

Characterizations of Bioflocculant Produced by *Bacillus coagulans* 8B and Application in Domestic Wastewater Treatment

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Abstract

Forty eight isolates belonged to *Bacillus* spp. were isolated from soil and water samples. Twenty two *Bacillus* isolates revealed a mucous phenotype when grown on nutrient agar plates. Only seven isolates of *Bacillus* were grown on starch broth media and gave flocculating activity for kaolin suspension. Isolate B8 (isolated from soil) gave the highest flocculating activity for kaolin suspension (37%). Biochemical tests for this isolate showed that B8 strain of *Bacillus coagulans*. The bioflocculant produced by *B. coagulans* B8 was extracted by organic solvents. Thin layer chromatography (TLC) analysis for *B. coagulans* B8 bioflocculant hydrolyzed by HCl with solvent system showed two brown spots when using phenol-sulphuric acid reagent with $R_f = 0.41$ and 0.58 which resembled to galactose and glucose, and only one red-violet spot when using ninhydrin reagent ($R_f = 0.68$) which may be resembled with phenylalanine. Domestic waste waters were of treated with *B. coagulans* B8 bioflocculant. Results showed that turbidity removal (flocculating activity) was reduced to 62.7%.

Keywords: Bioflocculant, bacillus coagulant, wastewater

خصائص الملبد الحيوي المنتج بواسطة *Bacillus coagulans* واستخدامه لمعالجة مياه المجاري المنزلية

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خلاصة

تم عزل 48 عزلة بكتيرية تعود الى جنس *Bacillus*، جمعت من عينات الماء والتربة. اعطت اثنان وعشرون عزلة صفة اللزوجة عندما زرعت على وسط الاكار المغذي، اظهرت سبعة عزلات فقط من *Bacillus* نمواً في وسط النشاء المغذي واعطت فعالية التلبد في عالق الكاولين. اعطت العزلة B8 (المعزولة من التربة) اعلى فعالية تلبد في عالق الكاولين (37%). اظهرت الاختبارات الكيموحيوية والفحص المظهري لهذه العزلة انها تعود الى نوع *Bacillus coagulans*. استخلص الملبد الحيوي من العزلة *B. coagulans* B8 بأستعمال المذيبات العضوية. اظهر التحليل الكروماتوغرافي للطبقة الرقيقة (TLC) للملبد الحيوي المتحلل، ان المنتج من *B. coagulans* B8 يحوي جزء كاربوهيدراتي وجزء بروتيني، حيث ظهرت بقعتان بنية اللون عند استخدام الفينول - حامض الكبريتيك لتحري عن الكاربوهيدرات ($R_f = 0.41, 0.58$) التي ماثلت الكالكوتوز والكلوكوز، وظهرت بقعة واحدة حمراء غامقة عند استخدام النينهيدرين لتحري عن الاحماض الامينية ($R_f = 0.68$) التي ماثلت الحامض الاميني الفنيل الانلين . درست قابلية مادة التلبد على ازالة مياه المجاري المنزلية، كانت القدرة على ازالة العكورة (فعالية التلبد) فقد اختزلت الى 62.7%.

كلمات مفتاحية: الملبد الحيوي، عزلات تلبد *Bacillus*، مياه المجاري.

Introduction

Microbial flocculants (MBFs) are special natural organic macromolecule substances that can flocculate suspended solids, cells, colloidal solids, etc. [1]. Monomers of polyacrylamide (organic flocculant) are potent carcinogen and neurotoxic to humans and other animals [2]. Several bioflocculants from different microorganisms have been reported. Flocculants produced by *Bacillus subtilis* [3], *Bacillus licheniformis* [4] and *Rhodococcus erythropolis* [5], are predominantly protein in nature; whereas, those produced by *Bacillus firmus* [1]. Bioflocculants possess several advantages: such as safety, strong effect, biodegradable and harmless to humans and the environment, so they may potentially be applied in several industrial and waste water treatment processes such as pharmaceutical, fermentation, food industries, drinking and

