Assessment of *Bacillus pumilus* Isolated from Fresh Water Milieu for Bioflocculant Production

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Abstract: A bioflocculant produced by a *Bacillus* species was assessed with regards to its physiochemical properties and flocculating efficiency. Identification of the bacteria through 16S rDNA sequencing revealed it to have 99% similarity to *Bacillus pumilus* strain ZAP 028. The optimum culture conditions for bioflocculant production by the bacterial strain were inoculum size of 4% (*v/v*), maltose as a sole carbon source, multiple nitrogen source (yeast extract, urea and ammonium sulfate) and medium initial pH 7. The bioflocculant was thermostable with high flocculating rate for kaolin suspension at low dosage 0.1 mg/mL over a wide pH range (3–11). Fourier-transform infrared (FTIR) spectroscopy analysis result of the purified bioflocculant showed that hydroxyl, amino and carboxyl groups were the main functional moieties in its molecular structure. The bioflocculant was composed of sugar (75.4%), protein (5.3%) and uronic acid (15.4%). Scanning electron microscopy (SEM) showed a dendritic bioflocculant structure and the energy dispersive X-ray spectroscopy (EDX) analysis revealed that the purified bioflocculant had weight fractions of elements as follows: 22.71% of C, 11.56% of N, 41.60% of O, 0.51% of S and 7.98% of P. The bioflocculant produced had strong flocculating activity and high thermal stability, which affords its utilization in industrial processes.

Keywords: *Bacilllus pumilus*; bioflocculant; flocculating activity; thermostable

1. Introduction

Bioflocculants are natural product metabolites of microorganisms that have capacity to flocculate various suspended solids including colloidal solids and cells [1,2]. Chemical flocculants have been extensively applied in various industries owing to their cost-effectiveness and high flocculating efficiency [3]; however, many researchers are investigating the use of bioflocculants as a result of health problems implicated in the use of chemical flocculants. Polyaluminium chloride (PAC) and polyacrylamide are among chemical flocculants that have been used extensively in downstream processing, wastewater treatment, food and fermentation industries [3]. However, polyaluminium salts have been implicated in Alzheimer's disease [4] and evidence also exists associating polyacrylamide monomers with neurotoxicity and carcinogenicity in humans [5]. Consequently, the use of these chemical flocculants is now a restricted cause of concern. These inevitable disadvantages of chemical flocculants have shifted the focus on investigating a variety of microorganisms for their flocculant-producing potentials [1,6].

Unlike chemical flocculants, bioflocculants have additional benefits such as being environmentally friendly, biodegradable, free of the risk of secondary pollution, and nontoxic and harmless to humans,

animals and the environment [1,7,8]. Bioflocculant-producing microorganisms have been isolated from almost all kinds of different environments such as soil, wastewater, activated sludge and rivers [9–12]. Recently, bioflocculants have also been produced by microorganisms isolated from unusual environments such as sputum [13] and human saliva [14].

Bioflocculants are not only useful in wastewater treatment but have a wide range of applications. For example, bioflocculants produced by *Sobacillus silvestries* W01 and *Paenibacillus* sp. AM49 were used to harvest marine microalgae *Nannochloropsis oceanica* and *Chlorella vulgaris*, respectively [12,15]. The practical applications of bioflocculants in industries however, have thus far been limited due to their high cost of production as well as high dosage requirements [1]. To overcome this challenge, utilization of a number of cost-effective substrates for bioflocculant production has been investigated [16]. As an example, dairy wastewater was used as a cheap substrate for bioflocculant production by *Klebsiella mobilis* [16]. In addition, a facultative oligotrophic *Klebsiella* sp. PB12 produced an exopolysaccharide in nutrient-poor medium thus reducing production costs [17]. The need for novel bioflocculants that are thermostable with enhanced bioflocculation efficiency and yield has therefore become apparent.

In this current study, the effect of culture conditions on bioflocculant production by a freshwater bacterium identified as *Bacillus pumilus* was assessed and the purified bioflocculant characterized. From our literature search, no report has been documented on the implication of *Bacillus pumilus* in the production of bioflocculant.

2. Materials and Methods

2.1. Sample Processing

Rock scraping samples were obtained from Thyume River in Alice, Eastern Cape, South Africa. The samples were transported to the Applied and Environmental Microbiology Research Group (AEMREG) laboratory and processed in accordance to the description of Jensen et al. [18] with minor modifications. About 0.5 g of rock scraping sample was dissolved and serially diluted in sterile distilled water (5 mL). Each dilution (100 μL) was spread on M1 agar plates with a sterile glass spreader and thereafter incubated at 28 °C for 120 h. The purity of the isolates was then verified by plating them on nutrient agar.

2.2. Growth Media for Bioflocculant Production

The activation medium was composed of beef extract (3 g/L), tryptone (10 g/L), and NaCl (5 g/L) in distilled water [19]. The components for screening medium were glucose (20 g/L), K_2HPO_4 (5 g/L), KH_2PO_4 (2 g/L), urea (0.5 g/L), yeast extract (0.5 g/L), $(NH_4)_2SO_4$ (0.2 g/L), and $MgSO_4 \cdot 7H_2O$ (0.2 g/L) in sterile distilled water [20]. Production medium was composed of maltose (20 g/L), K_2HPO_4 (5 g/L), KH_2PO_4 (2 g/L), urea (0.5 g/L), yeast extract (0.5 g/L), $(NH_4)_2SO_4$ (0.2 g/L), and $MgSO_4 \cdot 7H_2O$ (0.2 g/L) and the pH of the medium was adjusted to 7.0 with either NaOH (0.1 M) or HCl (0.1 M). Both media were autoclaved at 121 °C for 15 min.

2.3. Isolation of Bioflocculant-Producing Bacteria

A loop full of the bacterial colonies was inoculated into activation medium and incubated in a rotary shaker (160 rpm) at 28 °C. After 24 h of incubation, 1 mL (2% *v/v* inoculum) of the culture broth was used as a seed culture and inoculated into 50 mL of freshly prepared production medium in 250 mL Erlenmeyer flasks and incubated in a rotary shaker (160 rpm) for 120 h at 28 °C. After the incubation period, 2 mL of cell-free supernatant of the fermented broth was obtained by centrifuging at 4000 × g, for 30 min in order to separate the cells. This cell-free supernatant was analyzed for flocculating activity in accordance with the description of Kurane et al. [21]. The isolate F45 which showed the highest flocculating rate was preserved on nutrient agar slants and in glycerol stock (20%) and stored at −80 °C until further use.

2.4. Determination of Flocculating Activity

The determination of flocculating rate of the isolate was carried out in accordance with the method of Kurane et al. [21] using kaolin suspension (4 g/L) as a test material. Two milliliters of the culture supernatant and 3 mL of 1% CaCl$_2$ (*w/v*) were added into 100 mL of kaolin clay suspension (4 g/L) in 250 mL Erlenmeyer flask, the mixture was agitated for 60 s and left to sediment at room temperature for 5 min. The control was also prepared following the same procedure except that culture supernatant was substituted with freshly prepared broth. The turbidity in the upper layer was read at 550 nm with a spectrophotometer (Helios Epsilon, NY, USA) and the flocculating rate was estimated as follows:

$$\text{Flocculating rate} = \{(A - B)/A\} \times 100\% \tag{1}$$

where A and B are the optical densities of the control and the sample at 550 nm, respectively. Triplicates of the experiment were performed and the mean value determined.

