

Purification and Characterization of Biofloculant produced by *Bacillus amyloliquefaciens* ABL 19 Isolated from Adeti Stream, Ilesa, Osun State, Nigeria

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Abstract: A biofloculant-producing bacterium was isolated from Adeti Stream in Ilesa, Osun State. The isolate was identified by the 16S rRNA sequencing technique. The BLAST (Basic Local Alignment Search Tool) analysis of the nucleotide sequence obtained for the bacterium showed a 98% similarity with *Bacillus amyloliquefaciens*. The biofloculant was extracted with cold acetone and purified using gel-filtration chromatography. The bacterium produced biofloculant optimally in glucose and peptone as sole sources of carbon and nitrogen. The biofloculant functioned effectively over a pH range of 4-11 in the presence of calcium chloride. The optimum temperature for the purified biofloculant was 60°C. Chemical composition of the biofloculant revealed it to be a glycoprotein and the molecular weight was estimated at 60.8 kDa. The above properties of this polymer suggests it to be a better alternative to more expensive organic and inorganic flocculants. This study revealed a purified biofloculant with low dependence on calcium chloride and low dosage requirement.

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1. Introduction

Biofloculants are compounds secreted by microorganisms which promote flocculation by causing colloids and other suspended particles in liquid to aggregate or form a floc (Abd-El-Haleem *et al.*, 2008). Generally, flocculants are classified into three groups: inorganic flocculants, organic synthetic flocculants, and naturally occurring biofloculants (Shih *et al.*, 2001). The first two groups are commonly utilized in different industrial processes such as wastewater, drinking water purification and dredging due to their effectiveness and low cost but have been known to cause environmental and health problems, but biofloculants are eco-friendly, biodegradable and not harmful to the environment (Choi *et al.*, 2001; Shih *et al.*, 2001; Gong *et al.*, 2003; He *et al.*, 2009).

Despite the obvious advantages that synthetic or chemical flocculants provide, their status has not been favorable currently and there have been concerns on their safety. Most of high molecular weight polymers are recalcitrant; for example, monomers of polyacrylamide are potent carcinogen and neurotoxic to humans and other animals (Vanhoric *et al.*, 1983). They have detrimental effect on flora and fauna. Naturally occurring polymeric materials have been shown to cause flocculation, and they are mostly derived from plants but a cheap and easy alternative, which is currently gaining popularity, is microbes (Canilha *et al.*, 2005).

The interest in biotechnological methods for the production of biofloculants lies in the possibility of

using different organisms to synthesize extracellular substances with different compositions (Taniguchi *et al.*, 2005; Gong *et al.*, 2008, Zheng *et al.*, 2008). Natural flocculants having a plant or microbial origin, may be composed of polysaccharide, proteins, lipids, lipoproteins and lipopolysaccharide and flocculation by them involves the polymer chain sticking to multiple particles making an aggregate large enough to settle down.

Microbes, especially bacteria have shorter generation times, are versatile and can produce extracellular polymeric material. Several groups of microorganisms such as algae, bacteria, actinomycetes and fungi, have been reported to produce biofloculants (Takagi and Kadowaki, 1985; Zhang *et al.*, 1999; Huang *et al.*, 2005). *Rhodococcus erythropolis* produced biofloculant NOC-1 with an efficient activity that caused flocculation of a wide range of suspended solids (Kwon *et al.*, 1996) while *Phormidium* J-1, a benthic filamentous cyanobacterium isolated from drainage channel was found to produce a high molecular weight polymer which can flocculate bentonite particles from suspensions. *Archuadendron* sp. TS-49 also produced a biofloculant which effectively flocculated various microorganisms and organic/inorganic materials (Li *et al.*, 2003) and *Aspergillus parasiticus* produced a biofloculant with flocculating activity for kaolin suspension and water-soluble dyes (Deng *et al.*, 2005).

In spite of these array of biofloculants thus identified, few of them have been applied practically in

industry because some require further enhancement of flocculating capability in order to make dosage requirements cost-effective (Gao *et al.*, 2006). The importance of flocculation capabilities presented by these bioflocculants has continued to prompt research into screening, characterization and structural identification of these compounds produced by microorganisms, hence, this study.

2. Materials and methods

Isolation of bioflocculant-producing strains

The wastewater, soil and activated sludge samples were collected from Ile-Ife and Ilesa, Osun State. Bioflocculant-producing microorganisms were preliminarily screened on yeast extract, peptone and glucose (YPG) agar medium composed per litre of glucose 20 g, peptone 20 g and yeast extract 10 g at pH 6.5. The samples were serially diluted (10^{-1} - 10^{-6}) and inoculated on yeast extract, peptone, glucose (YPG) agar plates. The YPG agar plates were incubated at 37 °C for 24 h. Distinct colonies were selected randomly according to their different morphological characteristics and further purified by repeated streaking on nutrient agar. These isolates were stored on sterile YPG agar slants in McCartney bottles at 4 °C.

2.1. Production of bioflocculant

Standardized culture (0.5 ml) of each isolate was inoculated into 50 ml (1:100 v/v) of YPG broth in 250 ml flasks and incubated on a rotary shaker at 25 °C at 120 rpm for 72 h. The fermentation broth was centrifuged at 5000 rpm for 30 min to remove the cells and the supernatant was examined for flocculating activity.

