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Isolation, Identification and Characterization of Bioflocculant-Producing Bacteria from Activated Sludge of Vulindlela Wastewater Treatment Plant

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Abstract: The low microbial flocculant yields and efficiencies limit their industrial applications. There is a need to identify bacteria with high bioflocculant production. The aim of this study was to isolate and identify a bioflocculant-producing bacterium from activated sludge wastewater and characterise its bioflocculant activity. The identification of the isolated bacterium was performed by 16S rRNA gene sequencing analysis. The optimal medium composition (carbon and nitrogen sources, cations and inoculum size) and culture conditions (temperature, pH, shaking speed and time) were evaluated by the one-factor-at-a-time method. The morphology, functional groups, crystallinity and pyrolysis profile of the bioflocculant were analysed using scanning electron microscope (SEM), Fourier transform infrared (FTIR) and thermogravimetric (TGA) analysis. The bacterium was identified as *Proteus mirabilis* AB 932526.1. Its optimal medium and culture conditions were: sucrose (20 g/L), yeast extract (1.2 g/L), $MnCl_2$ (1 g/L), pH 6, 30 °C, inoculation volume (3%), shaking speed (120 rpm) for 72 h of cultivation. SEM micrograph revealed the bioflocculant to be amorphous. FTIR analysis indicated the presence of hydroxyl, carboxyl and amino groups. The bioflocculant was completely pyrolyzed at temperatures above 800 °C. The bacterium has potential to produce bioflocculant of industrial importance.

Keywords: bacteria; bioflocculant; flocculating activity; optimisation

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1. Introduction

Water is one of the most important components of survival and thriving to carbon-based life forms [1]. Water is polluted when compromised as the result of anthropogenic impurities, which could be agricultural, industrial and domestic wastes [2]. The consumption of polluted water has caused the increase in waterborne diseases in communities [3]. Flocculation falls among alternative physico-chemical methods of choice for the elimination of organic matter and contaminations from wastewater [4]. Its purpose is to aggregate or agglomerate dispersed fine particles together to form large flocs that are quick to sediment [4]. Flocculating agents are classified as inorganic, organic and naturally occurring flocculants, called microbial bioflocculants [1,5].

Inorganic (polyaluminium chloride, ferric chloride, etc.) and organic synthetic flocculant (polyacrylamide and its derivatives) continue to dominate as flocculation mediators in water treatment processes [6,7]. These flocculating agents have shown a great potential for removing of pollutants from wastewater; thus, they are noted as being cost-effective, and they have high flocculating efficiencies. However, some disadvantages have been noted as well, one of which is that they have been reported to cause Alzheimer's disease, cancer and other debilitating illnesses [8]. Furthermore, acrylamide and amine polymers are

resistant to biodegradation [9], with all attempts at degradation resulting in the reduction of the polymers to monomeric units, which may eventually percolate into sediments or water beds and possibly seep into underground waters [6–10]. The presence of acrylamide monomeric units and amine polymers derivatives in the environment constitutes a serious major health concern [10]. However, bioflocculants have not been associated with any negative health effects [11]. The enormous advantages associated with bioflocculants makes them interesting. In addition, the industrial application of bioflocculants in the wastewater treatment process has been limited by low efficiencies and bioflocculant yields and high production cost [12,13]. The imperative to identify microbial bioflocculants has propelled the exploration of extreme environments for microbial species with enhanced ability to produce bioflocculant in high yields and with improved flocculation efficiencies.

Microorganisms from various taxonomic genera, including actinobacteria phylum (*Streptomyces*, *Brachybacterium* and *Cellulomonas*) [6,14] as well as *Arthrobacter* and *Scenedesmus* [10–14], have all been found to produce bioflocculants in a variety of terrestrial and aquatic environments. The composition of some of the identified bioflocculants has contained polysaccharide, uronic acids, sugar and protein [15,16]. The chemical composition and flocculating efficiency of bioflocculants are influenced by a variety of factors, including the nature of the environment in which the bioflocculant-producing microorganisms were isolated, the medium composition in which the microorganisms were cultivated and the bioflocculant's functional groups, morphology and crystallinity, as well as its pyrolysis profile. Activated sludge wastewater is considered to be a potential source of microbes with novel metabolites. In addition, this environment contains macromolecule compounds which are essential in bioflocculants that include proteins, polysaccharide nucleic acids and cellulose. However, the exploration of this isolated environment of bioflocculant-producing microorganisms is still very limited [17,18]. Therefore, the continued exploration of diverse environments for novel microorganisms with improved bioflocculant production yields has been a focus of researchers.

In this study, a bioflocculant-producing bacterial strain was isolated from the activated sludge wastewater from the Vulindlela Wastewater Treatment Plant in the University of Zululand, Republic of South Africa. The bacterium was screened for bioflocculant production and identified by16S rRNA gene sequencing analysis. The medium composition and culture conditions known to influence bioflocculant production were optimized using the standard one-factor-at-a-time method. Lastly, the morphology, functional groups and pyrolysis profile of the extracted bioflocculant were characterised using scanning electron microscope (SEM), Fourier transform infrared (FTIR) and thermogravimetric (TGA) analysis.

2. Materials and Methods

2.1. Isolation of Bioflocculant-Producing Microorganisms

Activated sludge water sample was collected aseptically from secondary sedimentation tanks from the Vulindlela Wastewater Treatment Plant at the University of Zululand, RSA. In the laboratory, 10-fold serial dilutions of the water sample were conducted with 0.85% of saline solution [16]. About 1 mL of sludge water sample was transferred into 9 mL of sterile saline solution and agitated for 30 s. From these, serial dilutions (1×10^{-1} and 10^{-2}) were made; 100 µL of the serially diluted and undiluted samples was aseptically plated on Nutrient agar plates [19]. Nutrient agar plates were incubated at 37 °C for 3 days. The colonies were counted as colony forming units per millilitre (CFU/mL) and expressed as population density. The colonies were selected based on divergence in morphology, size and colour. Thereafter, pure sub-cultured colonies were screened for bioflocculant production.

2.2. Screening of Bacteria for Bioflocculant Production

The standard production medium that composed the ingredients shown in Table 1 was prepared according to the method described by Mathias et al. [20]. The production medium

was prepared by dissolving the ingredients in 1 L of filtered activated sludge water. The medium was poured into a conical flask (100 mL) and sterilized by autoclaving at 121 °C for 15 min. A loop full of pure bacterial strain was inoculated into sterile production media. Each flask with the mixture was placed in a shaking incubator (LAB-Consumables, Durban, South Africa) at a speed of 160 rpm and temperature of 30 °C. After 72 h of incubation, 2 mL of broth culture was centrifuged at 8000 rpm for 30 min at 4 °C to remove bacterial cells. The cell-free supernatant was used to evaluate bioflocculant activity [19,21].

Table 1. Production medium for screening of bacteria for bioflocculant production.

Ingredients	Amount (g)
Glucose	20.0
K_2HPO_4	5.0
K_2PO_4	2.0
Urea	0.5
Yeast extract	0.5
$MgSO_4 \cdot 7H_2O$	0.2
$(NH_4)_2SO_4$	0.2
NaCl	0.1
Activated sludge water (filtered)	1 Litre

2.3. Determination of Bioflocculant Activity

The bioflocculant activity test was conducted following the procedure used by Ugbenyen et al. [22]. Kaolin solution (4.0 g/L) was utilized to determine bioflocculating activity. Prior to the determination of the bioflocculant activity, 3 mL of 1% (*w*/*v*) $CaCl_2$ solution and 2 mL of cell-free supernatant were mixed with 100 mL of kaolin clay suspended solution in a 250 mL conical flask. The mixture was vigorously shaken for 60 s and transferred into a 100 mL graduated measuring cylinder. The mixture was allowed to sediment for 5 min at room temperature. A control was prepared similarly with the exception of the bioflocculant, which was replaced by the freshly prepared production media. The optical density of the clarification solution was measured at 550 nm wavelength using a spectrophotometer (Unic-7230, Shanghai Lianhua Company, Shanghai, China). The flocculating activity was calculated using the following equation [22]:

$$\text{Flocculating activity, FA \%} = \frac{[(A - B)]}{A} \times 100$$

where A represents the optical density measured at 550 nm (OD_{550} nm) of the control, and B represents the optical density measure at 550 nm (OD_{550} nm) of a sample.

2.4. Identification of a Bioflocculant-Producing Bacterium

2.4.1. Morphological Identification of the Bioflocculant-Producing Bacterium

The bioflocculant-producing strain with the most promising bioflocculant activity (70%) was identified. The pure bacterial colony was subjected to Gram staining to assess its morphological physiognomies, such as shape and Gram stain reaction. Briefly, the isolate was spread on the glass slides and heat fixed using a Bunsen burner (LAB-Consumables, Durban, South Africa). Crystal violet stain was used as a primary stain, followed by iodine solution. It was decolourised by ethanol and washed with tap water. The slide was flooded with safranin and rinsed with tap water. Lastly, the slide was viewed at 1000× magnification using a compound bright- field microscope. (Labotec (Pty) Ltd., Midrand, South Africa).