2.5. Optimization of Culture Conditions for Bioflocculant Production

2.5.1. Effect of Carbon Sources on Bioflocculant Production

Carbon and nitrogen sources are critical factors that greatly affect bioflocculant production by microorganisms [22]. The assessments for the effect of carbon and nitrogen sources on the production of bioflocculant were done following the procedure of Lachhwani [23]. The bacterial strain was inoculated into growth media contained in separate flasks and each supplemented with different carbon source(s) such as glucose (20 g/L), sucrose (20 g/L), starch (20 g/L), lactose (20 g/L), maltose (20 g/L), fructose (20 g/L), and/or phthalate (20 g/L) and incubated in a rotary shaker (120 rpm) at 28 °C for a period of 5 days. Flocculating rate was calculated as previously described.

2.5.2. Effect of Nitrogen Sources on Bioflocculant Production

For optimization of the effect of nitrogen sources on production of bioflocculant, individual sources of nitrogen (1.2 g/L) were incorporated into production medium contained in separate flasks. The nitrogen source candidates included the organic nitrogen sources such as yeast extract, urea, peptone and tryptone; inorganic nitrogen sources such as ammonium sulfate, and also a mixed nitrogen source composed of yeast extract, ammonium sulfate, and urea. Flocculating activity was determined as previously described.

2.5.3. Effect of Inoculum Size on Bioflocculant Production

The assessment of the influence of inoculum size on the production of bioflocculant was conducted in accordance with the method of Zhang et al. [20], this experiment was performed by using different inoculum sizes ranging from 0.5% to 2% (*v/v*) to inoculate the production medium.

2.5.4. Effect of Initial pH of Growth Medium on Bioflocculant Production

Using pre-determined optimum culture conditions for nitrogen and carbon sources, the pH of each individual medium contained in separate flasks was adjusted to range from pH 3–11 with either NaOH (0.1 M) or HCl (0.1 M) prior to inoculating each medium with the seed culture. The culture medium was then incubated in a rotary shaker (160 rpm) at 28 °C for a period of 5 days and flocculating activity determined as previously described [10].

2.5.5. Effect of Cations of Flocculating Activity of Crude Bioflocculant

To test for the influence of cations on flocculating activity, solutions (1% *w/v*) of KCl, NaCl, MnCl$_2$, MgCl$_2$, FeSO$_4$ and AlCl$_3$ were prepared, and the flocculating activity was determined as elaborated above but replacing CaCl$_2$ with each of the different cation solutions [10].

2.6. Time Course of Bioflocculant Production

The time course experiment was carried out following the method of Gao et al. [24] with minor modifications. The bacterial strain was cultured under optimal culture conditions determined from previous experiments. For the preparation of growth medium, the following components were mixed in distilled water: maltose (20.0 g/L), KH_2PO_4 (2.0 g/L), KH_2PO_4 (5.0 g/L), $(NH_4)_2SO_4$ (0.2 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L), urea (0.5 g/L) and yeast extract (0.5 g/L) [20].

For inoculum preparation, the isolate was cultured in 50 mL activation medium and incubated at 28 °C in a rotary shaker (160 rpm) for 24 h. After 24 h of incubation, the optical density (OD_{600}) of the culture broth was adjusted to give 0.1 using 0.85% (w/v) sterile distilled water. The diluted culture broth (4% v/v) was used as a seed culture to inoculate 200 mL of growth medium in 250 mL Erlenmeyer flasks. About 20 mL of medium samples was withdrawn at 12 h intervals and monitored for each of the following parameters: pH, cell growth, cell count and flocculating activity. At each withdrawal, 2 mL of culture broth was centrifuged at 4000 × g, 4 °C for 30 min and the supernatant was used to determine flocculating activity of the produced bioflocculant. At time interval of 12 h, the optical density of the fermented culture was measured with spectrophotometer (OD_{600}) and bacterial cell count was done by a standard plate technique in order to monitor the bacterial growth.

2.7. Extraction and Purification of Bioflocculant

The bioflocculant was purified according to the procedure described by Okaiyeto et al. [25] using media composition based on the optimum culture conditions determined in previous experiments. Briefly, after 24 h of fermentation, the fermented broth was transferred to centrifuge bottles and centrifuged at 4000 × g at 4 °C for 30 min to remove bacterial cells. The supernatant was mixed with one volume of distilled water and centrifuged again at 4000 × g, 4 °C for 15 min to remove insoluble substances. Two volumes of ethanol were added to the supernatant, stirred and left to stand at 4 °C overnight. The supernatant was discarded and the precipitate was vacuum-dried to obtain the crude biopolymer. The crude product was then dissolved in distilled water and mixed with one volume of chloroform/n-butyl alcohol (5:2 v/v). After stirring, the mixture was left to stand at room temperature for 12 h. The upper phase was separated, centrifuged at 4000 × g for 15 min at 4 °C and the supernatant was dialyzed against distilled water overnight. The dialysate was then vacuum-dried to obtain a purified bioflocculant.

2.8. Chemical Composition Analysis of Bioflocculant

The bioflocculant total sugar content was determined by the phenol–sulfuric method as described by Chaplin and Kennedy [26] using glucose as a standard solution. The protein content of the bioflocculant was determined using the Folin–Lowry method as described by Lachhwani [23] with bovine serum albumin (BSA) as a standard. The uronic acid content of the bioflocculant was determined using the carbazole–sulfuric acid method [26].

2.9. Fourier Transform Infrared Spectrophotometry (FTIR) Analysis

Fourier transform infrared spectrophotometer (Perkin Elmer System 2000, FT-IR, Middlesex, England) was used to determine the functional moieties of the bioflocculant. The bioflocculant was ground with KBr salt at 25 °C and pressed into a pellet for FTIR spectroscopy over a wave number range of 4000-370 cm^{-1} [27].

2.10. Scanning Electron Microscopy Imaging (SEM)

The surface morphology structure of the purified bioflocculant was attained with a scanning electron microscope (SEM) (JSM-6390LV, JEOL, Tokyo, Japan). Images of bioflocculant powder, kaolin clay before and after flocculation were scanned. For elemental analysis of the bioflocculant, energy dispersive X-ray (EDX) analysis was conducted using a JEOL (JSM-6390 LV SEM, Tokyo, Japan) and the elements were analyzed using the Noran system six software package (version 6.0, Thermo Electron corporation, Madison, WI, USA, 2012).

2.11. Optimization of Flocculating Activity of Purified Bioflocculant

2.11.1. Effect of Bioflocculant Dosage on Flocculating Activity

For bioflocculant dosage determination, the method of Wang et al. [28] was used with minor modifications. Different concentrations of bioflocculant solutions were prepared and used to determine the optimum bioflocculant dosage. Concentrations of 0.05, 0.08, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL were used. Two milliliters of the bioflocculant solution was mixed with 100 mL of kaolin suspension (0.4% *w/v*) containing 1% (*w/v*) $CaCl_2$ in 500 mL flasks. The solution was stirred rapidly and transferred into 100 mL graduated cylinder and left to settle for 10 min at room temperature. About 2 mL of the clear upper phase of the supernatant was removed for determination of flocculating activity. The flocculating activity was measured at 550 nm as previously described.