downstream processing [1]. Inorganic and organic synthetic polymer flocculants are frequently used in water and wastewater treatment because they are economical and highly effective [6]. However, their use often gives rise to environmental and health problems in that some of them are not readily biodegradable and some of their degraded monomers, such as acrylamide, are neurotoxic and even strong human carcinogens. Residual alum concentration in treated water can also impose health problems apart from the production of high amount of sludge [6]. The aims of this study were: Isolation and Identification of *Bacillus* with highly productivity of bioflocculant. Study of some physico-chemical properties of bioflocculant. Determination of bioflocculant role in Domestic wastewater treatment.

Materials and Methods

Collection Sample, isolation and quantitative screening bacteria

Four grams of each soil sample was suspended in 20ml of sterilized distilled water in sterile flasks, shake to homogenize and heated to 80°C for 10 min. Serial dilutions for each sample were set up, then 0.1 ml of each dilution was spreaded on a nutrient agar plates, and incubated aerobically at 37°C for 24 h [7]. For water samples, 10ml was taken from each sample by sterile syringe, centrifuged at 6000 rpm for 15 min and heated to 80°C for 10 min. 0.1 ml of each sample was spreaded on the surface of nutrient agar plates and incubated at 37°C for 24 hour. The growing colonies were streaked on nutrient agar and this step was repeated until pure culture was obtained. A loop-full of highly mucoid isolates appeared on nutrient agar was inoculated in to 5ml of nutrient broth and incubated at 37°C for 48 h, after incubation the absorbency at 600 nm for each culture was measured. A smear of bacteria was prepared from purified culture, stained with Gram stain and examined under light microscope (100X lens) and the morphological feature of bacterial was observed including Gram reaction, shape and spore forming [3]. Biochemical tests were used to identification the bacteria. Highly mucoid isolates appeared on Brain heart infusion plates were selected and subjected to further step of screening represented by B8.

Measurement of flocculating activity (flocculation test)

The flocculating activity was evaluated by measurement the turbidity of a kaolin suspension. Bacterial isolates were inoculated in 50 ml of starch broth medium and incubated at 37°C for 48 h, cells were precipitated by centrifugation at 6000rpm for 30 min, and 0.5 ml of cell free supernatant was added to 45 ml of kaolin suspension (containing 4.5 ml of CaCl₂ solution) in 100 ml beaker. The mixture was vigorously stirred

for 20s and left to stand, without shaking, for 5 min. The turbidity of the sample supernatant (A) was measured by the spectrophotometer at 550 nm. A control was prepared using the same method, but the cell free supernatant was replaced by distilled water (B). The flocculating activity was calculated according to the equation [8,11].

$$\text{Flocculating activity (\%)} = ((\text{O.DB} - \text{O.DA}) / \text{O.DB}) \times 100$$

Where:

O.DA: is the optical density of the sample experiment at 550 nm

O.DB: is the optical density of control experiment at 550 nm

Extraction of bioflocculant

Extraction with two ratio of 96% ethanol:

Cell free supernatant was mixed with 96% cold ethanol at a ratio of 1:2 v/v and left overnight at 4°C. The aqueous layer was removed and the precipitate was washed in 5 ml distilled water and the precipitate layer was collected in a glass petri dish and left to dry at 60°C till dryness. The dried bioflocculant was collected and preserved in glass vials as dried powder [9].

Analysis of bioflocculant by thin layer chromatography (TLC)

Separation and identification of *Bacillus* bioflocculant were performed by thin layer chromatography (TLC) using silica gel coated plate (TLC covered with silica gel 60) (20 × 20)cm, this method was applied as mentioned by [10].

Treatment of waste water with bioflocculant

Samples of domestic wastewater with pH 6.5 and optical density (0.38) at 550 nm, were collected by sterilized bottle. The bottle was filled leaving about 30 mm of empty space to allow mixing during laboratory analysis.