2.2. Measurement of flocculating activity

The suspension of kaolin clay was used to measure the flocculating activity according to the method described by Kurane *et al.*, (1986). A concentration of 5.0 g/L of kaolin clay at pH 7 was suspended in distilled water. A volume of 0.25 ml calcium chloride (100 mM) was added to 9 ml of kaolin clay suspension in a test-tube with 0.1 ml of the crude broth. The mixture was made up to 10 ml, stirred and allowed to settle for 5 min. The absorbance of the upper phase was measured at 550 nm using a colorimeter. The flocculating activity was measured and calculated as follows: Flocculating activity (%) = $(B-A)/B \times 100\%$

A and B were the absorbances at 550 nm for sample and control, respectively. A control in which distilled water was used instead of bioflocculant was also measured under the same conditions. Finally, the strain with the highest and stable flocculating activity for kaolin was selected for further studies.

2.3. Time Course of Bioflocculant Production

The medium for bioflocculant production was composed as follows: glucose (20 g), KH_2PO_4 (2 g), K_2HPO_4 (5 g), $(\text{NH}_4)_2\text{SO}_4$ (0.2 g), NaCl (0.1 g), urea (0.5 g) and yeast extract (0.5 g) per litre of distilled water with initial pH of 6.5 (Wang *et al.*, 1995). For the inoculum preparation, the selected strain was pre-subcultured in 50 ml of YPG broth in 250 ml conical flasks and loaded on a rotary shaker (120 rpm) at 25 °C. Medium samples of 5 ml volume were withdrawn at 3 h intervals from each flask to measure the pH, cell growth and flocculating activity.

2.4. Strain Identification

The preliminary identification of the bacteria isolate exhibiting the best bioflocculant production was carried out based on their morphological and biochemical characteristics according to the Bergey's Manual of Systematics Bacteriology (Holt *et al.*, 1994). The molecular characterization was carried out at the International Institute of Tropical Agriculture (IITA), Ibadan, using 16S rRNA gene sequencing technique.

2.5. Extraction of DNA and PCR amplification

The DNA extraction was carried out according to a modified process of Trindade *et al.*, (2007). The DNA extracted was then subjected to Polymerase Chain Reaction (PCR) amplification using the primer pair 27F and 1525R. The PCR amplification was performed in 25 μl containing 4 μl of the DNA solution, 0.4 μl of 10mM dNTPs, 2 μl of 25mM MgCl_2 , 1 μl of 10 pmol each of primer, 0.2 μl of 2.5 units of DNA polymerase (Promega, USA), and the 5 μl of 5 \times PCR buffer. Sterile DNase free water was added to make a volume of 25 μl .

Thirty five PCR cycles were conducted in automated thermal cycler. The thermal conditions were as follows: denaturation at 94 °C for 1 min (3 min for the first cycle), annealing at 56 °C for 1 min and extension at 72 °C for 1 min. This was followed by one cycle of final extension for 7 min at 72 °C. The PCR amplicon was analyzed by 1.5% agarose gel electrophoresis. The purified products were sequenced and obtained 16S rRNA gene sequences blasted into the internet (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for similarity search.

2.6. Effect of Carbon and Nitrogen Sources

The effect of different carbon and nitrogen sources on bioflocculant production was assessed with glucose, sucrose, lactose, starch, fructose representing the carbon sources and peptone, urea, ammonium chloride, ammonium sulphate representing nitrogen sources.

2.7. Effect of Concentration of Ca^{2+}

Calcium chloride concentration (100mM) was added to constant mixture of kaolin clay suspension and Ca^{2+} and the rate of flocculation was determined to obtain the optimum amount of calcium chloride required for the maximum flocculating activity.

2.8. Effect of various cations and initial pH on flocculating activity

Calcium chloride solution was replaced with salt solutions of magnesium chloride, sodium chloride, ferric chloride, potassium chloride and ferrous sulphate in the reaction mixture and their effect on flocculating activity assessed. The effect of initial pH of the production medium was also assessed in the range of 3–9 using 0.1 M H₂SO₄ and NaOH.

2.9. Extraction, purification of bioflocculant

Cells were harvested by centrifugation at 5000 xg for 20 min after 72 h of production and then subjected to acetone precipitation. Two volumes of acetone was mixed with one volume of the supernatant, stirred and left to stand for 12 h at 4 °C. This was centrifuged to obtain the precipitate and further purification was achieved by addition of one volume of mixed solution of chloroform and n-butyl alcohol (5:2 v/v). The mixture was stirred and left for 12 h and centrifuged to recover the precipitate. The precipitate was air-dried and thereafter applied to Sephadex G-100 column (1.0 cm × 48 cm) for further purification. The gel chromatography column was balanced with phosphoric acid buffer solution (0.02 mol/L, pH=7.0) which was used for elution and 1 ml fractions were collected at the flow rate of 12 ml/h. The flocculating activity of each eluent was assayed and the active fractions pooled for further characterization of the purified sample.