2.4.2. Molecular Identification of Bioflocculant–Producing Bacterium

The bioflocculant-producing strain was further identified using 16S rRNA gene sequencing analysis. Briefly, the extraction of the genomic DNA of the isolate was carried out using the Quick-DNA ™ fungal/bacterial miniprep kit (Zymo Research) (Omega

Bio-Tek, Inc., Norcross, GA, USA). This was performed to obtain DNA from the bacterial strain. The PCR amplification was carried out utilising universal primers, forward primer 5′-AGAGTTTGATCMTGGCTCAG-3′ and reverse primer 5′-CGGTTATTGTTACGACTT-3′, and DreamTaq™ DNA polymerase (Thermo Fisher Scientific™) was used to measure the 16S rRNA gene sequence. PCR product gels were extracted using Zymo Research, Zymoclean™ Gel DNA recovery kit. Thereafter, the extracted fragments were sequenced in forward and reverse direction using a Nimagen BrilliantDye™ terminator cycles sequencing kit V3.1, RB D3 -100/1000) and were purified using a Zymo Research ZR-96 DNA sequencing clean-up kit™. The purified fragments were analysed on the ABI 359XI Genetic Analyser, and the results were obtained using BLAST search [23].

2.5. Activation of the Isolate for Fermentation

An amount of 1 L of the production medium containing 3 g of beef extract, 10 g of tryptone and 5 g of sodium chloride was prepared. A total of 5 mL of the production medium was measured into different test tubes and autoclaved for 15 min at 121 °C. Afterwards, the bacterium was inoculated into the tubes and incubated for 25 h at 28 °C and 160 rpm in a rotary shaker. (LAB-Consumables, Durban, South Africa).

2.6. Optimisation of Bioflocculant Production Conditions

In order to enhance and increase the yields of bioflocculant production and flocculating activity, the parameters such as inoculation volume, carbon and nitrogen sources, metal ions, initial pH, shaking speed, temperature and time course were evaluated.

2.6.1. Determination of the Inoculation Volume

The optimum inoculation volume was determined using broth culture ranging from 1% (0.5 mL), 2% (1.0 mL), 3% (1.5 mL), 4% (2.0 mL) and 5% (2.5 mL). The broth cultures were inoculated into 50 mL of production media. Flasks were incubated at 30 °C for 72 h at 160 rpm. To determine the flocculating activities, 3 mL of 1% $CaCl_2$ (1% w/v) and 2 mL of supernatant from the centrifuged production medium were added to 250 mL conical flasks with 100 mL measuring cylinder and allowed to stand for 5 min at room temperature for sedimentation. An amount of 1 mL of clear supernatant was withdrawn, and the flocculating activity was evaluated using spectrophotometer at 550 nm [24].

2.6.2. Effect of Carbon and Nitrogen Sources on Bioflocculating Activity

Carbon and nitrogen sources have a significant impact on bioflocculant production by microorganisms [25]. Different carbon sources (20 g) such as glucose, fructose, sucrose, maltose, galactose, xylose, lactose, starch and the mixture of CHO (sucrose and glucose) were prepared in 1 L of filtered activated sludge wastewater. These parameters were evaluated according to Liu et al. [26]. The production media were prepared in separate flasks. The bacterial strain was inoculated into the prepared media. The media were supplemented with 20 g/L each of various carbon sources and incubated at 30 °C, 150 rpm for 3 days. To determine the influence of nitrogen on bioflocculant production, the different nitrogen sources such as organic and inorganic [viz yeast extract powder, casein, peptone, urea, NH_4Cl and $(NH_4)_2\ SO_4$)] and mixture of nitrogen (urea and yeast extract powder) were prepared by replacing 1.2 g/L of mixed nitrogen source [yeast extract (0.5 g), urea (0.2 g) and $NH_2\ SO_4$ (0.5 g)] into the production media in separate containers, and the flocculation activity was calculated according to Ugbenyen et al. [22].

2.6.3. Effect of Metal Ions on Bioflocculating Activity

Different cations such as Na^+, K^+, Li^+, Mn^{2+}, Ba^{2+}, Fe^{3+} and Al^{3+} were assessed for their effect on flocculating activities for the bioflocculant production. Three millilitres of $CaCl_2$ solution 1% (w/v) used as standard cation were replaced by the different cations (1% w/v) [5]. The control was prepared by adding kaolin solution and cell-free supernatant

without an addition of cation. The flocculating activities were determined following methods by Ugbenyen et al. [22].

2.6.4. Effect of Initial pH of the Production Media

To determine the effect of initial pH on bioflocculant production. The pH of the production medium was adjusted using 1 M NaOH and 1 M HCl prior to sterilization and inoculation [27]. The pHs used were 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12. The flocculating activity was thereafter determined.

2.6.5. Effect of Shaking Speed on Bioflocculant Production

The effect of shaking speed on the bioflocculant production was determined following method describe by Liu et al. [26]. Various shaking speed of 60–240 rpm were determined. An amount of 50 mL of production medium and 1.5 mL inoculation were prepared in 100 mL conical flasks. The flasks were incubated at different speeds ranging from 60 to 240 rpm at the temperature of 30 °C for the periods of 72 h. The flocculating activities were measured after 72 h of cultivation.

2.6.6. Effect of Cultivation Temperature on Bioflocculating Activity

The effect of temperature on bioflocculant production was evaluated using a method described by Maliehe et al. [28]. An amount of 50 mL of production medium with the optimum inoculation volume (v/v) of the bioflocculant solution was prepared in 100 mL conical flasks and incubated at different temperatures ranging from 20 to 60 °C at 160 rpm for 72 h. The flocculating activities were measured.

2.6.7. Time Course Assay

The effect of culture time was evaluated by first preparing the production medium made up of glucose (20 g), urea (0.5 g), yeast extract (0.5 g), NH_2SO_4 (0.2 g), $KHPO_4$ (2 g), K_2HPO_4 (5 g), NaCl (0.1 g) and $MgSO_4$ (0.2 g) in 1 L of filtered activated sludge water. The pH was adjusted to 6 with either 1 M NaOH or 1 M HCl. The mixture was autoclaved at 121 °C for 15 min. The bacterial strain was cultivated separately under optimal growth conditions. To standardise, 50 mL of sterile saline solution was inoculated with a loop of pure colonies of the isolate, vortexed and the suspension standardized to OD 660 nm 0.1. The standardised saline solution was used as seed culture for inoculation preparation. From the seed culture, the optimum inoculation volume of 1% (v/v) was incubated on a rotary shaker (120 rpm) at 40 °C. Samples were drawn every 12 h. Two millimetre of the sample was centrifuged at 8000× g for 15 min. The supernatant was used to determine the flocculating activity. For a period of five days, the optical density at 660 nm (OD 660 nm) and the pH of the medium were measured every 12 h [8].

2.7. Extraction and Purification of Bioflocculant

The extraction and purification were carried out according to Ugbenyen et al. [22] and Dlamini et al. [29], respectively. After 72 h of fermentation, the culture broth was centrifuged for 15 min at 8000 rpm at 4 °C. One volume of distilled water was added to the supernatant and further centrifuged for 15 min at 8000 rpm at 4 °C to remove all the insoluble substances. Two thousand millilitres of ice-cold ethanol was added to the supernatant, shaken vigorously and left at 4 °C overnight for precipitation. The supernatant was discarded and the precipitate was vacuum-dried to obtain a purified bioflocculant.

2.8. Chemical Analysis of the Purified Bioflocculant

2.8.1. Composition Analysis of the Purified Bioflocculant

The total sugar content analysis was evaluated using the phenol-sulfuric acid method, with glucose being used as a standard [30]. Briefly, 0.2 g of purified bioflocculant was added into a beaker containing 100 mL of distilled water. About 0.2 mL of phenol was added to the solution together with 1.0 mL of the concentrated sulfuric acid. The solution was allowed

to stand at room temperature for 10 min before being shaken vigorously. The absorbance was measured using a spectrophotometer at an optical density of 490 nm [12]. Total protein content of the bioflocculant was determined by Bradford assay with bovine serum albumin (BSA) as standard [31]. Briefly, 20 µL of each standard solution was withdrawn and transferred into the wells of a 96-well plate. About 180 µL of Bradford reagent was added to each standard dilution and was mixed by pipetting. The solution was allowed to stand at room temperature for 2 min. The solution was then measured using a spectrophotometer at 595 nm. To measure the uronic acid content, the carbazole-sulfuric acid method was used [21,32]. Briefly, 0.95 g of sodium tetraborate decahydrates was dissolved in 2.0 mL of hot water, after which 98 mL of ice-cold concentrated sulfuric acid was added. The solution was mixed carefully and cooled in an ice bath. About 0.05 mL of carbazole was added, mixed and reheated at 100 °C for 15 min. The mixture was allowed cool at room temperature and was measured at 525 nm using a spectrophotometer (Unic-7230, Shanghai Lianhua Company, Shanghai, China).