2.11.2. Effect of Temperature on the Flocculating Activity

The effect of temperature on flocculating activity of the purified bioflocculant was determined following the procedure of Wang et al. [29]. The desired concentration of the bioflocculant was prepared in 10 mL of distilled water. Two-milliliter aliquots of the solutions were transferred into different eppendorf tubes and heated for 30 min at different temperatures (50 °C, 80 °C and 100 °C). The residual flocculating activity was measured at room temperature.

2.11.3. Effect of pH on the Flocculating Activity

To examine the effect of pH on flocculating activity, the pH of the kaolin suspension was adjusted using either HCl or NaOH in the pH range of 3–11 in 250 mL flasks. The desired bioflocculant concentration was added to each flask and the flocculating activity was measured as previously described [27].

2.11.4. Effect of Cations on Flocculating Activity

The effect of various cations on the flocculating activity of the purified bioflocculant was also investigated. This was conducted by replacing the 1% (*w/v*) $CaCl_2$ solution with various salt solutions including KCl, NaCl, $MnCl_2$, $MgCl_2$, $FeCl_3$, and $AlCl_3$ at the same concentration [23]. Flocculating activity was measured as previously described using the optimum bioflocculant concentration.

2.12. Statistical Analysis

All data were treated in replicates and the standard deviation of the mean values was taken. Data were subjected to one-way analysis of variance (ANOVA) using MINITAB Student Release 12 statistical package for Windows 95/98 NT (Minitab Inc., State College, PA, USA, 2007).

3. Results and Discussion

3.1. Isolation of Bioflocculant-Producing Bacteria

About 13 bacterial isolates were obtained from rock scrapings and screened for bioflocculant production. Isolate F45 was among the isolates demonstrated a flocculating activity above 50% when tested against a kaolin suspension. The 16S rDNA analysis of the bacteria revealed it to have 99% similarity to *Bacillus pumilus* strain ZAP 028. The genus *Bacillus* is ubiquitous in nature and includes a variety of species that are commercially useful [30]. Compared with other industrially important Bacilli such as *B. subtilis* or *B. licherniformis*, *Bacillus pumilus* is known for high resistance for oxidative stress [31]. This natural potential and resistances displayed by *B. pumilus* could be a major benefit for the development of industrial production processes, since oxidative stress can take place in all phases of fermentation processes [32–34].

In another study, a novel *Bacillus pumilis* strain was used for the production of xylanase at alkaline pH and temperatures above 40 °C [31]. In bioflocculation, a number of Bacillus species have been documented for their bioflocculant-producing potential, including *B. licherniformis* [35],

B. mucilaginosus [36,37], *B. circulans* [38], *B. subtilis* [39] and *B. alvei* [40]. However, *Bacillus pumilus* has not been reported in literature as a bioflocculant producer. Continuous screening for new bioflocculant-producing strains is highly imperative because of the low flocculating activity and low yields of bioflocculants produced by microorganisms reported in the literature, which are some of the problems hindering their large-scale production. Hence, *Bacillus pumilus* reported in the studies is a new strain that has not been implicated in bioflocculant production before. The high flocculating activity observed with the bioflocculant produced by this strain is an added advantage over those reported in the previous studies.

3.2. Optimization of Culture Conditions for Bioflocculant Production

3.2.1. Effect of Carbon Source on Bioflocculant Production

The choice of carbon source has a great influence on the production of bioflocculants. The results of the effect of various carbon sources on bioflocculant production by the bacterial strain are shown in Figure 1.
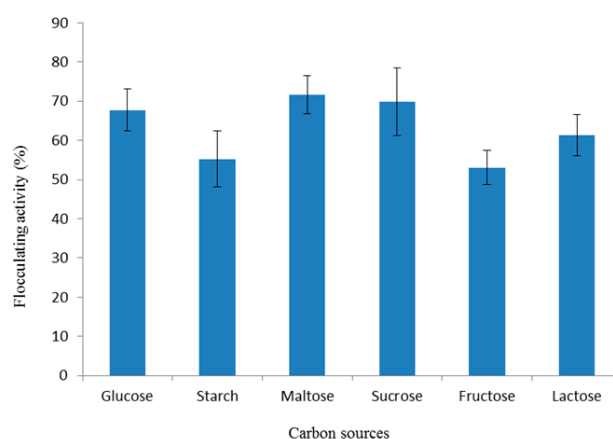

Figure 1. Effect of carbon sources on bioflocculant production.

Among the various sources of carbon tested, maltose was the preferred carbon source for the production of the bioflocculant by the test bacteria with flocculating activity of 71.7%, followed by sucrose with a flocculating activity of 69.8%. Glucose, lactose and starch supported bioflocculant production with flocculating activities of 67.8%, 61.3% and 55.3%, respectively. Fructose showed the least flocculating activity at 53.1%. Maltose was then used as a carbon source of choice for all subsequent experiments.

Similarly, Wan et al. (2013) reported that maltose was the preferred carbon source for bioflocculant production by *Solibacillus silvestris* exhibiting a flocculating activity of 88.7% [12]. He et al. [41] reported that glucose, sucrose and fructose were preferred carbon sources for REA-11 production, a bioflocculant produced by *Corynebacterium glutamicum*. It is well documented in the literature that many bioflocculant-producing microorganisms prefer organic carbon sources for optimum bioflocculant production [42]. However, the utilization of inorganic carbon sources for bioflocculant production is still scant in literature.

3.2.2. Effect of Nitrogen Source on Bioflocculant Production

Nitrogen sources play a crucial role in the production of bioflocculants [42]. Different microorganisms utilize either organic or inorganic nitrogen sources, or both, to produce bioflocculants [43]. The effect of organic, inorganic and multiple nitrogen sources on bioflocculant production by the test bacteria were evaluated. As shown in Figure 2, it was evident that the complex nitrogen source composed of yeast extract; urea and ammonium sulfate was the most preferred for bioflocculant production by

Bacilus pumilus strain as opposed to the individual nitrogen sources. Urea as a sole nitrogen source also supported bioflocculant production (54%) when compared with other single nitrogen sources such as peptone, yeast extract, tryptone and ammonium sulfate.
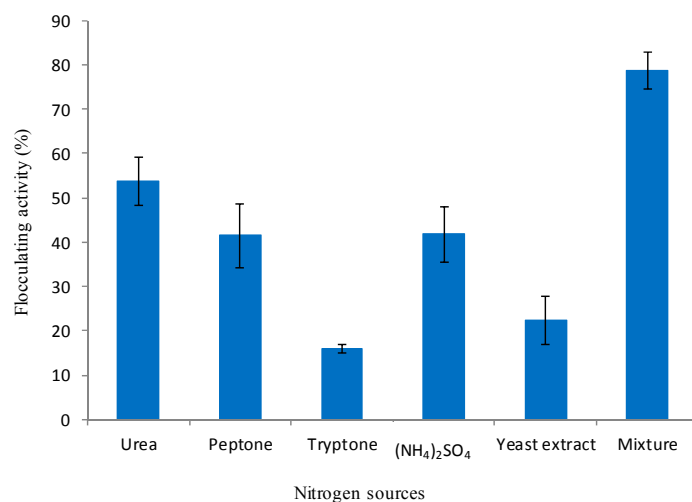

Figure 2. Effect of various nitrogen sources on bioflocculant production.