2.10. Characterization of bioflocculant

2.10.1. Molecular weight

The molecular weight of the bioflocculant was determined using the Sephadex G-100 column (1.0 cm × 48 cm) and phosphate buffer solution (0.02 mol/L, pH=7.0) for elution at the flow rate of 12 ml/h. The elution retention time of standard proteins: BSA (67 kDa), Ovalbumin (43 kDa), Peroxidase (40 kDa), α-Chymotrypsinogen A (24 kDa), Lysozyme (14.4 kDa) were used to prepare a calibration curve and blue dextran of a high molecular weight of 2×10⁶ Dawas used as molecular weight standard.

2.10.2. Temperature stability of crude and purified bioflocculant

This was assessed by incubating aliquots of the crude and purified bioflocculant at 4°C-100°C for 30 min and consequently determining the residual flocculating activity.

2.10.3. Effect of pH and flocculating dosage

The effect of pH and flocculating dosage was evaluated by adjusting the pH of the kaolin clay with H₂SO₄ (0.1 M) and NaOH (0.1 M) from pH 3-12. The flocculating dosage was carried out by varying the volume of culture broth and purified flocculant added to constant mixture of kaolin suspension and calcium chloride. The flocculating activities were subsequently determined to obtain the optimum dosage.

2.10.4. Carbohydrate and protein content of bioflocculant

The total carbohydrate content in the bioflocculant was determined by the phenol–sulfuric acid method by Dubois (1986) using alginate as standard while the protein content was measured by the Bradford method (1976) using bovine serum albumin as standards.

3. Results and Discussion

3.1. Screening and identification of bioflocculant-producing bacteria

A total of 48 colonies of bioflocculant-producing bacteria were isolated from wastewater, soil and activated sludge samples and the assay of their flocculating abilities revealed a bacterium exhibiting a high flocculating activity of 94%. This bacterium was Gram-positive rod, amylase-producing, endospore-forming and non-motile as shown in Table 1.1 and its identity was confirmed by the 16S rRNA sequencing method. The BLAST (Basic Local Alignment Search Tool) analysis of the nucleotide sequence obtained for the bacterium showed a 98% similarity with *Bacillus amyloliquefaciens*. It was therefore coded as *Bacillus amyloliquefaciens* ABL 19. Many reported microorganisms which could secrete biopolymer flocculant belongs to *Bacillus* sp. (Salehizadeh and Shojaosadati, 2001; Deng *et al.*, 2003; Kwon *et al.*, 1996; Suh *et al.*, 1997). *Bacillus* sp. gilbert (Piyo *et al.*, 2011), *Bacillus licheniformis* (Li *et al.*, 2009), *Virgibacillus* (Cosa *et al.*, 2011), *Bacillus firmus* (Salehizadeh and Shojaosadati, 2002), *Bacillus circulans* (Li *et al.*, 2009) and the like.

3.2. Time course of bioflocculant production

The growth curve in relation to the flocculating activity and cell number was shown in Fig. 1.0. The cell number increased gradually with culture time and bioflocculant production commenced at the 39 h. The peak of flocculating activity of 90% was recorded near the stationary phase at the 66 h which remained stable till 96 h. Several studies reported different culture times for maximum bioflocculant production by different bacteria. In *Bacillus firmus*, bioflocculant production peaked after 33 h (Salehizadeh and Shojaosadati, 2002). In *Agrobacterium* sp. M-503, production peaked after 48 h (Li *et al.*, 2010), while for *Vagococcus* sp. W31, production peaked after 60 h (Gao *et al.*, 2006). Also, in *Phormidium* strain J-1, production peaked after 96 h (Fattom and Shilo, 1984), while for *Halomonas* sp. OKOH (Mabinya *et al.*, 2011), and *Bacillus* sp. gilbert (Piyo *et al.*, 2011), maximum flocculating activities were attained in 135 h and 240 h respectively.

3.3. Effect of various concentration of calcium chloride

The effect of various concentrations of calcium chloride at dosage range of 0 – 0.6 ml was determined. The result showed a flocculating activity of 78% without the addition of calcium chloride after which there was further increment in flocculating activity until the optimum dosage was obtained at 0.25 ml (Fig. 1.1). The flocculating activity of *Bacillus amyloliquefaciens* ABL 19 became constant up to 0.6 ml. This observation was explained by the result from experiments by Nguyen, *et al.* (2007) which stated that the rate of the flocculation increases initially with the addition of calcium concentration and then approaches a steady-state at higher concentrations. This suggests that at a higher calcium concentration, saturation of the floc has occurred, and the rate of flocculation is independent of calcium concentration. It is also worthy of note that the bioflocculant obtained in this study has a low dependence on cations. The crude bioflocculant showed a flocculating activity of 78% without the addition of calcium chloride indicating that the addition of the cation only contributed 11.1% to attain maximum flocculation (88%).

3.4. Effect of cations

Divalent cations (Ca^{2+} and Mg^{2+}) greatly supported flocculation with flocculating activities of 94% and 87% respectively while the monovalent cations (K^{+} and Na^{+}) recorded a decline of flocculating activities of 66% and 67% respectively (Table 1.2). Iron salts, FeSO_4 and FeCl_2 inhibited flocculation at 38% and 0% respectively as reported in literature by (Forster, 1985; Higgins and Novak, 1997; Sobek and Higgins, 2002).