2.8.2. FTIR Analysis

FTIR was used to analyse functional groups of the purified bioflocculant using the Tensor 27, Bruker FT-IR spectrophotometer with a resolution of 4 cm^{-1} in the range of 4000–400 cm^{-1}. About 5 mg of the bioflocculant was mixed with potassium bromide powder and pressed into pellets [33].

2.8.3. Thermo-Gravimetric Analysis (TGA) of a Bioflocculant

The pyrolysis of the purified bioflocculant was analysed using a TGA analyser (PerkinElmer, Inc., Waltham, MA, USA) at a temperature range of 30–900 °C and heating rate of 10 °C per minute. This rate was kept constant under flow of nitrogen gas [29,34].

2.8.4. Elementary Analysis

To analyse the elements present in the purified bioflocculant, a scanning electron microscope (SEM) equipped with elemental analyser (JEOL USA, Inc., Peabody, MA, USA) was used. Five milligrams of the purified bioflocculant was added to a silicon-coated slide. The silicon-coated slide was fixed with a spin coater at 1000 rpm for 60 s and then inserted into the equipment for analysis [35].

2.9. Flocculation Characteristics of a Purified Bioflocculant

2.9.1. Effect of Dosage Concentration on Flocculating Activity

A method described by Cosa and Okoh [36] was followed to evaluate the effect of dosage concentration of the purified bioflocculant. Various concentrations of bioflocculant ranging from 0.2 and 1.0 mg/mL (w/v) were prepared. From each solution, 2 mL was mixed with 100 mL of kaolin clay suspension and 3 mL of 1% (w/v) $CaCl_2$ in a 250 mL conical flask. The mixture was shaken vigorously and transferred into a 100 mL measuring cylinder. The mixture was allowed to stand for sedimentation for 5 min at room temperature. Two millilitres of the supernatant was drawn from the upper part and used to determine flocculating activity.

2.9.2. Effect of pH on the Flocculating Activity of Purified Bioflocculant

The pH stability of the purified bioflocculant was evaluated using the Gomaa [37] method. An amount of 100 mL of kaolin solution (4 g/L) was adjusted in different flasks (250 mL), either 1 M NaOH or 1 M HCl in the pH range of 3–12. pH 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 were used to determine the flocculation activity of the bioflocculant. An amount of 2 mL of 0.4 mg/mL bioflocculant solution was mixed with kaolin solution from each flask and used for bioflocculating activity.

2.9.3. Effect of Metal Ions on Flocculating Activity

Different metal ions such as Na^+, K^+, Ba^{2+}, Mn^{2+}, Mg^{2+}, Fe^{3+}, and Al^{3+} were used to substitute 1% (w/v) $CaCl_2$ solution. Experimental control was prepared by adding free-cell supernatant in a kaolin solution with no cation being added. Thereafter, the effect of each cation on flocculating activity was measured as previously described [38].

2.9.4. Effect of Heat on Flocculating Activity

The effect of heat on flocculating activity was determined using a method described by Ntozonke et al. [10]. The purified bioflocculant was dissolved in distilled water to prepare an optimum concentration. Ten millilitres of the bioflocculant solution was heated at different temperatures ranging from 50 to 100 °C for 30 min. The residual flocculating activity of the purified bioflocculant against kaolin clay suspension (4 g/L) at room temperature was determined in order to find temperature stability for the purified bioflocculant.

2.10. Statistical Analysis

Experiments were conducted in triplicates. The means and standard deviations were determined using Graph Pad Prism Version 6. All p values ≤ 0.05 were considered as significant, while p values ≤ 0.05 were considered as very significant. Arrow bars represent the standard deviation, and values with different letters represent a significant difference ($p < 0.05$).

3. Results and Discussion

3.1. Isolation, Screening and Identification of Bioflocculant-Producing Bacterium

Optimisation of the growth process is performed to increase the production yield of the microbial by-product. The attempt to reduce the production cost of bioflocculants has been effective but not sufficient. Therefore, this has led to the search for microorganisms with better bioflocculant producing capacity. In this study, the number of CFU/mL of the isolates was investigated. The results show that wastewater from the activated sludge from the Vulindlela Wastewater Treatment Plant had 8×10^3 CFU/mL (Table 2).

Table 2. Bacterial population from the wastewater.

Sample	Un-Diluted (CFU/mL)	10^{-1} (CFU/mL)	10^{-2} (CFU/mL)
Wastewater	TN	TN	8×10^3

TN indicates too numerous to count.

The isolates were screened for bioflocculant production. Among the obtained screened isolates (32 isolates), the strain with the highest flocculating activity (70%) against kaolin solution (4 g/L) was selected and identified. On the nutrient ager plate, the isolate appeared circular, pale white and smooth, with 1 µm in diameter. The selected strain was found to be Gram-negative and rod-shaped. The bacterium was then identified using the 16S rDNA method, and the results were compared with the NCBI data base using the BLAST program. The result showed 99% similarity to the *Proteus mirabilis* sp. and was deposited as *Proteus mirabilis* with accession number of AB 932526.1. The literature has reported the ability of *Proteus mirabilis* to produce bioflocculant [34].

3.2. Optimisation of Culture Conditions

Optimisation of the growth process is done to increase the production yield of the microbial by-product. Bioflocculant production is influenced by several factors, such as medium composition (carbon and nitrogen sources) and environmental conditions (pH, metal ions, shaking speed, temperature and time). Furthermore, the inoculation volume has a significant impact on bioflocculant production [39]. Various microorganisms require distinct nutritional supplies and optimal fermentation conditions to properly synthesize novel cell materials and bioflocculants [9]. In the present study, various parameters were

optimized in order to increase bioflocculant yields from single and mixed bacterial cultures. Good flocculants have flocculating activity of greater than 90% [39].

3.2.1. Effect of Inoculation Volume on Bioflocculant Production

Inoculation volume plays an important role in bioflocculant production and cell growth [40]. A low inoculation volume prolongs the bacterial growth, while a large inoculation volume causes niches in which the organisms can overlap excessively, thereby hindering bioflocculant production. As shown in Table 3, inoculation volumes from 1% to 3% were favourable for bioflocculant production, as they produced more than 80% flocculating activity. A 3% (v/v) inoculation volume was more effective than others with an optimum flocculating activity of 90%. An increase in the inoculation volume above 3% (v/v) resulted in a decrease in flocculating activity. This might be due to the shortage of nutrient in the fermentation medium [40]. An inoculation volume of 1.5 mL (3%) was used for all of the tests that followed. Li et al. [41] found similar results whereby an optimum inoculation volume of 1% was favoured by *Bacillus licheniformis* X14. However, other studies found different results; the inoculation volume of 2% by *Aspergillus flavus* was obtained by Aljuboori et al. [7], and 4% by *Bacillus pumilus* was reported to produce the highest bioflocculating activity [39]. Ugbenyen et al. [42] preferred inoculation volumes ranging from 1% to 5% (v/v). As a result, the microorganism used in this study was cost-effective.

Table 3. Effect of inoculation volume, carbon sources, nitrogen sources and cations on flocculating activity.

Inoculation Volume (%)	FA (%) ± SD	Carbon Sources	FA (%) ± SD	Nitrogen Source	FA (%) ± SD	Cations	FA (%) ± SD
1	80.5 ± 0.41 ^{a}	Lactose	52.7 ± 0.92 ^{a}	Casein	52.4 ± 2.48 ^{a}	Control	46.2 ± 0.13 ^{a}
2	89.2 ± 0.14 ^{b}	Galactose	56.1 ± 0.98 ^{a}	Ammonium sulphate	64.3 ± 1.32 ^{b}	Li^{+}	65.7 ± 1.15 ^{b}
3	90.2 ± 0.14 ^{c}	Xylose	63.9 ± 1.34 ^{b}	Ammonium acetate	66.9 ± 0.17 ^{b,c}	K^{+}	66.7 ± 0.05 ^{b}
4	63.3 ± 0.15 ^{d}	Maltose	71.7 ± 4.45 ^{c}	Ammonium chloride	77.2 ± 0.57 ^{d}	Na^{+}	86.1 ± 1.86 ^{c}
5	49.4 ± 0.04 ^{e}	Fructose	83.4 ± 2.59 ^{d}	Urea	90.1 ± 2.39 ^{e}	Ba^{2+}	90.4 ± 0.08 ^{d}
-	-	Starch	83.8 ± 0.99 ^{d,e}	Yeast extract	91.4 ± 0.41 ^{e,f}	Ca^{2+}	90.8 ± 0.02 ^{d,e}
-	-	Glucose	89.3 ± 1.68 ^{d,f}	Peptone	92.1 ± 0.11 ^{e,g}	Mg^{2+}	93.1 ± 0.02 ^{e,f}
-	-	Sucrose	94.7 ± 3.10 ^{e}	Mixture of nitrogen	63.1 ± 0.44 ^{b,h}	Mn^{2+}	95.1 ± 0.25 ^{f,g}
-	-	Mixture (CHO)	68.9 ± 1. 3 ^{b,c,h}	-	-	Fe^{3+}	21.1 ± 1.01 ^{h}

FA indicates flocculating activity, while SD indicates standard deviation. Different letters (a, b, c, d, e, f, g and h) indicate statistical significance at ($p < 0.05$).