Similarly, Ugbenyen et al. [44] also observed that *Cobetia* sp. poorly utilized all tested inorganic and organic nitrogen sources when used individually as sole sources of nitrogen, but the nitrogen source composed of urea, yeast extract and ammonium sulfate resulted in high flocculating activity of 92.2%. In another study by Lixi et al. [45], the most favorable medium for production of bioflocculant by *Klebsiella* sp. contained three nitrogen sources (urea, ammonium sulfate, yeast extract), which supported an optimum flocculating activity of 94.7%. Gong et al. [46] observed that for *Serratia ficaria*, a multiple nitrogen source (beef extract and urea) supported a higher flocculating activity of 94.1% when compared to sole organic or inorganic nitrogen sources. These results suggest that it is necessary to test not only for the effect of individual nitrogen sources on bioflocculant production but for the effect of combined nitrogen sources as well. All inorganic nitrogen sources (ammonium sulfate, ammonium nitrate, and sodium nitrate) tested for bioflocculant production by *Chryseobacterium daeguense* W6 led to poor production of both the bioflocculant and cell growth, whereas the organic nitrogen sources proved to be better sources for bioflocculant production, for which tryptone was the most favorable exhibiting a flocculating activity of more than 90% [47]. Generally, it has been reported in the literature that organic nitrogen sources are more suitable for bioflocculant production and are more easily absorbed by the cells when compared to inorganic nitrogen sources [48].

3.2.3. Effect of Inoculum Size on Bioflocculant Production

Inoculum size is one of the factors that have been reported to have an effect on the production of bioflocculants by microorganisms [20]. The effect of inoculum size on bioflocculant production was studied using different inoculum sizes ranging from 1% to 5%. As the results are depicted in Figure 3, flocculating activity steadily increased with increasing inoculum size with an optimum activity attained at 4% (*v*/*v*) inoculum size, beyond which a steady decline in flocculating activity was observed.
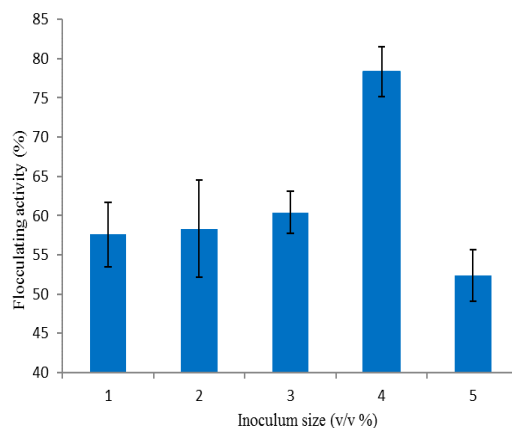
Figure 3. Effect of inoculum size on bioflocculant production.

As a result, an inoculum size of 4% (*v/v*) was used for all subsequent experiments. These results are in agreement with the phenomenon observed by Salehizadeh and Shojaosadati [49] that a small inoculum prolonged the lag phase of the strain, whereas a large inoculum made the niche of *Bacillus pumilus* strain overlap excessively and restrained bioflocculant production which means that at large inoculum size, the substrates in the production medium got depleted faster within a shorter period as a result of the bacterial population in the culture medium, generating high turbidity at an early stage of growth and this consequently affects bioflocculant production. A similar finding was reported by Xiong et al. [35] where it was observed that an inoculum size of 4% (*v/v*) was optimum for bioflocculant production by *Bacillus licherniformis*.

3.2.4. Effect of Initial pH of Production Medium on Bioflocculant Production

The initial pH of the culture medium does not only have an effect on the electric charge of the cells and the oxidation–reduction potential but also affects nutrient absorption and enzymatic reaction within cells [49]. Hence, the effect of initial pH of the culture medium on bioflocculant production was examined at pH values ranging from 3 to 11 and the results are presented in Figure 4. As can be seen from the Figure, the bioflocculant was significantly produced within a pH range of 7.0–9.0 with the optimum flocculating activity (80.6%) being observed at pH 7.
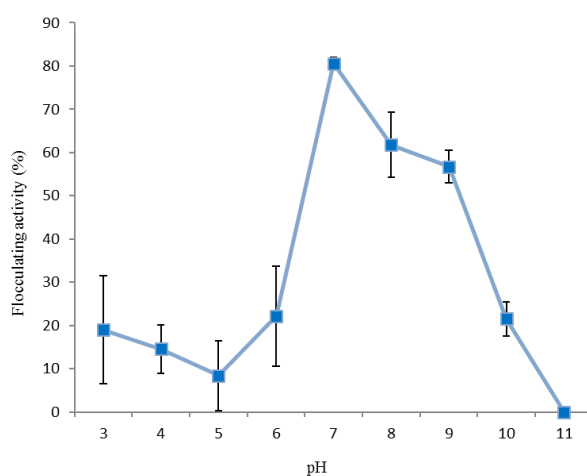

Figure 4. Effect of initial medium pH on bioflocculant production.

At both acidic pH (3–6) and high alkaline pH (10–11) ranges, flocculating activity was significantly reduced with no flocculating activity being observed at pH 11. Initial medium pH 7 was then used for

all subsequent experiments, which is a neutral pH suggesting that large amounts of acid and alkali used to adjust pH would be saved [22]. Consequently, production costs would be anticipated to be lower in the event of applying *Bacillus pumilus* in bioflocculant production.

Mabinya et al. [50] and Aljuboori et al. [51] observed similar neutral pH values for bioflocculants produced by *Halomonas* sp. OKOH and *Aspergillus flavus*, respectively. A bioflocculant produced by *Klebsiella* pneumonia also reached its highest flocculating activity at pH 7 [14]. However, the initial pH of the medium required for optimum bioflocculant production differs with different organisms. The optimum production of bioflocculants by *Bacillus* xn12 and *Streptomyces* xn17 strains was observed at pH 5 [52]. For bioflocculant production by *Arthrobacter* sp. B4, it was observed that the highest flocculation was attained at a pH value above 12 [53].

3.2.5. Effect of Cations on Flocculating Activity of Crude Bioflocculant

The effect of various cations on the activity of the crude bioflocculant was investigated and the results are depicted in Figure 5. The stimulating effect of cations in flocculation process is not only dependent on the concentration of the cations added but is also highly dependent on the valence ions [54]; hence, the effect of cations on the flocculating activity was investigated using cations with different valence ions (i.e., monovalent, divalent and trivalent). All the divalent cations tested enhanced the flocculating rate of the bioflocculant albeit to varying degrees as follows: Ca^{2+} (67.9%), Mg^{2+} (74.5%) and Mn^{2+} (82.9%) (Figure 5).
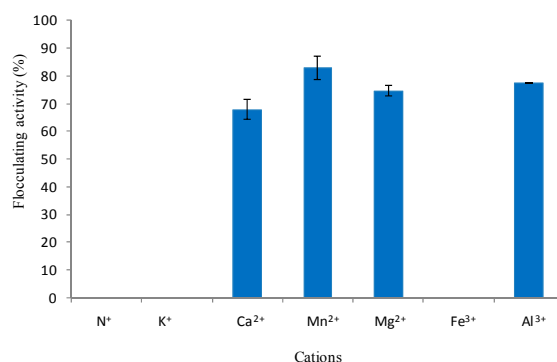

Figure 5. Effect of cations on the flocculating activity of the bioflocculant.