Bioflocculant produced was applied to deal with domestic wastewater. Various amounts (0.25, 0.5, 0.75, 1, 1.25 and 1.5 ml)

of cell free supernatant were added to 45ml of domestic wastewater containing 4.5 ml of CaCl_2 solution in 100 ml beaker, the pH was adjusted to 11 due to the bioflocculant work in base condition to obtain best activity [3].

The turbidity of wastewater supernatant was measured with a spectrophotometer at 550 nm and percentage removal was determined by comparing the estimated values to that of the control (wastewater without bioflocculant). The flocculating activity was calculated according to the previous equation [8,10].

Result and Discussion

Forty eight isolates were selected from 58 bacterial isolate, according to growth characteristic on nutrient agar and microscopic examination which belong to *Bacillus* spp. Fourteen isolates were isolated from water and 34 isolates from soil Table (1).

Pure cultures of *Bacillus* isolates were cultured on nutrient agar plates, for screening their ability to growth with mucoid appearance as indicator for flocculant production. Twenty two *Bacillus* isolates revealed a mucous phenotype (Figure 1).

The results showed that only seven isolates of *Bacillus* were grown on starch broth media and gave flocculating activity for kaolin suspension. Among them *Bacillus* B8 (isolated from soil) gave the highest flocculating activity for kaolin suspension (37%) (Figure 2). According to these results, the isolate B8 was selected for further study.

Bacillus B8 isolate was obtained from sample (water, soil), subjected to further biochemical tests. The results are shown in Table 2.

Bioflocculant produced by *B. coagulans* B8 was extracted by organic solvents in three methods. The best extraction method was by ethanol at a ratio of 1:2 v/v, bioflocculant dried weight was 0.145 g/100 ml. While when extracted with acetone at a ratio 1:3

v/v and ethanol at a ratio of 1:4 v/v the dried weight were 0.078 g/100ml and 0.117 g/100 ml respectively, (Figure 3).

Bioflocculant contents produced by *Bacillus coagulans* B8 were analyzed by TLC to determine its components such as carbohydrate and protein.

Bioflocculant produced by *B. coagulans* B8 was hydrolyzed by 6N HCl at 100°C for 5 h before application on TLC [9], standard sugars (glucose, galactose, sucrose and fructose) and standard amino acids (phenylalanine and glutamic acid) were used as markers.

The *B.coagulans* B8 bioflocculant carbohydrate was detected on TLC by phenol-sulphuric acid reagent, the presence of sugar was observed as brown spots on TLC plate, two spots were observed with ($R_f = 0.41$ and 0.58), which resembled to galactose and glucose (Figure 4). This result may indicate the presence of two kinds of sugars that may form basic and functional component of the bioflocculant.

Results showed that the optimal amount of bioflocculant of *Bacillus Sp.* for domestic wastewater was 0.5ml/50ml; the flocculating activity (turbidity removal) was 62.7% with pH 11 (Figures 6, 7]

From this study, it can be concluded the Ability of some *Bacillus* isolates obtained from soil or water can produce bioflocculant, and *Bacillus coagulans* B8 was the best in this field. The bioflocculant of *B. coagulans* B8 can be efficiently extracted with ethanol: water (1:2 v/v). The bioflocculant *B. coagulans* B8 is a glycoprotein made of polysaccharide (85%) and protein (7%) and its active ingredient is polysaccharide. The polysaccharide moiety of the bioflocculant contains glucose and galactose, while the protein moiety of the bioflocculant contains phenylalanine. The bioflocculant produced by

B. coagulans B8 has a satisfactory level of flocculating activity. These results suggest that this bioflocculant can be successfully applied for the clarification of wastewaters under various environmental conditions. Because the bioflocculant was formed in fermentation solution which bacteria and culture medium were also included, the lower or higher amount than optimal one would induce the increasing of turbidity. So, it was important to confirm the optimal amount [11]. Matter in domestic wastewater may be broadly classified according to its origin as inorganic mineral matter or organic carbonaceous material. Substances producing turbidity are often inorganic, while those causing taste, odour, and colour are generally organic compounds.