3.5. Effect of carbon and nitrogen sources on bioflocculant production

Constituents of the culture medium and culture conditions have been well documented to have effect on the production of bioflocculants (He *et al.*, 2004; Xia *et al.*, 2008). The results obtained in this study showed that glucose was best utilized by *Bacillus amyloliquefaciens* ABL 19 (94%) followed by sucrose, starch, fructose and lactose with flocculating activities of 92%, 92%, 81% and 83% respectively (Table 1.3). In the same light, peptone presented the best nitrogen source with optimum activity of 94%, followed by ammonium sulfate, ammonium chloride with flocculating activities of 91% and 92.7% respectively as shown in Table 1.4. On the other hand, the organism was unable to utilize urea as an organic carbon source with zero activity recorded.

3.6. Effect of initial pH

According to Nakata and Kurane (1999) and Salehizadeh and Shojaosadati (2001), the initial pH of the culture medium determines the electric charge of the cells and the oxidation-reduction potential which can affect nutrient absorption and enzymatic reaction. *Bacillus amyloliquefaciens* ABL 19 produced

bioflocculant within the pH ranges 4-8. As depicted in Fig. 1.2, the optimal activity (96%) was found at slightly acidic pH 6 after which flocculating activity declined, indicating that isolate ABL 19 is a neutrophile. Mitchell and Slaughter (1989), proposed that organisms (*Bacillus sp.*) that can grow at optimum pH in the range of 4 to 9 are named neutrophiles. pH requirements differ for individual organisms, for example, the strains *Streptomyces griseus* and *Aspergillus sojae* produced flocculating substances under acidic conditions (Shimforuya *et al.*, 1995). Bioflocculant production by *Rhodococcus erythropolis* was higher at alkaline pH values (8.0–9.5) (Kurane *et al.*, 1994), while in another study it showed activity at neutral pH (Takagi and Kadowaki, 1985). *Virgibacillus sp.* Rob preferred high alkaline pH (12.0). *Bacillus licheniformis* X14 optimally produces a bioflocculant ZS-7 under alkaline conditions.

3.7. Purification and identification of bioflocculant

After centrifugation, 1.67 g precipitate was recovered from culture broth (1 L). It is brown in color and the yield was compared with different yields of bioflocculant from other microorganisms as shown in Table 1.5. Information on the polarity of this bioflocculant showed high solubility in water in contrast to the butanol-chloroform mixture indicating a polar molecule.

The further purification of bioflocculant on Sephadex G-100 revealed a major independent elution peak (Fig. 1.3). From the UV spectrum analysis at 280 nm, protein was elucidated at fractions 11-18 and phenol-sulfuric acid test of the pooled fractions confirmed the presence of carbohydrates at 0.937 mg/ml. The molecule was inferred to be a glycoprotein which explains its high activity. As reported in literature, polysaccharides were suggested to play a major role in flocculation due to their capacity to form bridges between their negatively charged groups and divalent cations available in sludge (Flemming and Wingender, 2001; Korstgens *et al.*, 2001; Higgins and Novak, 1997).

The contribution of protein to flocs binding strength is explained by hydrophobic interactions and polyvalent cation bridging, both enhancing the stability of the biopolymer network (Jorand *et al.*, 1998). Similar bioflocculants have been reported before (Madla *et al.*, 2005; Lungmann *et al.*, 2007), and glycoprotein flocculants can be applied in various fields including biotechnology and nanotechnology (i.e. silicon wafers, lipid films and liposomes) (Lungmann *et al.*, 2007).

3.8. Molecular weight

The estimated molecular weight of the bioflocculant was 60.8 kDa from the calibration curve. The molecular weight of the bioflocculant was lower than some of the bioflocculants reported in literature.

For example, *P. elgii* has a molecular weight of 3.5×10^6 Da which was much higher than that of bioflocculant from *Bacillus licheniformis* X14, which was only 6.89×10^4 Da (Li *et al.*, 2009). The molecular weights of EPS produced by *S. phocae* PI80 and *E. faecium* MC13 were estimated to be 2.8×10^5 Da and 2.0×10^5 Da. It was higher than the EPS (2.8×10^4 Da) produced by *L. fermentum* TDS030603 (Fukuda *et al.*, 2010) and lower than the EPS from *L. pentosus* (2.0×10^6 Da), *L. rahmnosus*JAAS8 (9.1×10^5 Da) and *Lactococcus lactis* subsp. *lactis* 12 (6.9×10^5 Da) (Rodriquez-Carvajal *et al.*, 2008; Yang *et al.*, 2010; Pan and Mei, 2010).

