neutralization and destabilization of residual negative charges of carboxyl groups in an acidic polysaccharide, forming bridges which bind kaolin particles to each other.

Effects of initial temperature and pH of the clay mixture on bioflocculant activity

As shown in Table 4, the effect of the temperature in a kaolin clay mixture was tested using 4mg/L of bioflocculant. When the temperature range was 5–100˚C, flocculating activity was above 80%, the highest flocculating activity (96.2%) was achieved at 75˚C. Flocculating activity decreased significantly when the temperature increased. This can be explained by denaturalization of proteins in the bioflocculant and an increase in hot movement of kaolin particles. However, it was observed that the flocculating activity being over 82.0% in a wider pH range (pH 5.0–11.0) but the highest flocculating activity being 91.67%, at pH 1.0 and 89.19% at pH 3.0. It means that the biopolymer is stable at a wide range of pH. This is may be due to the functional groups of bioflocculant, the acidic carboxyl and hydroxyl groups could keep flocculating ability of bioflocculant in acidic pH. It was previously reported that the flocculating activity of *Citrobacter* sp. TKF04 was maintained at high levels in an acidic pH range of 2.0–6.0, however, there was no activity in basic pH ranges [39]. The bioflocculant produced by *Corynebacterium glutamicum* was relatively stable at moderate acidic conditions (pH 3.0–6.0), and pH lower than 3.0 or higher than 6.0 resulted in significant decreases in flocculating activity [17]. Also , M-1 achieved flocculating activity of over 74.0% in a wider pH range (pH 3.0–11.0) but the highest flocculating activity being 92.67%, at pH 5.0 [10].

CONCLUSIONS

A bioflocculant-producing strain was isolated from sludge sample collected from the inlet of an industrial wastewater plant identified as *Pseudomonas* sp strain 38A. The optimal conditions for bioflocculant production by strain 38A were an initial pH of 7, in fermentation medium containing L-glutamic as carbon and energy source, a temperature of 25 °C, 15 mg/L bioflocculant dosage, 5 ml/L $CaCl_2$ and a fermentation period of 24 h. Chemical analysis revealed an extracellular polysaccharide composed of 94.61% carbohydrates and 2.85% of protein. The carbohydrate was composed of a 95.5% of glucose, while the protein part was rich in glutamic acid, aspartic acid and Glycine, respectively. The bioflocculant was effective for flocculating Kaolin clay suspension in the range of pH 1–11 and temperature 5–100 °C. Consideration its excellent flocculating activity and harmlessness toward humans and the environment, the bioflocculant produced by strain 38A is expected to be a potential replacement of conventional synthetic flocculants and widely applied in water treatment and downstream processing of food and fermentation industries.

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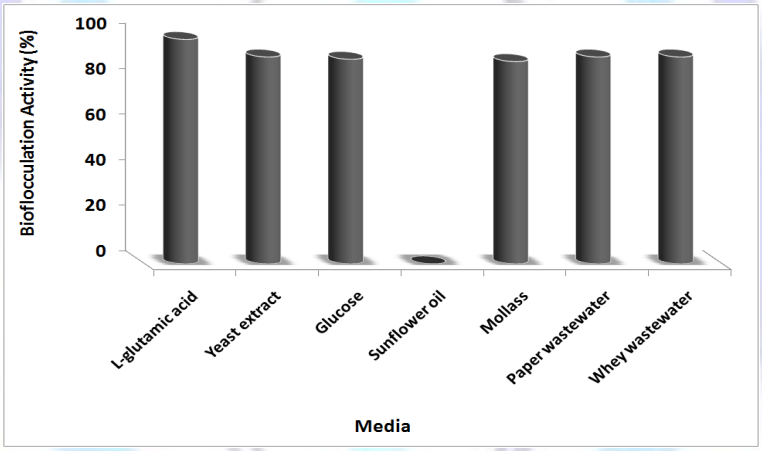

Figure (1): Effect of different carbon sources on flocculating activity.

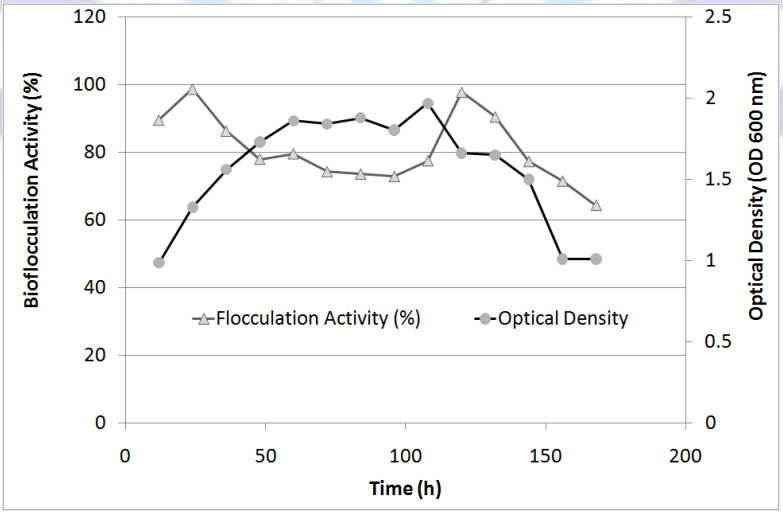

Figure (2): Effect of time course on bioflocculating activity and the growth of strain 38A.