Table (1): Numbers and percentages of *Bacillus* isolated from soil and water

Source of isolation	No. of samples	No. of <i>Bacillus</i> isolates	*Percentage of <i>Bacillus</i> spp. isolates from each sources%
Soil	40	34	58.62
Water	12	14	24.13
Total	52	48	82.75

*The percentage above was calculated according to total isolates (58 isolates)

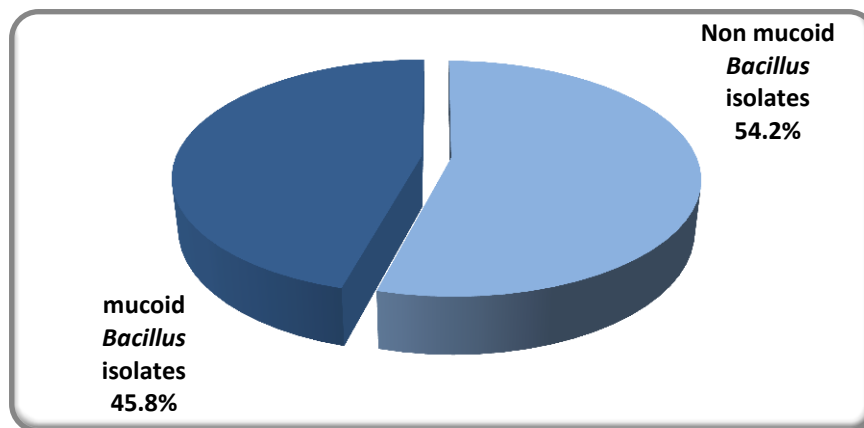


Figure (1): Percentage of muroid *Bacillus* isolates

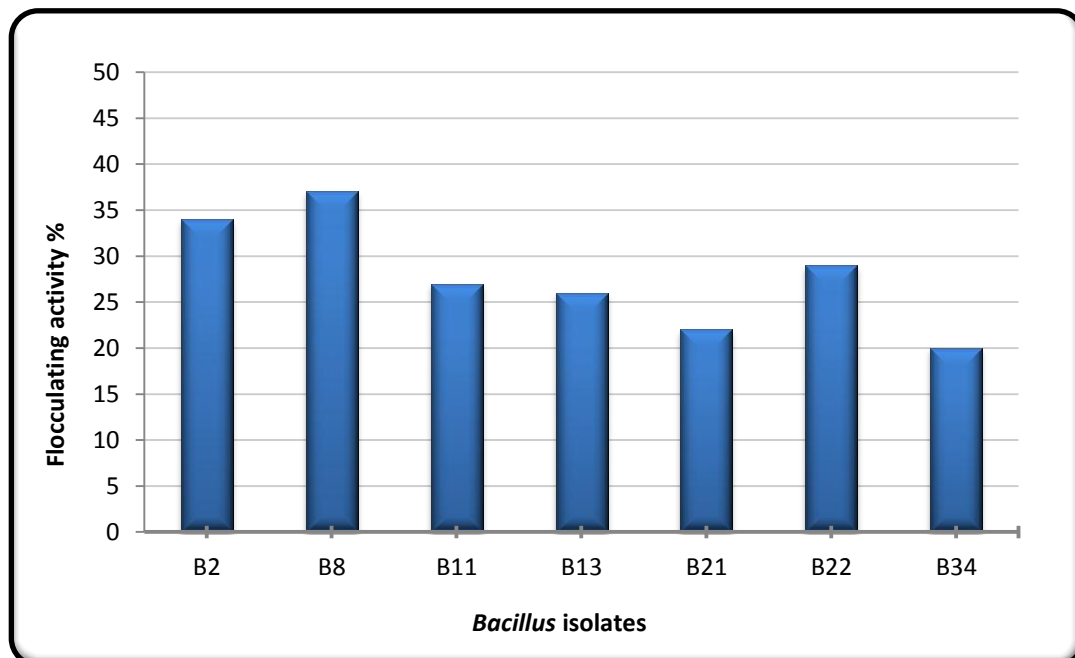


Figure (2): Production of bioflocculants by *Bacillus* isolates cultured in starch broth medium, pH 8 and incubated at 37°C for 48h.