3.2.2. Effect of Carbon Source on Bioflocccculant Production

Carbon sources are important for the growth of microorganisms as they provide energy and serve as building blocks for all components in the bacterial cells [43]. Different sugars such as sucrose, glucose, maltose, lactose, xylose, galactose, fructose and starch were investigated for optimum bioflocculant production by *Proteus mirabilis* AB 932526.1, and the results are shown in Table 3. In addition, the bioflocculant synthesis was examined in the presence of a carbohydrate mixture (CHO) containing sucrose and glucose. It was observed that among the different sugars studied, sucrose had the highest flocculating activity of 95%. Moreover, a mixed carbohydrates source resulted in a flocculating activity of 69%. Sucrose was followed by glucose, starch, fructose, maltose and xylose with flocculating activities of 89%, 84%, 83%, 72% and 64%, respectively. Flocculating activities below 60% were observed for galatose and lactose, with flocculating activities of 56% and 54%, respectively. Several studies revealed sucrose to be more effective for bioflocculant production, which is similar to this research finding. Zheng et al. [44] reported sucrose to be the most desirable

carbon source for bioflocculant production by *Bacillus* sp. F9. However, these may vary with different microbes. As an example, soluble starch as a carbon source was optimal for *Sarangium cellulosum* [45]. In the case of *Klebsiella* sp., maltose was observed to be the best carbon source [46]. The maximum flocculating activity of 90.1% was obtained when mixed carbohydrate (sucrose and glucose) was used [24]. Therefore, sucrose was used in all experiments to follow.

3.2.3. Effect of Nitrogen Sources on Bioflocculant Production

Various microorganisms utilise nitrogen for the synthesis of amino acids, proteins, and nucleic acids [47]. The effect of organic (urea, peptone, yeast and casein) and inorganic (ammonium sulphate, ammonium acetate and ammonium chloride) nitrogen sources on bioflocculant production by *P. mirabilis* AB 932526.1 was evaluated, and the results are shown in Table 3. In addition, the bioflocculant synthesis was examined in the presence of a nitrogen mixture containing urea and yeast extract. Among the various nitrogen sources, peptone proved to be the best nitrogen source, with the highest flocculating activity of 92%. It was followed by yeast extract (91%), urea (90%), ammonium chloride (77%), ammonium acetate (67%), ammonium sulphate (64%) and a mixture of nitrogen (63%). Casein was the least favourable nitrogen source, with a flocculating activity of 52%. Similar findings were reported by Cosa et al. [48], whereby peptone was found to yield the optimum flocculating activity for *Bacillus amyloliquefaciens*. However, since there were no statical differences between the three nitrogen sources (peptone, yeast extract and urea), any of them could have been used for bioflocculant production. However, in this study, yeast extract was preferred because of its physical and chemical properties, as well as because of its availability. Bioflocculant production by *Penicillium* sp. showed highest flocculating activity when yeast extract was used as a sole nitrogen source [49]. In the study done by Ntsangani et al. [50], yeast extract was reported to have resulted in maximum flocculating activity of 78% for the bioflocculant produced by *Bacillus* sp. F 19. On the other hand, Tawila et al. [24] obtained maximum flocculating activity of 72.1% when a mixed nitrogen source was used. Researchers have reported that organic nitrogen sources are easily absorbed by the cells and that they are mostly favourable for bioflocculant production when compared with inorganic nitrogen sources [51]. This hypothesis was observed in this study.

3.2.4. Effect of Metal Ions on Bioflocculant Production

Cations enhance the flocculating rate by neutralising and stabilising the residual negative net surface charge of functional groups of bioflocculants [52]. In this study, various metal ions were examined (Table 3). Most of the cations used stimulated the flocculation process and resulted in over 60% flocculating activity. Monovalent and divalent cations favoured bioflocculant production, with divalent Mn^{2+}, Mg^{2+}, Ca^{2+} and Ba^{2+} being more effective, as they resulted in the high flocculating activities of 95%, 93%, 91% and 90%, respectively. The profound effect of divalent cations of flocculation was followed by that of monovalent cations—namely, Na^{+}, K^{+}, and Li^{+}, with flocculating activities of 86%, 67% and 66%, respectively. The trivalent Fe^{3+} and blank (no cation used) were less effective, as they resulted in the lowest flocculating activities of 21% and 46%. Thus, the results corroborate with those of Agunbiade et al. [53], whereby flocculating activity was stimulated by monovalent and divalent cations, while trivalent cation inhibited the flocculating activity. Therefore, Mn^{2+} was selected as the cation for all experiments that followed.

3.2.5. Effect of pH on Bioflocculant Production

The initial pH of the fermentation medium plays an important role in the production of bioflocculant [54]. The effect of pH varying across the range of 3 to 12 in growth medium was evaluated, and the results are presented in Figure 1. The increase in pH for *Proteus mirabilis* AB 932526.1 was observed from pH 3 to pH 6, with the optimum bioflocculating activity of 83% being observed at pH 6. The increase in pH above 6 led to a decrease in bioflocculating activity. The cultivation of the microorganisms at pH below or above

6 resulted in low bioflocculating activity [22]. A drastic decrease in bioflocculating activity was observed from pH 7, which may be due to the disfunction or degradation of the bioflocculant molecule under the neutral and alkaline conditions. Similarly, Liu et al. [26] reported pH 6 to be more effective in bioflocculant production. However, other studies have reported pHs other than pH 6 to enhance bioflocculant production [55,56]. Consequently, pH 6 was used in all experiments to follow.

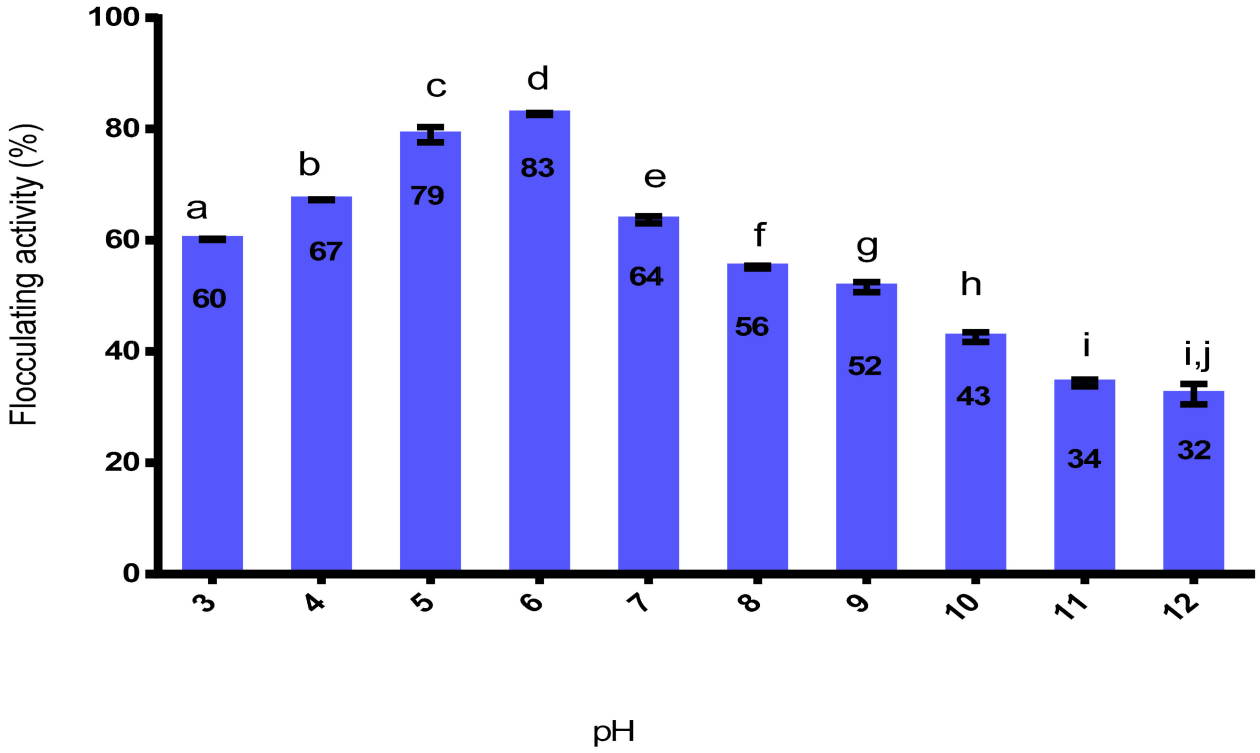

Figure 1. Effect of pH on bioflocculant production by *Proteus mirabilis* AB 932526.1. Flocculating activity (%) with different letters (a, b, c, d, e, f, g, h, i and j) are significantly different ($p < 0.05$). Error bars indicate standard deviation.