3.9. Flocculating dosage

Dosage optimization in water treatment technologies is another aspect to be taken into consideration. It is widely recognized that a lower dosage of bioflocculants with a high performance in flocculating activity will contribute towards cost effectiveness. The optimum dosage of both crude and purified bioflocculant was (0.9 ml/L) and (0.45 ml/L) respectively (Fig.1.4 and 1.5). This dose was lower than that of the bioflocculant produced by UPMB13 as reported by Zufarzaana *et al.*, (2012) which was shown to have high flocculating activity at a low dosage input of 0.5% (5 ml/L). In the same light, the reduction in flocculating activity which occurred with further addition of bioflocculant was explained by (Chan and Chiang, 1995), that when the optimum concentration is exceeded, the aggregated particles can re-disperse and this disturbs particle-settling. This has been attributed to an increase in the repulsive energy between the flocculants and the microorganisms, which causes hindrance in floc formation (Mishra *et al.*, 2004).

3.10. Temperature stability

The crude bioflocculant produced by *B. amyloliquefaciens* ABL 19 could withstand 4 °C-80 °C (Fig.1.6) in contrast to the purified bioflocculant which could only withstand up to 60 °C (Fig. 1.7). This may be attributed to the presence of protein molecules in this bioflocculant. According to Decho, (1990) and Hoagland, (1993), proteins in the microbial EPS play an important role in stabilizing the tertiary polymer structure and to some extent help in adhesion of cells to surfaces.

He *et al.*, (2004) reported that heating bioflocculant REA-11 (a purified bioflocculant) to 100 °C causes it to completely lose activity. Another bioflocculant (As-101) lost 50% of its flocculating activity when heated at 100 °C for 15 min (Salehizadeh *et al.*, 2000). Yokoi *et al.*, (1995) also reported that almost all residual flocculating activity was lost when the bioflocculant of *Bacillus* PY-90 was heated at 100 °C for 40 min. The lower flocculating activity of the bioflocculant at higher temperatures above 80 °C may be due to the breakdown of the polysaccharide chain

which led to the low potential to form bridges with the kaolin particles (Liu *et al.*, 2010).

3.11. Effect of pH

The pH conditions suitable for flocculation process by the bioflocculant was carried out and the crude broth functioned well between pH ranges of 4 – 10 as shown in Fig. (1.8) while Fig. 1.9 showed the effectiveness of the purified sample at acidic pH of 4.0 and alkaline pH range of 7-9 respectively. This demonstrated the effectiveness of the bioflocculant over a wide pH range. However, its flocculation efficiency slightly reduces at highly acidic (pH<5) and highly alkaline (pH>9) circumstances. This may be returned to the biopolymer shows different electric states at different pH, in turn affects the bridging efficiency of the biopolymer for clay powder (Yong *et al.*, 2009). According to Salehizadeh *et al.*, 2000, bioflocculation by *Bacillus* sp. As-101 was more prevalent in acidic conditions, while biopolymer flocculant produced by *Bacillus licheniformis* CCRC12826 was effective in neutral pH range (Shih *et al.*, 2001). Similar finding was also reported by Liu *et al.* (2013), for bioflocculant produced by *Chryseobacterium daeguense* W6 which preferred conditions of low acidic to low alkaline with the same pH range of 4.0–8.0. For *P. elgii* reported by Li *et al.*, (2010), the flocculation activities in a wider pH range (pH 3.0 – 11.0) were measured and a relatively stable and high level (over 80%) was found, which means that the bioflocculant produced by *P. elgii* can be used with all kinds of wastewater.

Conclusion

Table 1.1: Biochemical and Physiological Characteristics of *Bacillus amyloliquefaciens* ABL 19 Isolated from Adeti Stream, Ilesa, Osun State.

Isolate	ABL 19
Colony color	White
Colony shape/edge	Filamentous/Fimbrate
Colony surface	Dry/Flat
Opacity	Opaque
Gram reaction/Shape	+/Rod
Spore Staining	+
Anaerobic test	+
Starch Hydrolysis	+
Nitrate Reduction	-
Citrate Utilization	+
Indole Test	-
Catalase Test	+
Motility Test	-
Glucose fermentation	+
Lactose fermentation	+
Mannitol fermentation	-
Arabinose fermentation	-
Triple Sugar Iron Agar	+
Oxidase test	+
Urease test	+
H ₂ S test	-

Key: + = Positive result; - = Negative result

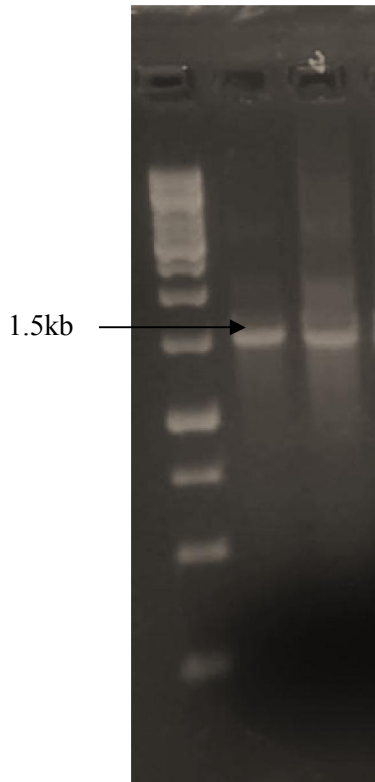


Plate 1: Agarose gel picture of 16S rRNA amplicon (~1.5 kb) of *Bacillus amyloliquefaciens* ABL 19 (in duplicate) with the ladder (first lane).