Among these cations, Mn^{2+} gave the highest flocculating activity and was therefore used as a replacement for Ca^{2+} in all subsequent experiments. On the other hand, in the previous studies where the flocculating activity was around 80%, the sole carbon source was glucose, whereas, when the flocculating activity was 67.9%, the sole carbon was maltose. This shows that the synergistic effect of cations differs for different bioflocculants, which means that the chemical composition of the bioflocculant produced from glucose-containing medium differ from the one produced sucrose-containing medium. This might be the reason why a decline in flocculating activity was observed in the bioflocculant produced from maltose-containing medium. However, when the bioflocculant was produced from optimal culture conditions in the presence of maltose as the carbon source, the flocculating activity was 89.7% with Mn^{2+} as the flocculating aid (Figure 5).

Furthermore, it was noticed that Na^+, K^+, Fe^{3+} completely inhibited the flocculating activity of the bioflocculant while Al^{3+} stimulated the second highest flocculating activity of 77.2% (Figure 5). The high flocculating activity observed might be due to ionic density of Al^{3+} that increases the ionic strength of the suspension, and therefore, the electrostatic repulsive forces among charged groups of the bioflocculant chain decrease notably and consequently increases the flocs formation as a result of electrostatic attraction between the bioflocculant and the suspended particles. In addition, $AlCl_3$ is an inorganic flocculant like polyaluminum chloride and ferric chloride ($FeCl_3$) that are highly charged and conventionally used in water treatment. The flocculating activity of the bioflocculant was enhanced in the presence of Al^{3+} and inhibited in the presence of Fe^{3+}. The enhanced flocculating activity noticed

in the presence of Al^{3+} might be due to the synergistic effect of Al^{3+} with the bioflocculant and the poor flocculation observed with Fe^{3+} might be due to antagonistic effect of Fe^{3+} with the bioflocculant thus lead to high flocculating activity for the Al^{3+} and poor flocculation in the presence of Fe^{3+} when the effects of trivalent cations on the flocculating activity of the bioflocculant were compared.

Furthermore, it could be safer to say that Fe^{3+} might possibly alter the surface charge of kaolin surfaces and cover the adsorb sites [46]. The competition of the positively charged particles and less adsorb sites might induce the antagonist effect of Fe^{3+} that resulted into poor flocculation. Moreover, even though both Al^{3+} and Fe^{3+} are trivalent cations, they belong to different group in the periodic table with different features. For example, Al^{3+} has electronegativity of 1.61 and first ionization energies of 577.5 KJ/mol, whereas Fe^{3+} has electronegativity of 1.832 and first ionization energies of 762.5 KJ/mol. This implies that the electrostatic repulsive forces between Fe^{3+} and charged groups of the bioflocculant will be higher as compared with Al^{3+} and this consequently leads to the inhibition of flocculating activity of the bioflocculant in the presence of Fe^{3+}.

Monovalent cations could produce bonds that are loose in structure resulting in decreased floc density and size, which in turn caused reduction in flocculating activity [55]. This might probably account for the poor flocculating activity observed in the presence of Na^+ and K^+. Excessive adsorption of the ions might be the cause of inhibition of the flocculation process in the presence of Fe^{3+} [55].

The flocculating activity of the bioflocculant produced by the consortium of *Cobetia* sp. and *Bacillus* sp. was enhanced by all of the divalent metal ions tested (Ca^{2+}, Mg^{2+}, and Mn^{2+}) but it was completely inhibited by Li^+ and K^+ [56]. The flocculating activity of BFB4 bioflocculant produced by *Arthrobacter* sp. B4 was Ca^{2+}-dependent and unaffected by other metal ions tested, no flocculation occurred in the absence of Ca^{2+} ions [53].

On the other hand, Liu et al. [47] reported a cation-independent bioflocculant MBF-W6 produced by *Chryseobacterium daeguense* since it was observed that none of the cations added could evidently improve its flocculating rate. The function of cations is to stimulate the flocculating activity by neutralizing and destabilizing the residual negative charge of functional groups and to form bridges between particles [10]. However, metal ions have also been reported to inhibit flocculating activity or have no effect at all on the flocculating activity of bioflocculants [53,56].

3.3. Time Course of Biofloccculant Production

The time course profile of bioflocculant production by the bacterial strain was investigated over a growth period of 120 h and the results are depicted in Figure 6.
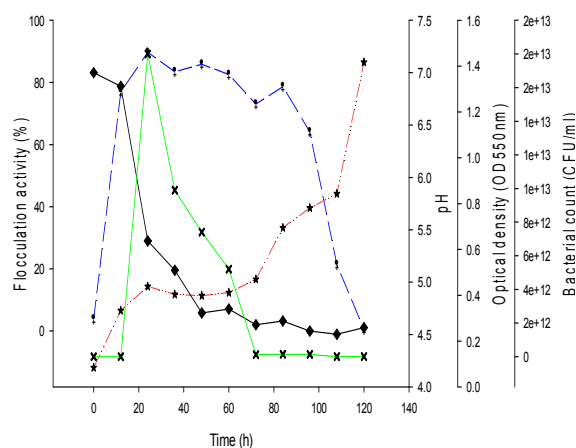

Figure 6. Time course profile for bioflocculant production by *Bacillus pumilus*. Bacterial count (CFU/mL)—Green line, pH—Black line, Optical density measured at 660 nm (OD)—Red line, and Flocculating activity (%)—Blue line.

There was a sharp increase in flocculating activity between 0 and 24 h of fermentation after which a steady decline was noticeable up to 96 h, beyond which a very sharp drop in flocculating activity was observed. Maximum flocculating activity of 89.7% (Figure 6) was attained at early stationary phase (24 h) and 2.5 g of bioflocculant was recovered from 1 L of fermentation broth. The reason why the flocculating activity was higher during the time course of bioflocculant production was because an optimal culture conditions were used in this experiment compared to the previous study. The bioflocculant was produced at optimal fermentation conditions and hence, an increase in flocculating activity was observed with the flocculating activity. The decline in flocculating activity observed between 96 and 120 h could be associated with the production of bioflocculant-degrading enzymes by the microorganism and cell autolysis [57]. There was a corresponding increase in bacterial growth with flocculating activity up to 24 h after which a sharp decline was observed. These results suggest that the bioflocculant was as a result of biosynthesis during its growth and not by cell autolysis [46]. Based on these results, a culturing period of 24 h was adapted for subsequent experiments using the test strain. The short time (24 h) recorded for optimum bioflocculant production by *Bacillus pumilus* strain suggest that bulk production of the bioflocculant may be achieved over a short period of time, which implies cost-containing possibilities on an industrial production scale.