3.2.6. Effect of Shaking Speed on Bioflocculant Production

Shaking speed determines the concentration of the dissolved oxygen, which can also affect nutrient absorption and enzymatic reaction [41]. Thus, the effect of shaking speed on the production of bioflocculant produced by *P. mirabilis* AB 932526.1 was evaluated in the range of 0–240 rpm. In Figure 2, it can be seen that the flocculating activity constantly increased when the shaking speed ranged from 0 rpm to 120 rpm, and the maximum flocculating activity of 98% was obtained at 120 rpm. However, the increase in shaking speed above the optimum caused a significant decrease in flocculating activity. The results were in conformity with the study by Zulkeflee et al. [57], who reported a shaking speed of 120 rpm to be the most effective for the bioflocculant production by *Bacillus subtilis*. The similarity may be due to the same oxygen demand by bacteria at different growth stages during bioflocculant production. Adebayo-Tayo et al. [58] reported shaking speeds of 140 rpm and 160 rpm to be optimal for bioflocculants production. Thus, in this study, the shaking speed of 120 rpm was optimum and was used in all subsequent tests.

3.2.7. Effect of Temperature on Bioflocculant Production

Fermentation temperatures have an impact on bioflocculant production. Figure 3 Illustrated the effect of cultivation temperatures on flocculating activity. There was a constant increase in flocculating activity from 20 °C to 30 °C. The highest flocculating activity of 96% was observed when the temperature reached 30 °C. Further decrease in flocculating activity was observed at temperatures above 30 °C. The decrease might be due

to denaturation of enzymes responsible for production of bioflocculant at higher temperatures [59]. Similarly, Ayangbenro et al. [60] reported 30 °C as an optimum temperature for bioflocculant production by *Pantoea* sp. The optimum temperature of 40 °C was observed by Ambarsari et al. [61] for bioflocculant production by *Chromobacterium violaceum*. Thus, 30 °C was selected and used as an optimum temperature.

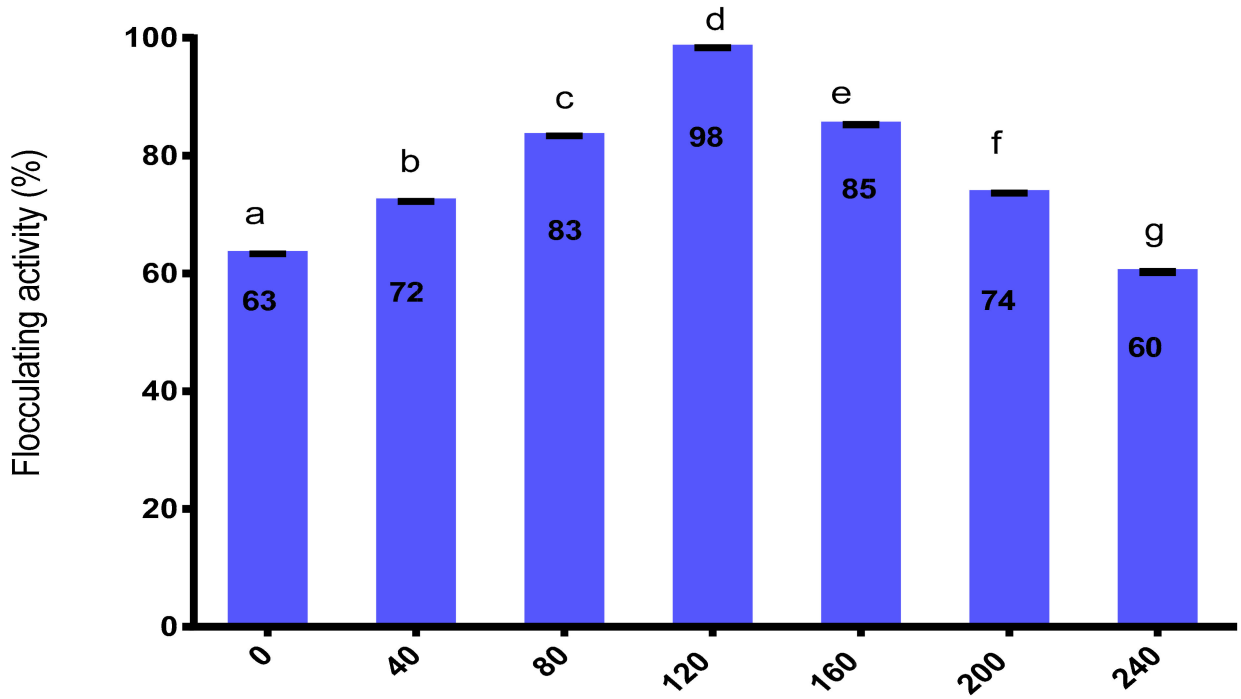

Figure 2. Effect of shaking speed on bioflocculant production by *Proteus mirabilis* AB 932526.1. Flocculating activity (%) with different letters (a, b, c, d, e, f and g) are significantly different ($p < 0.05$). Error bars indicate standard deviation.

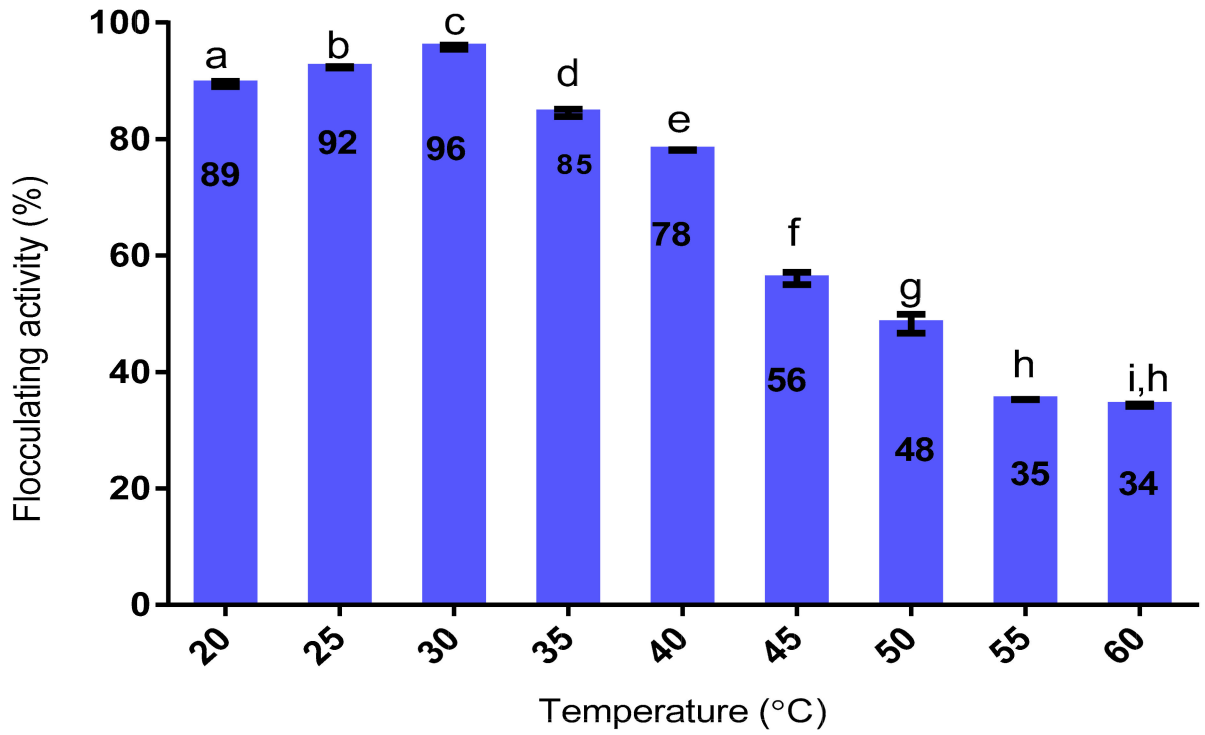

Figure 3. Effect of temperature on bioflocculant production by *Proteus mirabilis* AB 932526.1. Flocculating activity (%) with different letters (a, b, c, d, e, f, g, h and i) are significantly different ($p < 0.05$). Error bars indicate standard deviation.

3.2.8. Time Course on Bioflocculant Production

The effect of time on bioflocculant production by *P. mirabilis* AB 932526.1 in correlation with flocculating activity (FA), optical density (cell growth; OD) and pH was evaluated at the cultivation period of 120 h (Figure 4). The pH of the production medium remained constant in the first two days of the experiments. It then decreased from pH 5.76 to pH 5.16 during 60 to 120 h. The decrease in pH was perceived to be the result of organic acid being produced by *P. mirabilis* AB 932526.1 during the metabolism process [59,62]. The highest flocculating activity of 92% was observed at 72 h of fermentation period. The slight increase in flocculating activity was observed during lag phase (24 to 60 h). A rapid decrease in flocculating activity was observed after 72 h of fermentation, which was an indication of the shortage of nutrients for microbial growth [40]. The increase in cell growth was noticed from the first three days of fermentation. A slight decrease in cell growth was observed at 84–120 h. As the cells increased, so did the flocculating activity. Hence, the parallel increase in cell growth and flocculating activity indicates that the bioflocculant was produced through biosynthesis [24]. The results were in close conformity with the study by Xia et al. [25], who reported the maximum flocculating activity of 93% by the bioflocculant produced by *Proteus mirabilis* TJ-1 under optimised conditions. In this study, the optimisation of the culture conditions and medium composition significantly increased the flocculating activity by 22%. Thus, the obtained and preferable optimum conditions were employed during the production of the bioflocculant by *P. mirabilis* AB 932526.1, which was later extracted from the broth culture and partially purified.