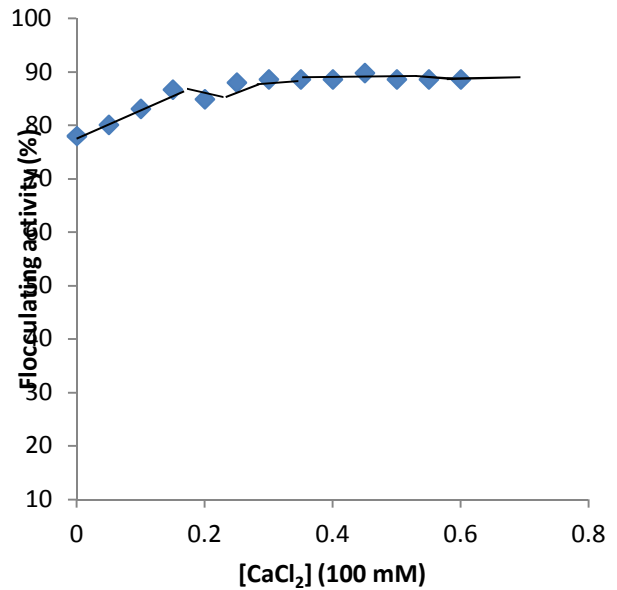


Figure 1.1: Effect of CaCl₂ concentration on flocculating activity of bioflocculant produced by *B. amyloliquefaciens* ABL 19 at 25 °C.

Table 1.2: Effect of Different Cations on Flocculating Activity of *Bacillus amyloliquefaciens* at 25 °C.

Cations	Flocculating activity (%)
Calcium chloride	94.4
Magnesium chloride	86.8
Potassium chloride	66.3
Sodium chloride	68.7
Ferric chloride	-
Ferrous sulphate	38.0

Table 1.3: Effect of Carbon Source Variation on Flocculating Activity of *Bacillus amyloliquefaciens* ABL 19 at 25 °C, pH 6.5 after 72 h.

Carbon sources	Flocculating activity (%)
Glucose	94.4
Fructose	81.3
Sucrose	92.2
Starch	92.2
Lactose	83.7

Table 1.4: Effect of Nitrogen Source Variation on Flocculating Activity of *Bacillus amyloliquefaciens* at 25 °C, pH 6.5 after 72 h.

Nitrogen sources	Flocculating activity (%)
Peptone	94.4
Ammonium chloride	92.8
Ammonium sulphate	91.0
Urea	-

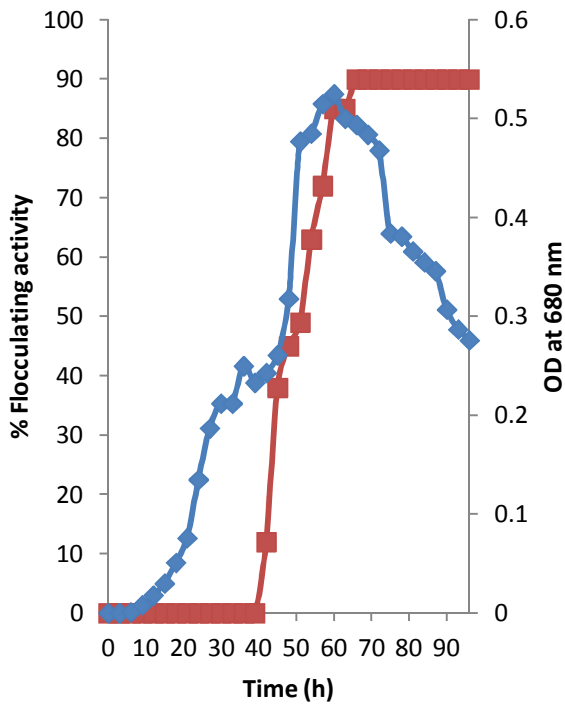


Fig. 1.0: The growth profile of *Bacillus amyloliquefaciens* ABL 19 in production medium at 25 °C, pH 6.5 after 96 h.

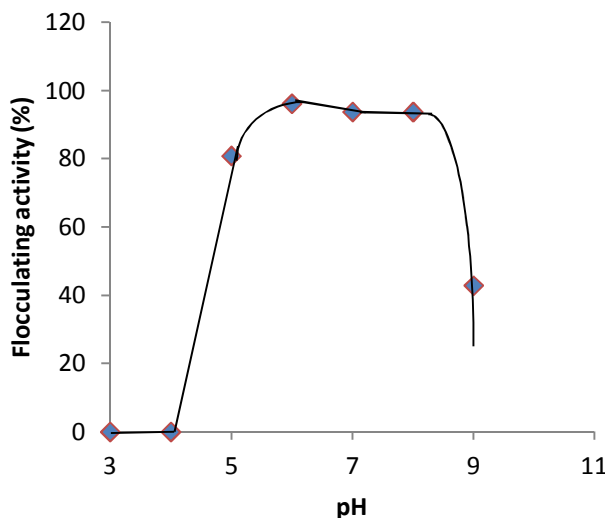


Fig. 1.2: Effect of pH variation of YPG medium on bioflocculation production by *Bacillus amyloliquefaciens* ABL 19 at 25 °C after 72 h.