Similarly, *Bacillus mojavensis* reached its maximum flocculating efficiency in the early stationary phase which was also recorded at 24 h, while the cell production reached its maximum in the stationary phase (at 72 h) suggesting that the produced bioflocculant was as a result of biosynthesis [58]. The optical density curve which may also be representative of cell growth also showed a similar trend to that of flocculating activity curve until 72 h of fermentation where it started to increase. During the first 24 h, the OD had a similar behavior as the flocculating activity because as the bacteria utilize the nutrient in the medium for cell growth, the bacterial cells increase in population and also produce bioflocculant gradually in to the medium in the presence of abundant nutrient. This process increases the turbidity of the culture with increase in cultivation as it was noticed in this study. When the nutrient was depleted, the metabolic waste products accumulate as well as the bacterial cells (bacterial count) decreases in the presence of low nutrient. On the other hand, the metabolic waste products and dead bacterial cells increases the turbidity of the culture and OD only show the turbidity of the culture which cannot differentiate between the viable cells and dead cells whereas, the bacterial counts expressed as CFU/mL only count the number of viable cells, this is the reason why the cell count and OD are not concordant [58]. At 70 h, the nutrient has been depleted and bacteria have reached the death phase of growth and viable cells declined drastically whereas a sharp increase in OD was observed which might be the a result of an increase in metabolic waste products and dead cells that increases the turbidity of the medium.

The initial pH of the medium is reported to be a very important factor in bioflocculation process. The pre-determined optimum initial pH of the medium was pH 7. In contrast to flocculating activity, the pH decreased from 7.0 to 5.39 within 24 h, followed by a slow decline until it reached a pH of 4.57 at 120 h of incubation (Figure 6). The decline in pH may be indicative of the production of organic acids during metabolism by the microorganism [37,59] or may be attributed to the acidic nature of the bioflocculant produced by the bacteria [60].

A bioflocculant produced by *Bacillus licherniformis* X14 reached its maximum flocculating activity at 48 h while cell growth reached a maximum at 20 h [57]. Time course profiles for production of bioflocculants differ with different microorganisms. However, the majority of studies reported in the literature show a parallel relationship between bioflocculant production and cell growth with the maximum flocculating activity being reached in early stationary phase. This suggests that most bioflocculants are produced by biosynthesis through nutrient absorption during their growth, and not by cell autolysis [51,61,62].

3.4. Chemical Composition of Purified Bioflocculant

Biochemical analysis of the purified bioflocculant produced by *Bacillus pumilus* confirmed the presence of carbohydrates as a predominant component at 75.4% with uronic acid (15.2%) and

protein (5.3%) constituting the other significant proportions (Table 1). This finding correlates with the other reports of biochemical analyses of bioflocculants produced by other microorganisms, which were found to be mainly polysaccharide in nature [27,46]. Peng et al. [62] reported that a polymer flocculant produced by *Rhodococcus erythropolis* was predominantly of carbohydrate nature. Bioflocculants produced by a halophilic *Halomonas* sp. V3a [27] and *Serratia ficaria* [46] were also found to be polysaccharides. The low charge density as well as the size of polymers such as polysaccharides, have been reported to enhance their flocculating capabilities particularly in suspensions with high ionic strengths; hence, flocculation is often regarded as a function of molecular weight [27].

Table 1. Chemical composition of purified bioflocculant.

Component	Percentage (%)
Total sugar	75.4
Protein	5.3
Uronic acid	15.6

3.5. Fourier Transform Infrared Spectrophotometry (FTIR) Analysis

FTIR spectroscopy of the purified bioflocculant was performed to investigate the correlation between functional groups and the flocculating activity of the purified bioflocculant, and the results are depicted in Figure 7.
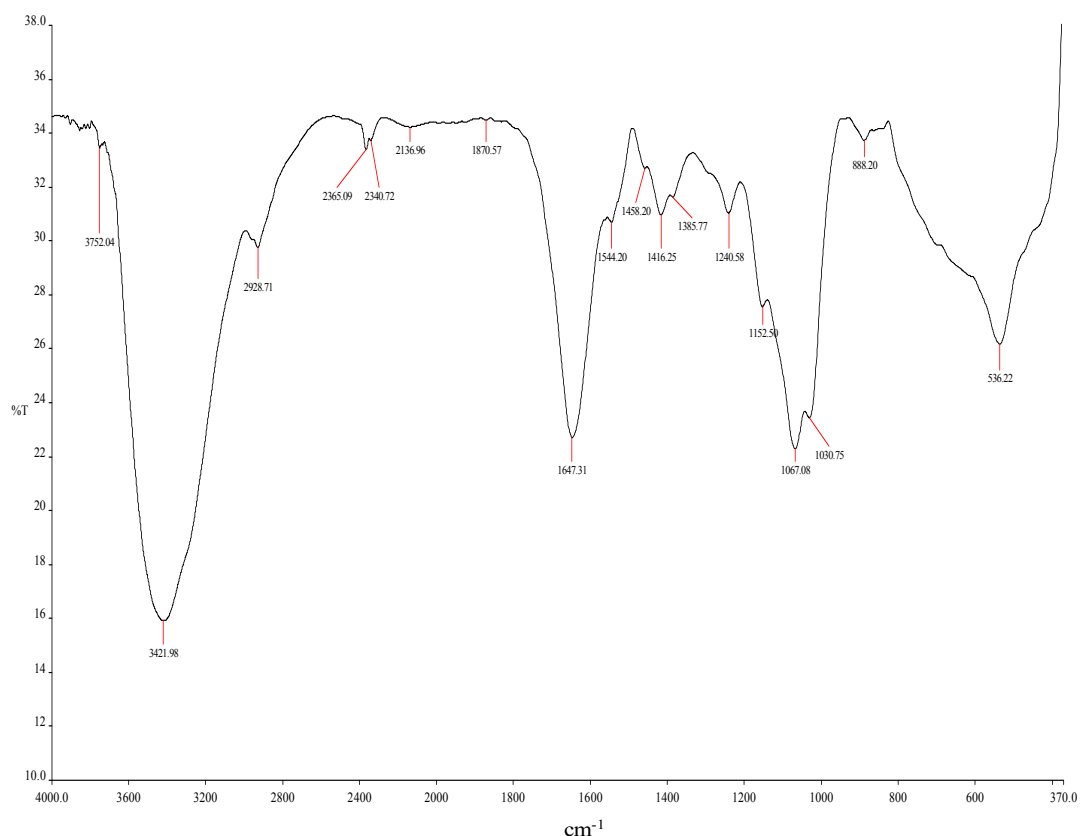

Figure 7. Fourier Transform Infrared Spectrophotometry (FTIR) spectra of the purified bioflocculant.

The infrared spectrum of the bioflocculant revealed a broad stretching peak at around 3429 cm^{-1}, which is representative of hydroxyl group, and a weak C–H stretching band at 2928 cm^{-1} [63]. Two typical absorptions of carboxyl groups were indicated by peaks at 1647 cm^{-1} (C=O asymmetric stretching vibrations) and 1416 cm^{-1} (C=O symmetric stretching vibrations) [64]. The symmetric

stretching vibration is also indicative of the presence of uronic acid in the bioflocculant molecule [47]. The absorption peaks displayed in the range from 1000 to 1200 cm^{-1} were known to be physiognomies of all sugar derivatives [46]. The FTIR spectrum of the produced bioflocculant was consistent with the results of other bioflocculants produced by different microorganisms [35,47,64]. The presence of hydroxyl and carboxyl groups serves as a further affirmation that the bioflocculant is predominantly a polysaccharide [13].