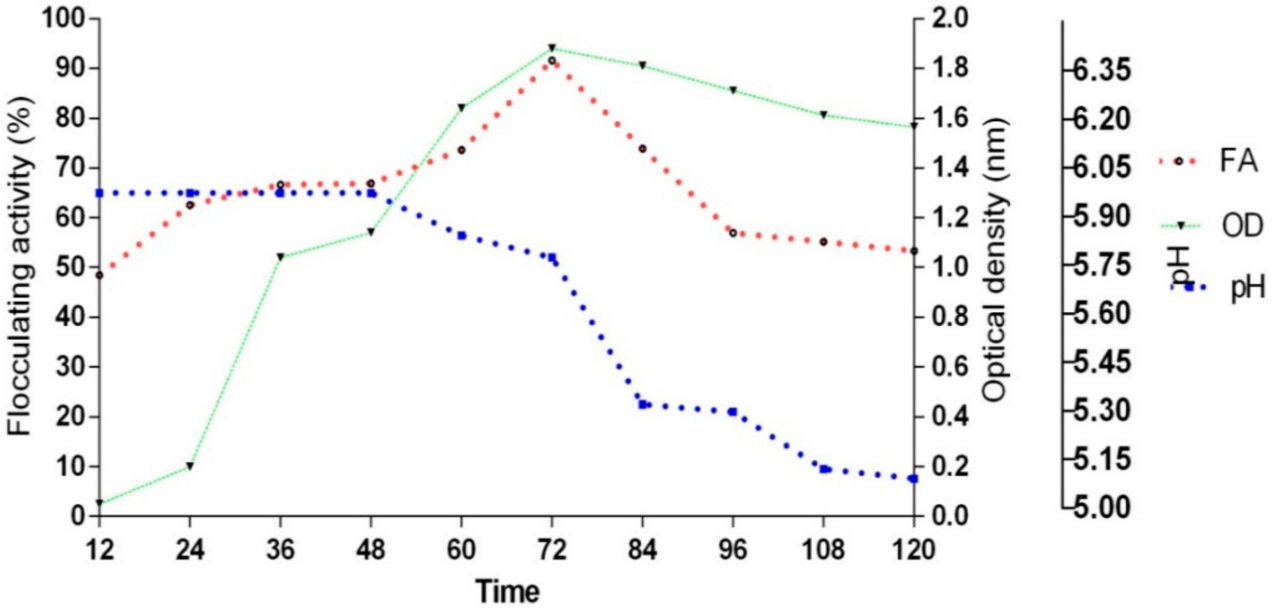

Figure 4. Effect of cultivation time on bioflocculant production.

3.3. Extraction and Purification of a Bioflocculant

Low yields of bioflocculants is a major challenge in bioflocculant production. A yield of about 3.8 g/L of purified bioflocculant was obtained in this study. The yield seems to be generally higher than most of the bioflocculants produced by pure bacterial strains; which are often less than 2 g/L [63]. For an example, a bioflocculant yield of 1.6 g/L from *Bacillus* sp. AEMREG7 was reported [64] and Ma et al. [65] also recorded a bioflocculant production by *Enterobacter* sp. to be 1.3 g/L. The high yield might be owed to the ability of *P. mirabilis* AB 932526.1 to efficiently metabolise the nutrients in the medium under optimum growth conditions and the polarity of the solvents used during extraction and purification processes. Nevertheless, the obtained yield was much lower in comparison with the yields of 4.52 g/L and 15 g/L produced by *Halomonas* sp. V3a and a consortium

of microorganisms, respectively [66]. Thus, it is suggested in the future to use *P. mirabilis* AB 932526.1 in bacterial consortia in order to further improve bioflocculant yield and flocculating activity.

3.4. Chemical composition of the Purified Bioflocculant

The chemical composition of the purified bioflocculant was assessed in order to determine the key components [67]. Several bioflocculants are reported to possess protein, polysaccharides or other extracellular polymeric substances. In this study, a chemical analysis of the purified bioflocculant was mainly composed of 64% (*w*/*w*) total sugar, 10% (*w*/*w*) total protein and 24% (*w*/*w*) uronic acid content (Table 4).

Table 4. Chemical compositions of the purified bioflocculant.

Sample	Concentration (%)
Total sugar	64.25%
Total protein	10.42%
Uronic acid	23.51%

The bioflocculant was predominately composed of carbohydrates, which made it heat stable. The results of this study are in agreement with most studies that have stated that the predominant component of bioflocculants is carbohydrates [11]. For an example, the bioflocculants produced by *Aspergillus parasiticus* [7], *Virgibacillus* sp. Rob [48] and *Klebsiela pneumonia* [68] are predominantly carbohydrates.

3.5. Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The Fourier transform infrared spectroscopy was used to determine the functional groups present in the molecular chain of the bioflocculant produced by *P. mirabilis* AB 932526.1 (Figure 5). The functional group of the bioflocculant revealed by IR spectrum displayed the presence of a strong broad stretching peak at 3317 cm^{-1}. This bond indicates the presence of both alcohol and amine groups within the bioflocculant chain. The presence of the hydroxyl group in the bioflocculant favours the possibility of hydrogen bonding with one or more molecules of water, which results in the exhibition of all bioflocculants in an aqueous solution [69]. A weak vibration at 1663 cm^{-1} indicates the presence of a carbonyl group, which indicates the presence of uronic acid in the bioflocculant and plays an important role in serving the functional moiety by spreading new polymers in dissimilar forms [70]. The peak at 1247 cm^{-1} showed a C–O bond that indicates the presence of sugars [71]. The absorption peak at 1167 cm^{-1} revealed the formation of glycosidic linkages, which makes a spectral change in a bioflocculant. A spectrum observed at 1067 cm^{-1} was indicative of carboxylic acid with an O–H bond. A small absorption peak at 586 cm^{-1} represents the alkyl halide. The occurrence of these functional groups has been reported to be effective for bioflocculant activities [72]. Similarly, Okaiyeto et al. [73] and Tlou [74] reported functional group such as carboxyl, hydroxyl and amino groups to be found in the bioflocculants produced by *Bacillus toyonensis* strain AEMREG6 and *Providencia rettgeri* KF534469.

3.6. Thermogravimetric TGA Analysis of the Purified Bioflocculant

A thermogravimetric analyser was used to determine the pyrolysis property of the purified bioflocculant produced by *Proteus mirabilis* AB 932526.1. As shown in Figure 6, a weight loss of about 23% was observed when the temperature was between 40 and 200 °C. This might be a result of a loss in moisture content [64]. The second weight loss of about 32% was observed at a temperature of around 400 °C. This could be related to decomposition of the biopolymer. A further increase in temperature resulted in more weight loss in the bioflocculant. From the obtained results, it can be deduced that the bioflocculant from *Proteus mirabilis* AB 932526.1 is thermostable.

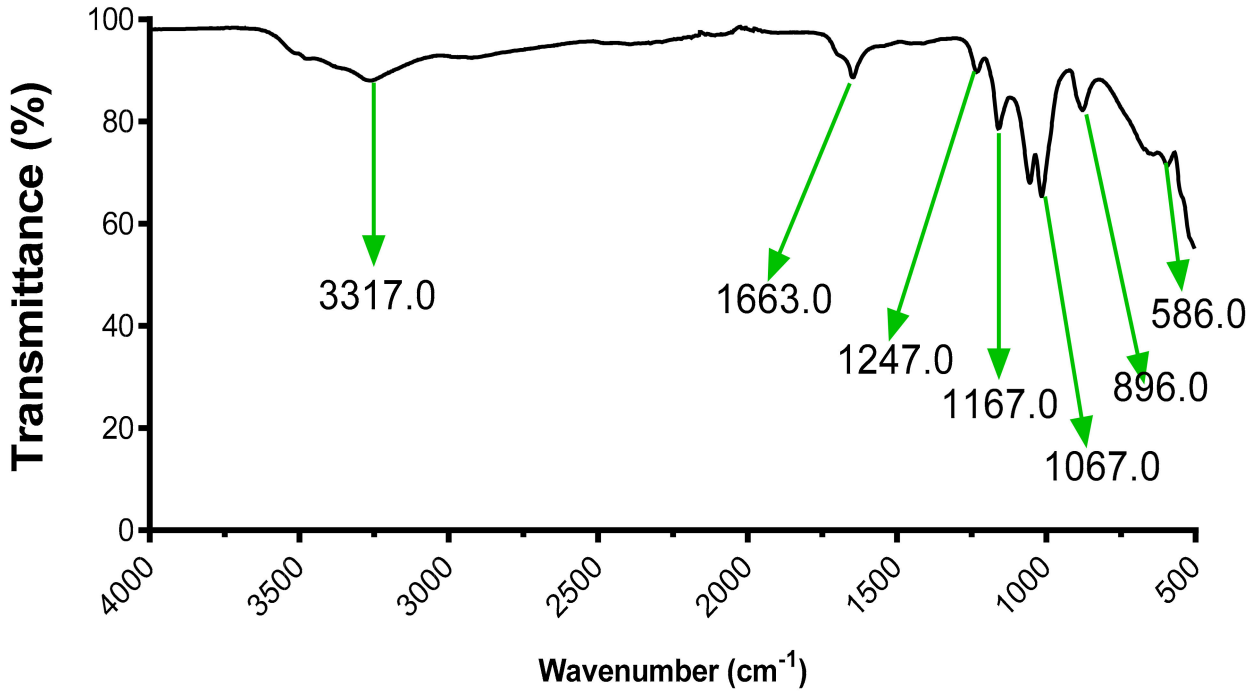

Figure 5. Effect of FTIR of the purified bioflocculant production by *Proteus mirabilis* AB 932526.1.