Table (2) Biochemical tests for identification of *Bacillus coagulans* B8 isolate

Tests	Result
Catalase	+
Oxidase	-
Gram stain	+
Starch hydrolysis	+
Methyl Red	-
Voges-Proskauer	+
Egg-Yolk Lecithinase	-
Citrate Utilization	+
Motility	+
Indole	-
Growth in 7% NaCl	-
Growth at 40°C and 50°C	+
Acid production from Carbohydrate fermentation	
Glucose	+
Mannitol	-
Lactose	+
Fructose	+
Xylose	+

(+) Positive result

(-) Negative result

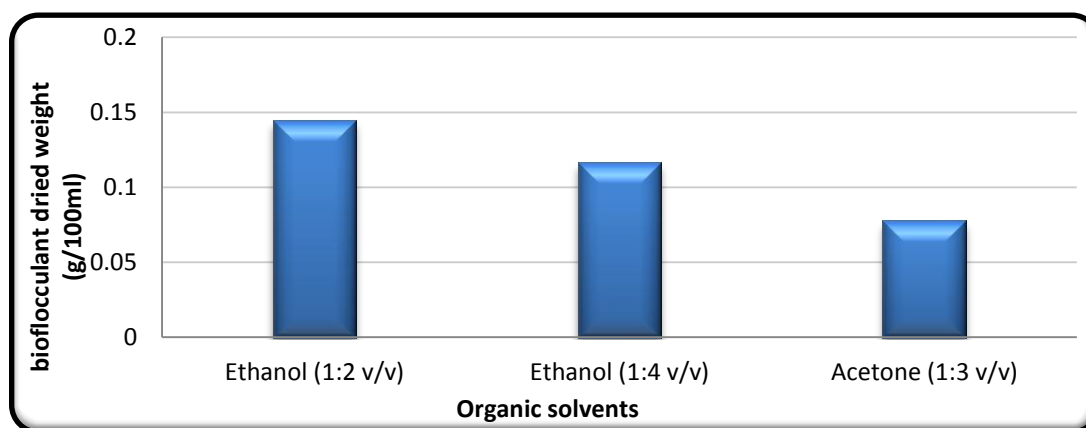


Figure (3): Bioflocculant extracted by different organic solvent

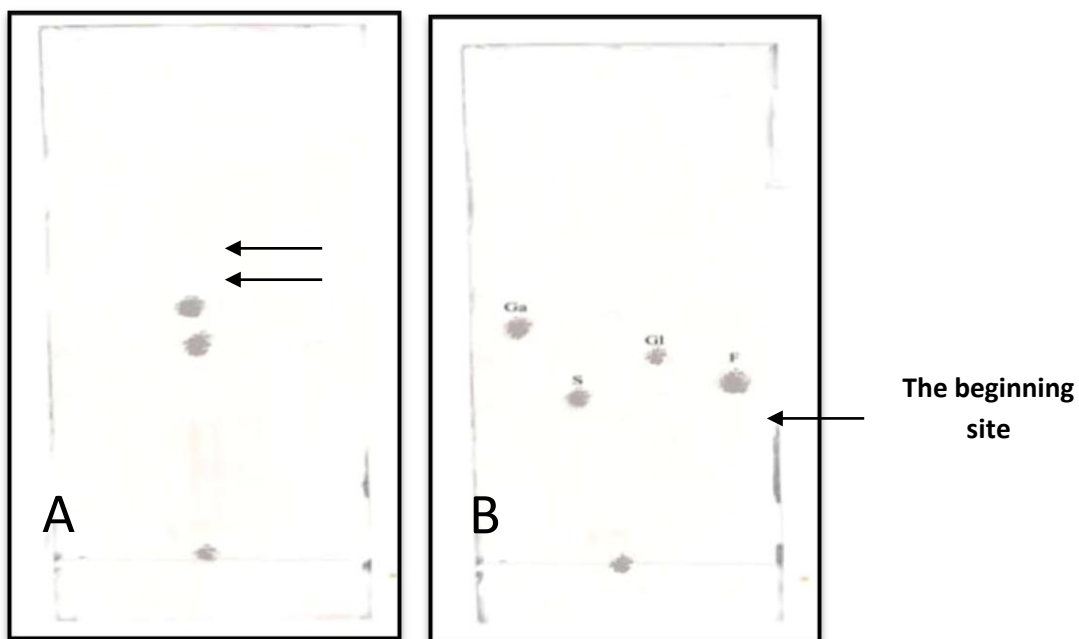