This study isolated a bioflocculant from *B. amyloliquefaciens* ABL 19 which has a unique property of low dependence on calcium chloride (cation). This discovery will initiate research into further characterization of this bioflocculant to obtain more information on its structure and properties. In the same light, as medium optimization is a crucial aspect of industrial fermentation, the use of cheap carbon and nitrogen sources will also be considered in further studies to improve the overall cost effectiveness of this bioflocculant.

Table 1.5: Comparison of the Purified Bioflocculant Produced by *Bacillus amyloliquefaciens* ABL 19 with other Bioflocculants.

Strain	Bioflocculant Yield (g/L)	Carbon/Nitrogen sources in fermentation	Components	References
<i>Enterobacter cloacae</i> WD7	2.27	Glucose/Sucrose, (NH ₄) ₂ SO ₄	acidic hetero-polysaccharide	Prasertsan <i>et al.</i> , 2006
<i>Proteus mirabilis</i> TJ-1	1.33	Glucose, peptone	Acid polysaccharide and protein	Xia <i>et al.</i> , 2008
<i>Bacillus firmus</i>	1.36	Glucose, peptone, yeast extract	Acidic polysaccharide	Chen <i>et al.</i> , 2002
<i>Bacillus</i> sp. Strain F19	1.47	Sucrose, yeast extract	Mainly polysaccharide	Zheng <i>et al.</i> , 2008
<i>Aeromonas</i> sp.	2.25	Sucrose, yeast extract, ammonium sulphate, urea	Not specified	Li <i>et al.</i> , 2007
<i>Vagococcus</i> sp. strain W31	2.3	Glucose, ammonium sulphate	Polysaccharide	Gao <i>et al.</i> , 2006
<i>Virgibacillus</i> sp. Rob	2.43	Glucose, peptone	Polysaccharide	Cosa <i>et al.</i> , 2011
<i>Bacillus amyloliquefaciens</i> ABL 19	1.67	Glucose, peptone and yeast extract	Polysaccharide and protein	Our study

Table 1.6: Purification Protocol of Bioflocculant produced by *Bacillus amyloliquefaciens* ABL 19.

Purification step	Total volume (ml)	Activity (%/ml)	Protein concentration(mg/ml)	Total units (%/ml)	Specific activity (units/ml)	Purification fold
Crude bioflocculant	75	900	0.1248	67500	7211	1.00
Acetone precipitate	7	10750	1.015	75250	10591	1.46
Sephadex G-100	43.75	3300	0.219	144375	15068	2.09

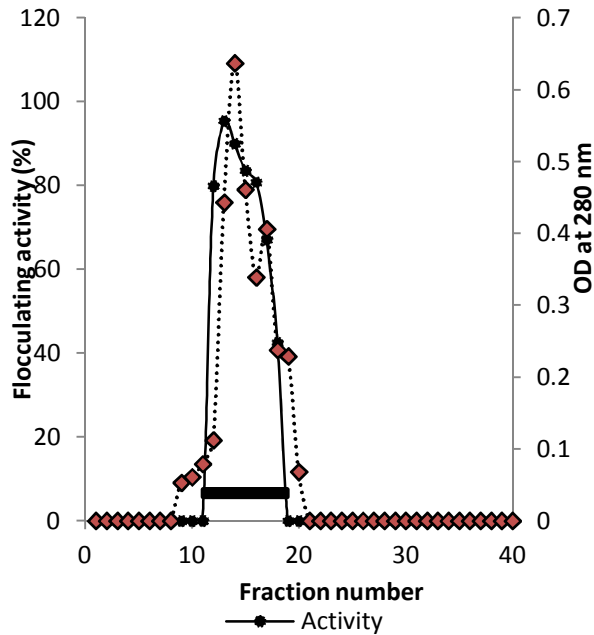


Fig 1.3: The elution profile of the bioflocculant produced from *B. amyloliquefaciens* ABL 19 on Sephadex G-100 column at 25 °C, pH 7.0 using 0.02 mol/L phosphate buffer as mobile phase.

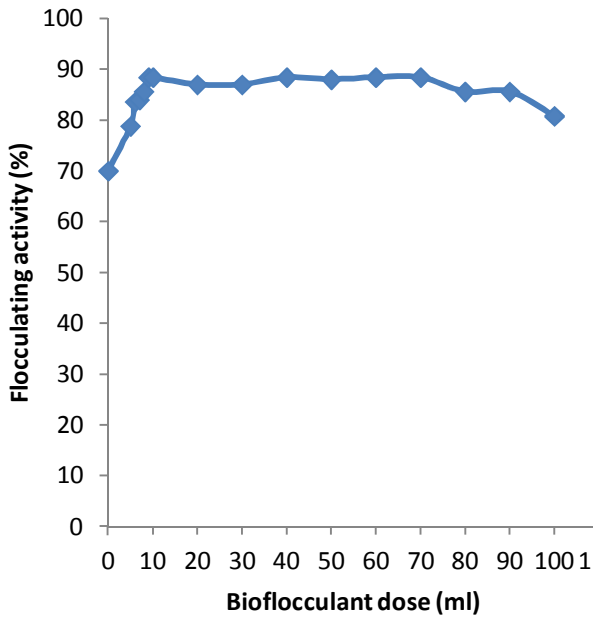


Fig. 1.4: Effect of flocculating dosage of crude bioflocculant on flocculation at 25 °C.