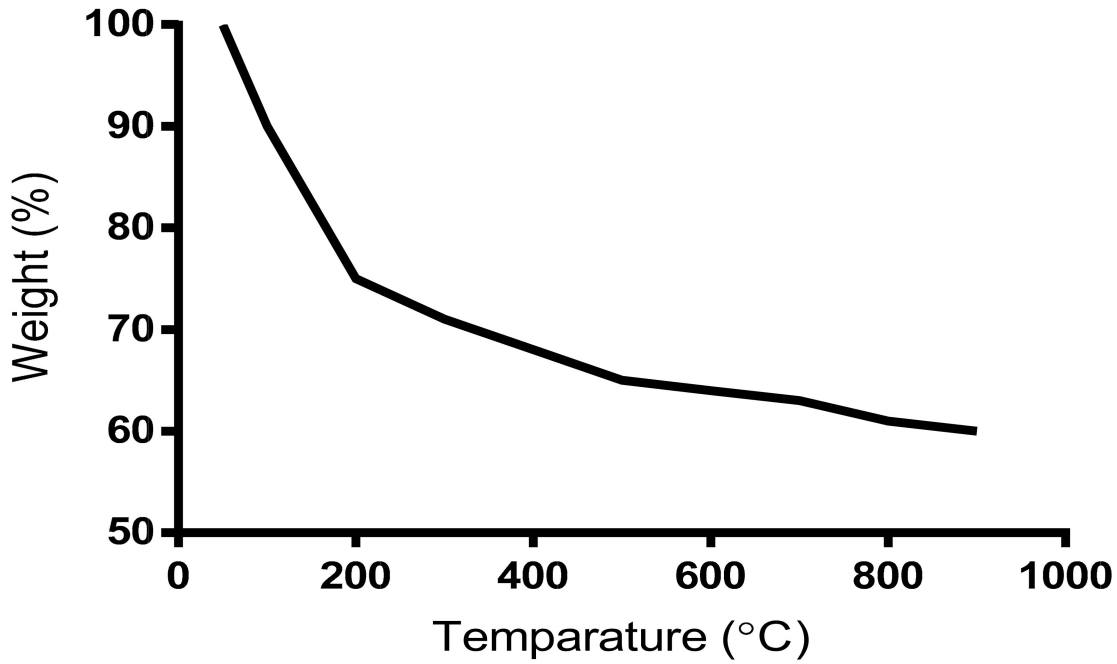

Figure 6. Effect of thermogravimetric (TGA) analysis of the purified bioflocculant production by *Proteus mirabilis* AB 932526.1.

3.7. Elemental Analysis of the Purified Bioflocculant

P. mirabilis AB 932526.1 produced a glycoprotein bioflocculant molecule. Thus, the elemental analysis of the bioflocculant was carried out to assess its structural make-up, and the results are presented in Figure 7. The bioflocculant revealed a composition of various elements in weight by percentage (% w/t). Elements such as O, C and Ca accounted for 47%, 22% and 12%, while elements such as Mg, Na, Mo, N, P and K account for about 19% combined. The presence of these elements in bioflocculant is perceived as having an

impact on its stability and flexibility [75]. The results seemed to be similar to the findings from other studies [16,75].

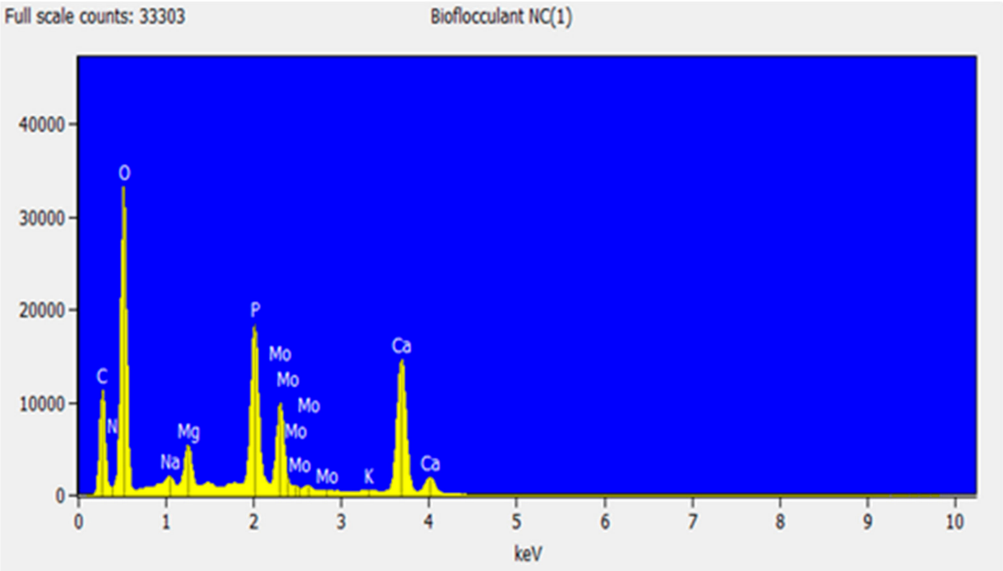

Figure 7. Elementary analysis of a produced bioflocculant.

3.8. SEM Analysis of the Purified Bioflocculant

SEM analysis was carried out to evaluate the surface morphological structure of the bioflocculant with its flocculation of kaolin clay powder. The purified bioflocculant (Figure 8A) seems to be amorphous in structure. A fine scattered uniform-size kaolin clay particle which is white in colour is observed in Figure 8B. In Figure 8C, the formation of big flocs is formed as the bioflocculant and the kaolin clay aggregated. This makes it easy to separate liquid and solids in water during the flocculation process. Okaiyeto et al. [73] and Tlou [74] observed similar results when aggregation of bioflocculants and kaolin clay were investigated.

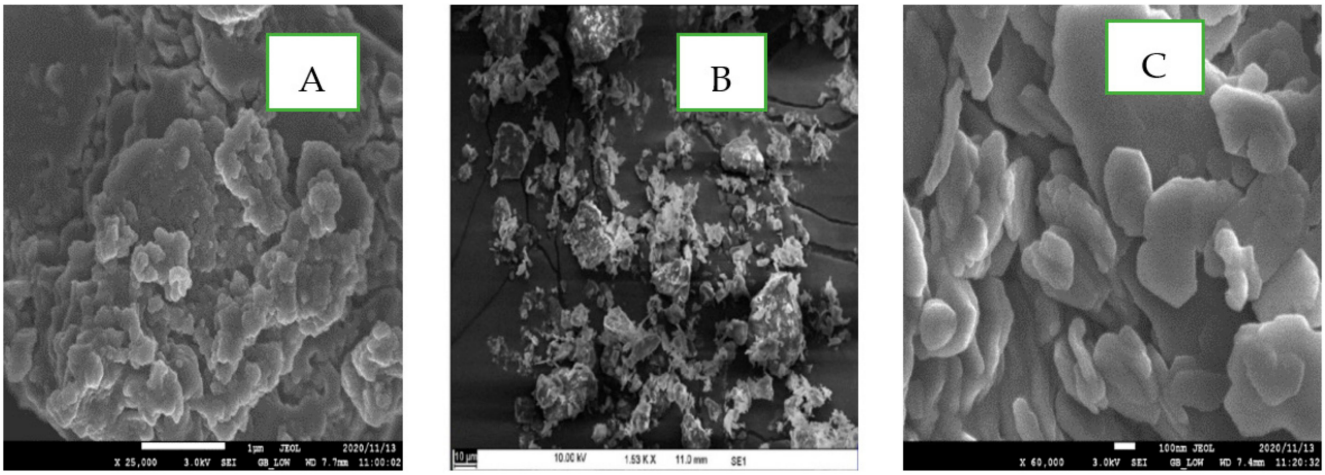

Figure 8. SEM images of a purified bioflocculant (**A**), kaolin clay (**B**) and kaolin clay suspension flocculated with a bioflocculant (**C**).