Figure (4): TLC analysis for detection of sugar of *B. coagulans* B8 bioflocculant by Phenol–sulfuric acid using silica gel plate with solvent system: butanol-acetic acid-water (3 : 1 : 1, w/w/w) and 96% ethanol-water (63 : 37, w/w) at room temperature: A-TLC plate showed two brown spots with $R_f = (0.58, 0.41)$
 B- Sugar standards (Gl: glucose, Ga: galactose, S: sucrose, F: fructose)

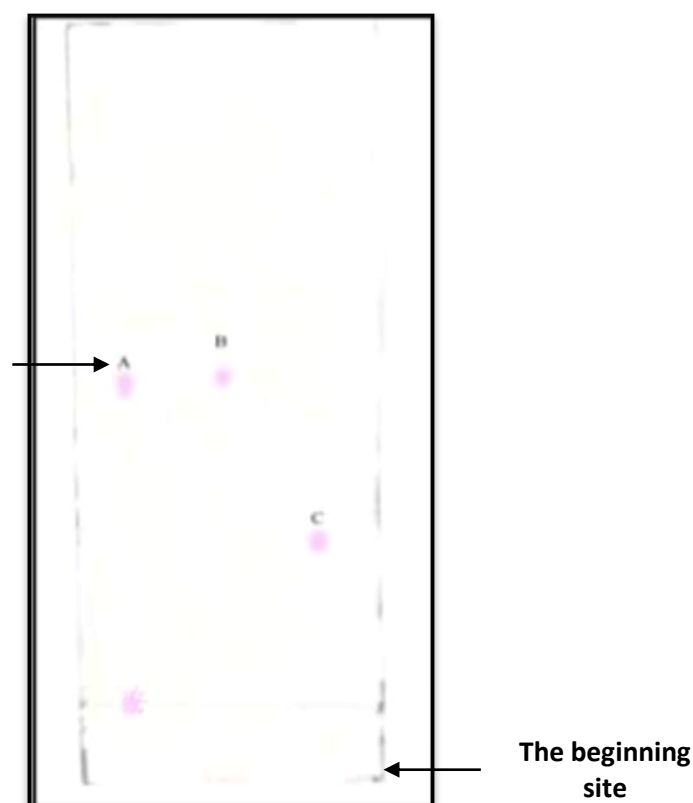


Figure (5): TLC analysis for detection of amino acids of *B. coagulans* B8 bioflocculant by ninhydrin using silica gel plate with solvent system : buthanol-acetic acid-water (3 : 1 : 1, w/w/w) and 96% ethanol-water (63 : 37, w/w) at room temperature. TLC showed red-violate spot with $R_f = 0.68$ (A: sample, B: phenylalanine, C: Glutamic acid)