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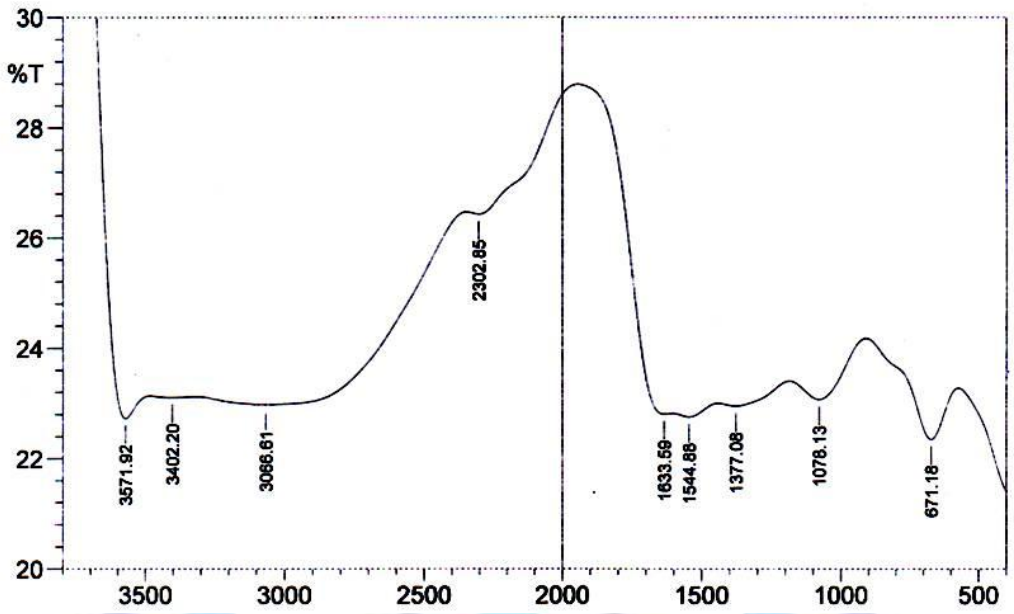

Figure (3): Infrared spectra of purified bioflocculant from pseudomonas sp.

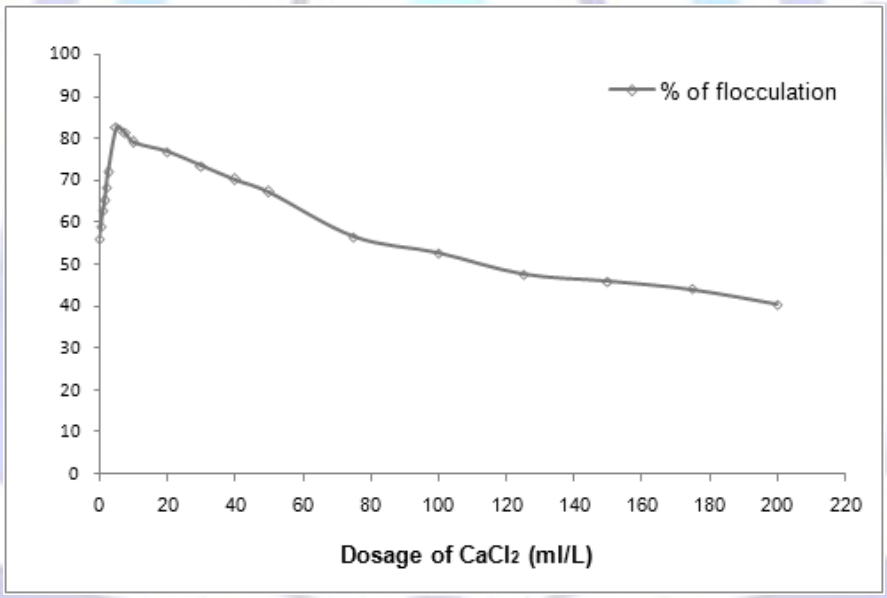

Figure (4): Effect of $CaCl_2$ concentration on flocculation activity.

Table 1: Prescreening results of bioflocculating bacteria isolated from sludge sample collected from the inlet of Burg El-Arab industrial wastewater treatment plant.

Isolate	Flocculation activity (%)
9B	76.29
10A	76.91
38A	97.30
38C	83.83

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Table 2: amino acids and sugars compositions of the bioflocculant

Amino acids	% (w/w)	Sugars	% (w/w)
Aspartic acid	6.52	xylose	6.38
Thereonine	1.67	fructose	4.36
Serine	1.61	glucose	95.47
Glutamic acid	59.58	sucrose	35.97
Proline	1.61	maltose	30.07
Glycine	4.94	lactose	23.91
Alanine	3.37	lactulose	24.76
Valine	2.54		
Methionein	0.80		
Isoleucine	2.81		
Leucine	3.35		
Tyrosine	0.60		
Phenylalanine	1.83		
Histidine	3.01		
Lysine	3.60		
Ammonia	0.71		
Arginine	1.37		

Table 3. Effects of dosage, temperature, pH and different metal ions on the flocculating activity

Metal ions	FA%	Dosage (mg/l)	FA%	pH of bioflocculant	FA%	Temp.˚C of bioflocculant	FA%
K	88.15	1	56.23	1	60.89	5	81.75
Na	87.22	2	67.22	3	70.57	25	82.68
Mg	20.67	4	85.66	5	77.65	40	79.51
Fe	27	5	92.73	7	82.68	60	76.91
Al	12.49	10	98.51	9	76.72	80	76.16
Zn	48.97	15	99.89	10	71.32	100	75.79
Ca	95.22	20	99.89	11	63.31		
Blank	56.05	25	99.89				
		30	99.89				
		35	99.89				
		40	99.89				
		45	99.89				
		50	56.23				

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Table 4. Effects of temperature and pH of a Kaolin clay mixture on the flocculating activity

Reaction temperature	FA%	Reaction pH	FA%
5	77.65	1	97.76
25	82.68	3	89.19
35	88.45	5	85.47
50	90.68	7	82.68
65	94.41	9	83.98
75	96.27	10	83.24
85	80.81	11	82.86
100	23.46		