3.9. Conditions for Flocculating Activity of the Purified Bioflocculant

3.9.1. Effect of Dosage Concentration on the Purified Bioflocculant

Dosage concentration is important in the bioflocculant for effective flocculation process [31]. It is widely recognized that a lower dosage of bioflocculants with high performance in flocculating activity contributes towards cost effectiveness. The effect of different bioflocculant concentrations within a range of 0.2–1.0 mg/mL was evaluated (Table 5). The optimum dosage size of the purified bioflocculant from *Proteus mirabilis* AB 932526.1 was 0.4 mg/mL. Above and below 0.4 mg/mL, the bioflocculant concentrations resulted in a decrease in flocculating activity. The dosage concentration (0.4 mg/mL) was lower than that of bioflocculant produced by *Arthrobacter humicola* [53], which was shown to have its highest flocculating activity at optimum dosage input of 0.8 mg/mL. Maliehe et al. [28] and Agunbiade et al. [53] reported 0.4 mg/mL and 0.2 mg/mL as the most preferable concentrations, respectively.

Table 5. Effect of dosage and pH on flocculating activity.

Dosage (mg/mL)	FA (%) ± SD	pH	FA (%) ± SD	FA (%) ± SD	Metal Ions
0.2	57.1 ± 5.78 ^{a}	3	81.1 ± 0.07 ^{a}	Na^{+}	63.3 ± 0.15 ^{a}
0.4	81.9 ±1.96 ^{b}	4	86.4 ± 1.35 ^{b}	Li^{+}	65.3 ± 0.13 ^{a}
0.6	80.8 ± 6.31 ^{b,c}	5	88.9 ± 0.48 ^{c}	K^{+}	64.0 ± 0.21 ^{a}
0.8	65.5 ± 1.75 ^{a}	6	97.4 ± 0.05 ^{d}	Ca^{2+}	70.2 ± 0.11 ^{b}
1.0	49.4 ± 1.62 ^{a,d}	7	93.3 ± 0.32 ^{e}	Ba^{2+}	77.2 ± 0.42 ^{c}
-	-	8	80.2 ± 0.23 ^{a}	Mn^{2+}	98.4 ± 0.13 ^{d}
-	-	9	78.2 ± 0.14 ^{a,f}	Mg^{2+}	98.3 ± 0.14 ^{d}
-	-	10	65.6 ± 1.86 ^{g}	Fe^{3+}	90.2 ± 0.16 ^{e}
-	-	11	60.4 ± 0.58 ^{h}	Control	43.3 ± 0.10 ^{f}
-	-	12	63.7 ± 0.45 ^{g,i}	-	-

FA% indicates flocculating activity, while SD indicates standard deviation. Different letters (a, b, c, d, e, f, g, h and i) indicate statistical significance at ($p < 0.05$).

3.9.2. Effect of pH on the Purified Bioflocculant

The pH of reaction mixtures plays a major role in the flocculating activity of bioflocculants [56]. In Table 5, the effect of pH on flocculating activity of the purified bioflocculant was evaluated by adjusting the pH of kaolin suspension over a range of 3–12, with 1 N NaOH and 1 N HCl prior to an addition of 0.4 mg/mL bioflocculant solution. The bioflocculant had a good flocculating activity at alkaline, neutral and basic pH. The optimal flocculating activity of 97% was observed at pH 6, while at pH 7 and pH 8 the flocculating activity seemed to slightly decrease. Nevertheless, the results indicate that the bioflocculant is stable at a wide pH range. The findings of this study were similar to those of other studies, whereby the optimum flocculating activity of bioflocculants was observed at a wide pH range (3–12) [68,76]. Studies reported in the literature suggest that a pH tolerance of a bioflocculant at a wide range could indicate that the bioflocculant can be applied to purify various wastewaters and other downstream processes without having to adjust the pH of the wastewater, consequently lowering the cost of the flocculation process [1].

3.9.3. The Effective Use of Metal Ions on the Purified Bioflocculant

The use of metal ions can improve flocculation through neutralisation and stabilisation of the residual negative charges of the functional groups of the bioflocculants [77]. In this study, monovalent, divalent and trivalent metal ions were used to evaluate the effect of cations on the flocculating activity of the bioflocculant by *P. mirabilis* AB 932526.1. All the tested cations enhanced the flocculating activity, with divalent being the most effective. The flocculating activity of 98% was obtained when both Mg^{2+} and Mn^{2+} were utilised (Table 5). These were followed by trivalent cation (Fe^{2+}), with 90% of flocculating activity. All other tested cations resulted in flocculating activities above 60%, except for control

(non-cation), with 41% flocculating activity. Divalent and trivalent cations are known to aid in the flocculation process due to more surface areas for absorption [24]. The same results were reported by Nwodo et al. [5], whereby divalent and trivalent cations greatly enhanced flocculating activity of the bioflocculant.

3.9.4. Effect of Heat on the Purified Bioflocculant

The effect of various temperatures ranging from 50 to 100 °C and 121 °C on the purified bioflocculant was evaluated, and the results are depicted in Figure 9. It was observed that the bioflocculant was thermostable at 50 °C, with the highest flocculating activity of 71%. However, the flocculating activities significantly decreased thereafter. The decrease in flocculating activity may perhaps be due to denaturation of the protein content of the bioflocculant. The thermal stability of a bioflocculant is due to the presence of uronic acid and hydroxyl group and the formation of a hydrogen bond in the structure of the bioflocculant [22]. Hence, this finding shows that the main backbone of the purified bioflocculant was glycoprotein. These observations were in line with the reports by Agunbiade et al. [53] and Gomaa [37], whereby bioflocculants flocculate best at low temperature. This could be more useful in terms of its application in water and wastewater treatment in industries [18].

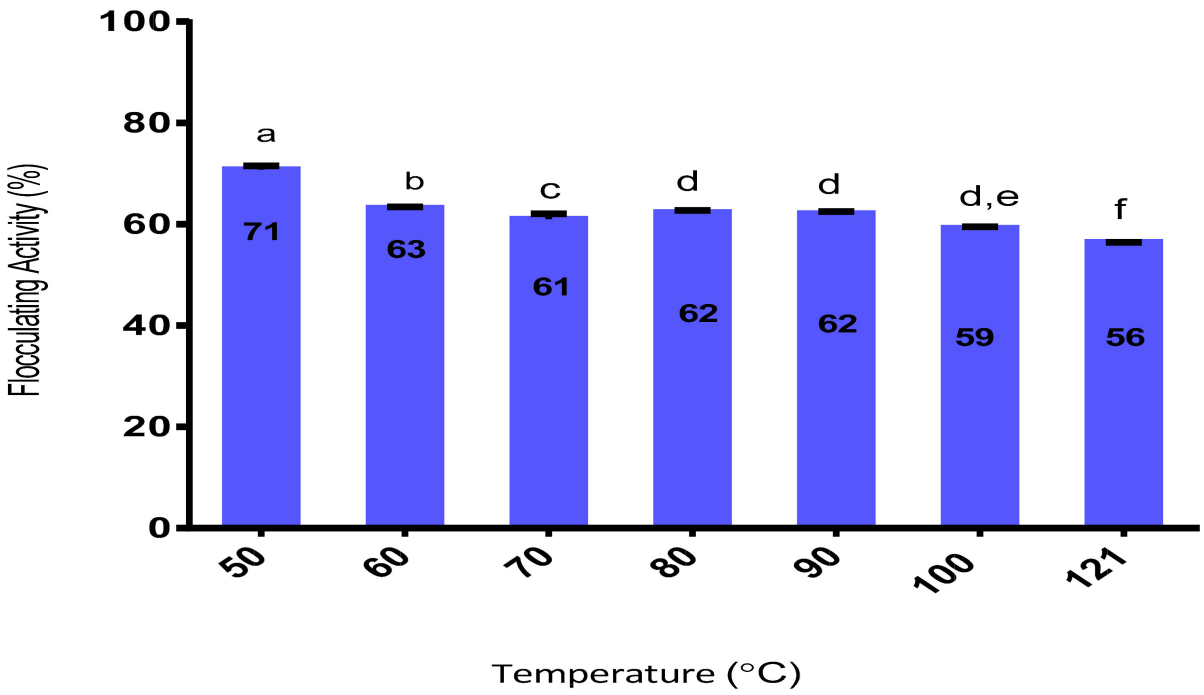

Figure 9. Effect of heat on flocculating activity. Flocculating activity (%) with different letters (a, b, c, d, e and f) are significantly different ($p < 0.05$). Error bars indicate standard deviation.

4. Conclusions

P. mirabilis AB 932526.1 was found to be a promising bioflocculant-producing bacterium. It gave a high bioflocculant yield of 3.8 g/L, which was produced under the optimal growth conditions and medium composition. The bioflocculant was composed of carbohydrates, protein and uronic acid. FTIR analysis revealed the presence of functional groups such as hydroxyl, amine and carboxyl groups. The pyrolysis property of the bioflocculant affirmed its thermal stability. Its morphological structure was revealed to be amorphous. The purified bioflocculant was effective at low concentration (0.4 mg/mL) and within a wide pH range (3–12). For further studies, *P. mirabilis* AB 932526.1 will be used in bacterial consortia in order to further improve bioflocculant yield and activity. Moreover, application of the bioflocculant in wastewater treatment and dye removal will also be considered.

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