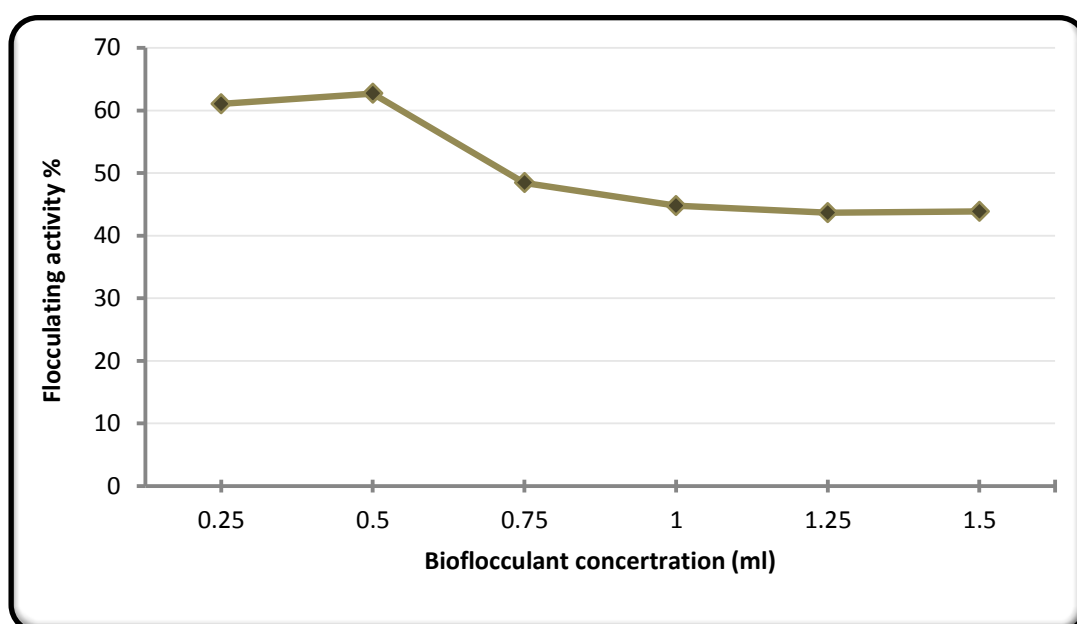


Figure (6): Effect of bioflocculant concentration of *B. coagulans* B8 on wastewater turbidity containing 1% CaCl_2 , pH 11

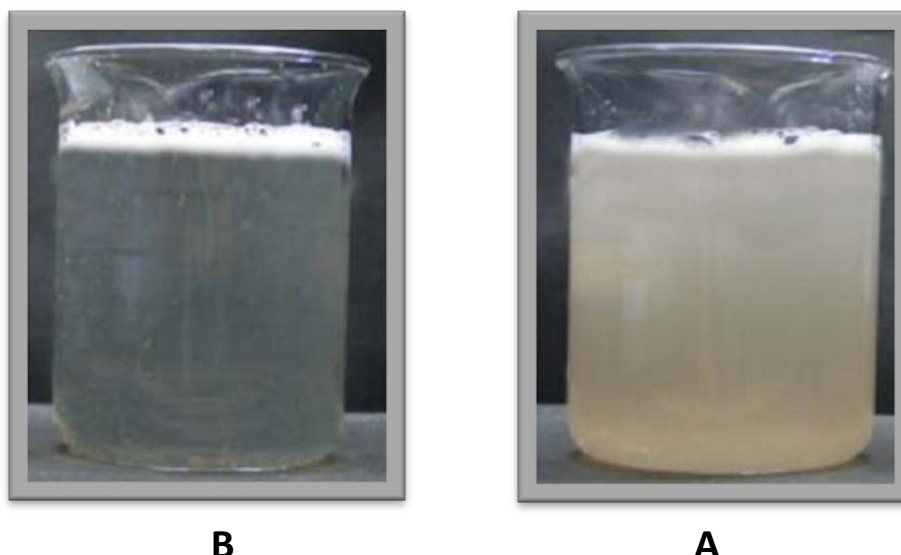


Figure (7): Effect of bioflocculant on the turbidity of wastewater containing 1% CaCl_2 , pH 11
 B: with bioflocculant A: without bioflocculant

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