3.6. Scanning Electron Microscopy Imaging (SEM)

SEM analyses of the bioflocculant and kaolin clay before and after forming a complex (flocculation) with the produced bioflocculant were performed to elucidate the surface morphology of the bioflocculant and its flocculation of kaolin clay. Figure 8a shows the SEM image of the bioflocculant, Figure 8b shows the kaolin clay before flocculation and Figure 8 (shows the flocs from precipitated kaolin clay after the addition of the bioflocculant.
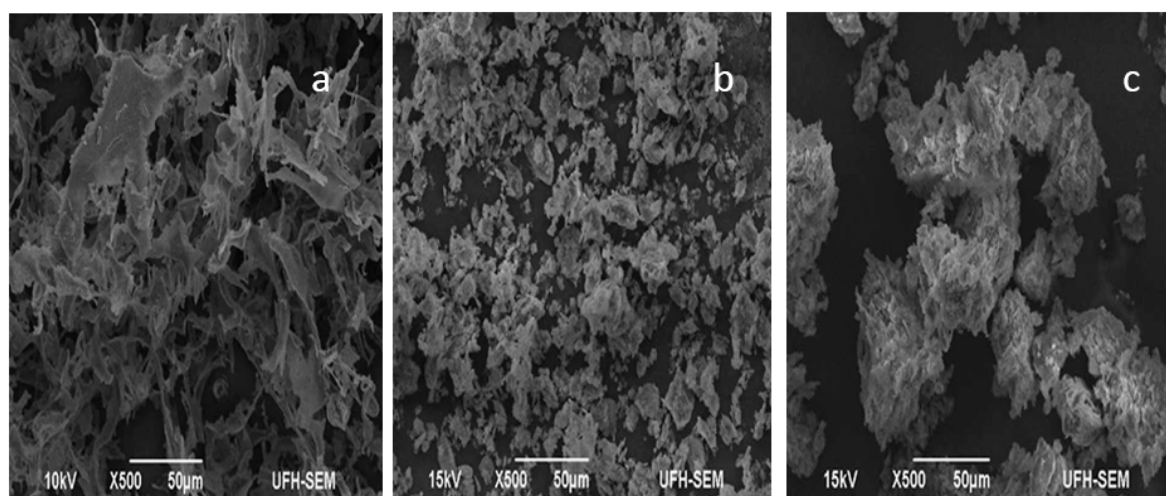

Figure 8. Scanning Electron Microscopy Imaging (SEM) of: bioflocculant (**a**); kaolin clay before flocculation (**b**); and kaolin clay flocculated by the bioflocculant (**c**).

The SEM image of the bioflocculant molecule revealed it to be dendritic in structure. Comparison of Figure 8b,c suggests that the bioflocculant molecule successfully connected the kaolin particles to form flocs, which settled down and separated from the suspension. These results reveal that the bioflocculant showed excellent flocculating properties of kaolin clay. It can also be deduced that bridging of the bioflocculant molecule played a significant role in the flocculation process [27].

3.7. Optimization of Flocculating Activity of Purified Bioflocculant

3.7.1. Effect of Bioflocculant Dosage on Flocculating Activity

The effect of dosage on the flocculating activity of the purified bioflocculant was investigated (Figure 9). The main objective of the bioflocculant dosage experiment was to determine the lowest amount of bioflocculant to be used while attaining the highest flocculating efficiency.
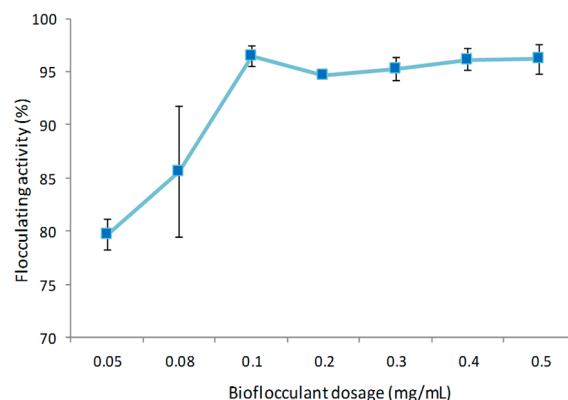
Figure 9. Effect of bioflocculant dosage on the flocculating activity of purified bioflocculant.

As depicted in Figure 9, the flocculating activity was above 90% within a dosage range of 0.1–0.5 mg/mL, and the maximum flocculating activity (96.5%) was achieved at an optimum bioflocculant dosage of 0.1 mg/mL. The flocculating activity was almost linear between 0.2 and 0.5 mg/mL, showing that there was no major difference in the flocculating activity as the bioflocculant dosage increased. The results in this study showed that the bioflocculant produced by bacterial strain exhibited high flocculating activity at very low dosage (0.1 mg/mL) which could be cost containing for industrial-scale applications. A lower dosage of bioflocculants with concomitant high flocculating activity will definitely reduce treatment cost [54]. The bioflocculant produced by *Paenibacillus mucilaginosus* achieved the highest flocculating activity at a dosage of 2.0 mg/L, and similar to the present study, higher or lower dosages reduced flocculating activity [64].

3.7.2. Effect of Temperature on the Flocculating Activity

The effect of temperature on the flocculating activity of the purified bioflocculant was investigated. The purified bioflocculant could maintain over 90% of its activity with only 4.8% decrease in flocculating activity after being exposed to heat (100 °C) for 1 h, thus exhibiting its thermo-stability characteristic (Figure 10).
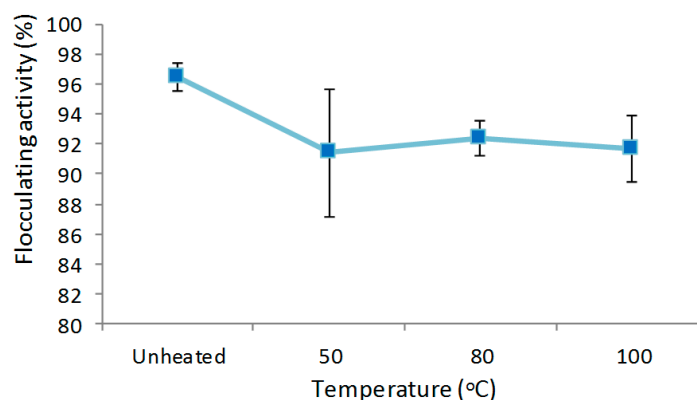

Figure 10. Thermal stability of the purified bioflocculant.

Generally, flocculants with peptide or protein backbone in their structure are thermally labile but those with predominant carbohydrate content are thermo-stable, thus it can be deduced that the thermal stability exhibited by the bioflocculant produced by *Bacillus pumilus* strain may be due to the presence of a polysaccharide backbone in its structure [49]. The results show that the bioflocculant produced by *Bacillus pumilus* strain has high thermal stability compared to the other bioflocculants reported in the literature [65,66]. A bioflocculant produced by *Aeromonas* sp. maintained its flocculating

activity with only 9.2% decrease in activity after being heated at 100 °C for 60 min, suggesting that it was thermo-stable [3].