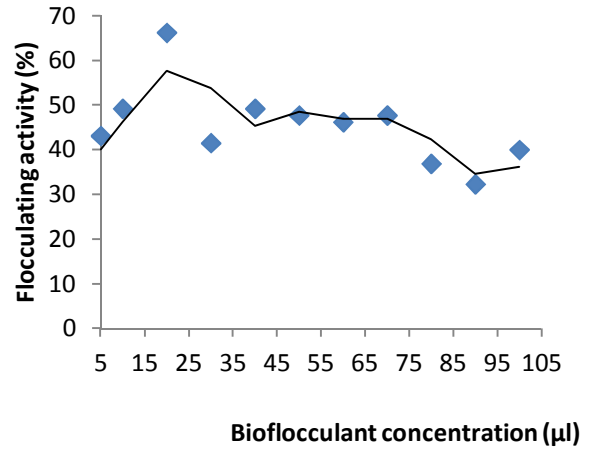


Fig. 1.5: Effect of flocculating dosage of purified bioflocculant on flocculation at 25 °C.

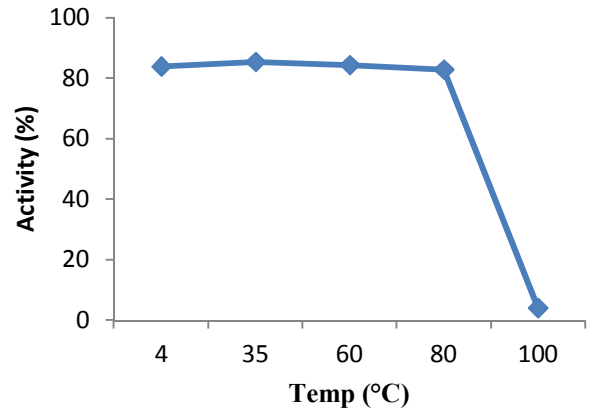


Fig. 1.6: Temperature stability of crude bioflocculant.

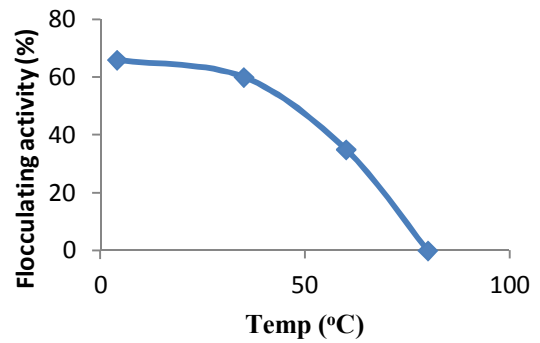


Fig. 1.6: Temperature stability of purified bioflocculant.

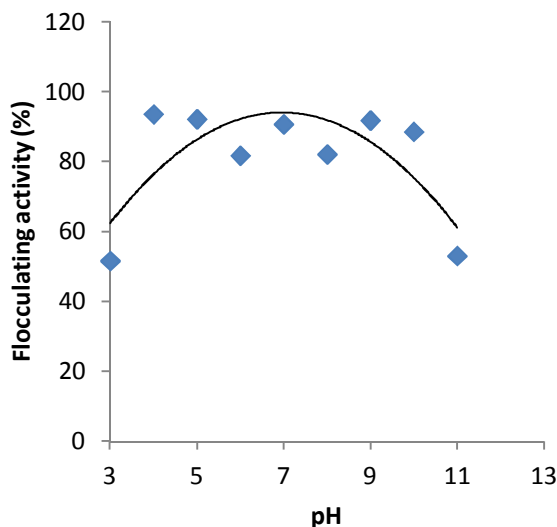


Figure 1.8: Effect of pH on flocculating activity of crude bioflocculant at 25 °C at different pH values.

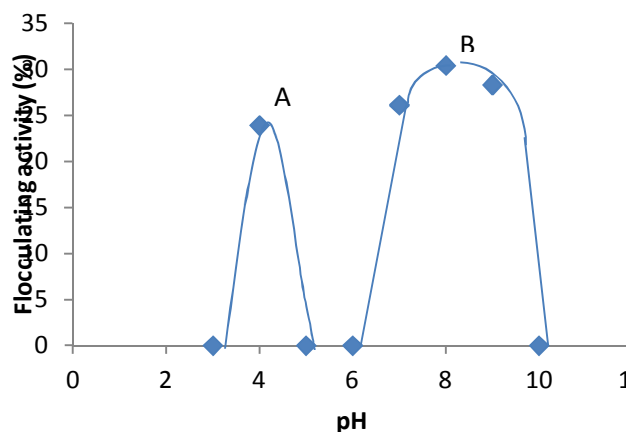


Figure 1.9: Effect of pH on flocculating activity of purified bioflocculant at 25 °C at different pH values. A-Flocculating activity at acidic pH, B-Flocculating activity at alkaline pH

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