3.7.3. Effect of pH on the Flocculating Activity

The effect of pH on the flocculating efficiency of the purified bioflocculant was examined. The results show that the bioflocculant was active over a wide pH range and demonstrated excellent flocculating rate (above 80%) at either strong acidic or alkaline pH conditions (Figure 11).
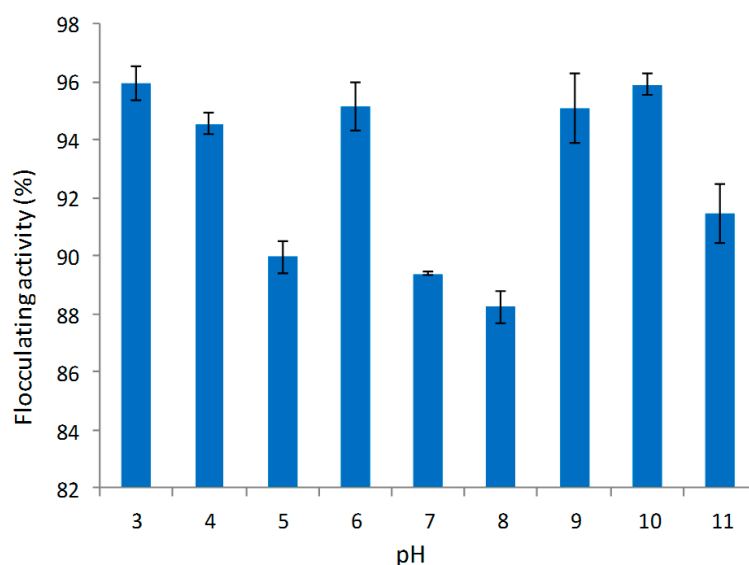

Figure 11. Effect of pH on the flocculating activity of the purified bioflocculant.

This may be attributed to the fact that the electric states of the bioflocculant differ at different pH conditions and this in turn affects the flocculation efficiency of the bioflocculant for the kaolin particles [67]. The highest flocculating activity of 95.9% was attained at pH 10. There was inconsistency in bioflocculant activity at acidic region noticed by a decline in flocculating activity at pH 5. This may be accounted for by the fact that the spatial arrangement of surface charge is not only pH dependent but is also contingent on temperature [68]. Therefore, it can be concluded that the spatial charge arrangements for bioflocculation were not ambient at pH 5 and at neutral pH. The pH tolerance of the bioflocculant at a wide pH range suggests that it could be applied in various industries to treat various waters or wastewaters without having to adjust the pH of the water thus rendering the bioflocculant cost-effective [69]. Similar findings were reported for MBF-5 bioflocculant, which showed high flocculating activity under both acidic and alkaline conditions [13]. The bioflocculant produced by *Halomonas* sp. V3' had a wide pH range of 3–11 with a flocculating activity above 80% [27]. Bioflocculant NU-2 produced by *marine myxobacterium* was tolerant under a wide pH range of 2–13 [70].

3.7.4. Effect of Cations on the Flocculating Activity

The effect of cations on the flocculating activity of the purified bioflocculant was investigated and the results are depicted in Figure 12.
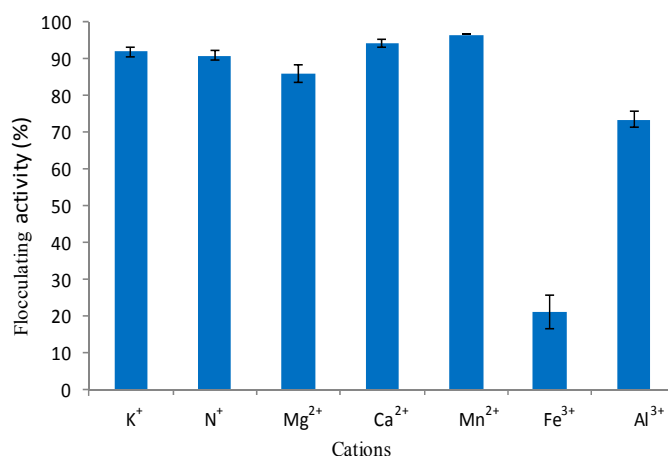
Figure 12. Effect of cations on the flocculating activity of the purified bioflocculant.

It was found that all tested cations stimulated the bioflocculant activity, albeit to varying degrees except for Fe^{3+}, which only showed a flocculating activity of 21%. The flocculating activities observed with all the other cations were above 70% (Figure 12). The divalent cation Mn^{2+} demonstrated the highest flocculating activity (96.5%) for the purified bioflocculant when compared with all the other cations tested (Figure 12). The synergistic effect of cations was optimal with Mn^{2+}, which showed that it helps to neutralize negative charges on the bioflocculant and the suspended kaolin particles, shortening the distance between them, increasing the initial adsorption of the bioflocculant onto the kaolin particle and thus leading to floc formation and sedimentation [57,71]. Increasing the number of valence ions in suspension increases the ionic strength, which in turn decreases the electrostatic repulsive forces among bioflocculant charged groups. In the event of the above phenomenon, the conformation of the bioflocculant becomes altered into a more closely-packed structure which is not able to form bridges [69]. This might account for the reduced flocculating ability of the bioflocculant in the presence of Fe^{3+} and Al^{3+} compared to monovalent and divalent cations. However, when comparing the two trivalent cations, it was evident that the synergistic effect of Al^{3+} led to a high flocculating activity compared to the antagonistic effect of Fe^{3+}. In addition, aluminum salts have, on their own, been used in industries as flocculants [3].

From our results for the effect of cations on the flocculating activity of both crude and purified bioflocculant, we observed that monovalent cations completely inhibited the flocculating activity of the crude bioflocculant. The experiment for the effect of cations on the flocculating activity of crude bioflocculant was carried out at pH 7 (pH of the kaolin suspension was adjusted to neural). On the other hand, the effect of cations on the flocculating activity of purified bioflocculant was carried out at the optimal pH 10 (Figure 11).

The pH of the reaction mixture in a flocculation process is one of the key factors that determine flocs formation and the stability of suspended particles [72]. Furthermore, this shows that the ionization of the functional groups in the molecular chain of the bioflocculant is pH-dependent [73]. It will be safe to assume that the spatial charge arrangements for flocculation were not advantageous for the crude bioflocculant with the monovalent cations at pH 7 and that was why the flocculating rates were completely inhibited. Conversely, for the purified bioflocculant, the synergistic effect of the monovalent cations was highly enhanced with the monovalent cations at alkaline condition pH 10 (Figure 11). Thus, this is an indication that the bioflocculant exhibits different electric states at different pH conditions and consequently affected the flocculating efficiency of both crude and purified bioflocculant for kaolin particles.

Similarly to our findings, Ugbenyen et al. [44] also reported that Mn^{2+} was the most preferred metal ion for the flocculating activity of the bioflocculant produced by *Cobetia* spp. The bioflocculant activity of MBF-3 produced by *Bacillus* sp. was stimulated by Al^{3+}, Mg^{2+}, Ca^{2+}, K^+, and Na^+, with Al^{3+} being the most effective cation [74].

4. Conclusions

This study has shown that the bacterial strain *Bacillus pumilus* is an excellent producer of bioflocculant. To the best of our knowledge, there are no reports in the literature on bioflocculant production by this *Bacillus pumilus* strain. The bacteria produced a thermostable polysaccharide optimally when maltose and multiple nitrogen sources (yeast extract, urea and ammonium sulfate) were used as sources of carbon and nitrogen in the production medium, respectively. The bioflocculant had strong flocculating efficiency for kaolin suspension over a wide pH range (3–11) with a low requirement of dosage (0.1 mg/mL). The excellent thermal stability of the bioflocculant produced by *Bacillus pumilus* suggests that the bioflocculant produced could be utilized as an alternative for harmful synthetic chemical flocculants being used in water/wastewater treatment.

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Conflicts of Interest: The authors declare no conflict of interest.

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