



Exploration of biofloculant production of endophytic bacterium: assessment of characteristics and efficiency of its biofloculant in wastewater treatment

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Abstract

Endophytic bacteria are promising sources of potent biofloculants, yet their diversity, biosynthetic potential and utility remain underexplored. This study focused on isolating biofloculant-producing endophytes from *Adansonia digitata*, exploration of biosynthetic gene clusters and application of its biofloculant in wastewater treatment. The standard method and 16S rRNA analysis were used for isolation and identification of endophytic bacteria. The polymerase chain reaction was employed to detect the presence of Type I Polyketide Synthase (PKS-I KS), Type II polyketide synthase and non-ribosomal peptide synthetase gene clusters. The infrared spectrometry, energy-dispersive X-ray and X-ray diffraction analysis were employed for identification of functional units, elements and crystallinity. Its efficiency was evaluated in wastewater treatment using the Jar test. Amongst the 18 isolates, Isolate Gt18, which was identified as *Bacillus albus*, had the highest biofloculant production with flocculating activity of 99%. It revealed the presence of PKS-II. Its biofloculant composed of hydroxyl, amine and carbonyl functional groups. It had carbon (1.48%), oxygen (40.47%), phosphate (17.44%) and potassium (28.58%). It revealed crystalline nature. It reduced chemical oxygen demand and turbidity by 70 and 99.8% for sewage wastewater and 57 and 75% for brewery wastewater, respectively. The biofloculant from *B. albus* illustrated high potential applicability in wastewater treatment.

Keywords *Adansonia digitata* · Biofloculant · *Bacillus albus* · Biosynthetic gene clusters · Characterisation · Removal efficiency

Introduction

Microbial biofloculants are extracellular polymers (EPS) secreted by bacteria, algae and fungi during substrate metabolism, growth phase, cell lysis and degradation of chemical components (Pu et al. 2020). Amongst the identified microbial

floculant producers, bacterial strains are the most common producers, owing to their high metabolic capabilities for producing biofloculants (Chen et al. 2017). Biofloculants are made up of proteins, lipids, glycoproteins, polysaccharides and nucleic acids (Alias et al. 2022). As compared to their chemical counterparts, they are more environmentally benign and innocuous to humans (Okaiyeto et al. 2020). According to

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Yang et al. (2025), although biofloculants have many advantages, their industrial applications in wastewater treatment have been constrained by poor yields and low efficiencies, which often translate into high production costs.

The drawbacks of low biofloculant yield have prompted the exploration of untapped environments in search of high biofloculant-producing bacteria with enhanced biofloculant efficiencies. The rhizosphere is perceived to be a habitat rich in a diversity of microorganisms, including bacteria (Hakim et al. 2021). The bacteria are mostly found on the surface of plants (epiphytes) as well as in the interior of the plant tissues (endophytes) (Costa et al. 2012). Endophytes reside within the host tissue without harming the health and functionality of the host plant (Hardoim et al. 2008). Lodewyckx et al. (2002) and Singh et al. (2022) indicated that bacterial endophytes secrete various types of secondary metabolites, which can be applied in a variety of industries, including environmental contamination control such as water bioremediation (Manganyi et al. 2024). However, research on the use of endophytes in environmental science, particularly in the production of biofloculants, is still limited.

Most studies tackle the challenge of low biofloculant yields mainly through the identification of potent biofloculant producers and optimisation of their culture conditions. Although these strategies do improve biofloculant yields to some extent, biofloculant production is mainly influenced by the expressed flocculation-related biosynthetic gene clusters and genes (Salehizadeh et al. 2018). Based on literature, many non-flocculating extracellular functional genes have been reported. However, there are scant reports on the biosynthesis genes of biofloculant producers (Qi et al. 2019; Fu et al. 2020). The exploration of genes expressed during biofloculant production can ease the engineering of high biofloculant-producing strains, consequently overcoming the uncertainties in reproducing these biopolymers (Fu et al. 2020).

The majority of endophytic bacterial research, according to Etminani and Harighi (2018), focuses on agricultural plants. However, there is a lack of studies on the function, diversity and interactions of endophytic bacteria associated with forest trees and plants, with *Adansonia digitata* not being the exception. Literature has reported on endophytes from *A. digitata* as potential reservoirs of bioactive compounds possessing medicinal attributes (Kumar et al. 2023); however, there is a lack of research, particularly in biofloculant production for water and wastewater treatment. Hence, we isolated an endophytic bacterium with high biofloculant production from the roots of *A. digitata* and explored its biosynthetic genes responsible for biofloculant production.

The characterisation of biofloculants is done through advanced techniques to better understand their physical, chemical, thermal and structural properties (Rajivgandhi et al. 2021). These various techniques include Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy

(SEM) and X-ray diffraction (XRD) (Srinivasan et al. 2023). FTIR is used to determine the functional groups such as carbonyl, hydroxyl, amine and phosphate groups in biofloculants by producing an infrared absorption spectrum (Botirova et al. 2023). SEM is an electron microscope that uses a high-energy beam of electrons to divulge the surface morphology (shape and size) of biofloculants (Kumar et al. 2023; Roy and Mohanty 2020). XRD is a conventional technique employed to determine the lattice structure of a crystalline substance. Moreover, it provides cell dimensions and bond angles of biofloculants (Roy and Mohanty 2020). These methods were used in this study to characterise the produced biofloculant.

Therefore, in this study, we aimed to isolate and identify endophytic bacterium from the roots of *A. digitata*. We further identified the functional flocculation-related biosynthetic gene clusters to better understand the biological processes that underpin biofloculation production. Lastly, we characterised the extracted biofloculant in terms of its physical–chemical properties.

Materials and methods

Sample collection

The roots of *A. digitata* were obtained from the University of Limpopo grounds (23°53'20" S, 29°44'20" E) (Fig. 1). The roots were cut into 10 to 15 cm pieces, then packaged into sterile lockable plastic bags and transported to the microbiology laboratory, where the isolation procedure was performed immediately upon arrival.

Surface sterilisation of the roots

The roots of *A. digitata* were washed thoroughly under running tap water to remove any dirt and debris and then air dried. Afterwards, the surfaces of the roots were disinfected by dipping them in 75% ethanol for 2 min, followed by dipping in 5% sodium hypochlorite solution for another 2 min to remove epiphytes. They were then rinsed six times in sterile deionised water to remove any last traces of the chemical sterilants. To confirm the disinfection process, aliquots of the sterile water used in the final rinse were plated onto the biofloculation production medium (BPM), which was composed of constituents listed in Table 1. The medium was supplemented with 1 mL/L of funginex antifungal agent. Subsequently, the inoculated agar plates were incubated at 30 °C for 7 days and observed for bacterial growth (Nxumalo et al. 2020).

Isolation of endophytic bacteria

Isolation of endophytic bacteria was performed using the method by Khumalo et al. (2025), whereby a sterile mortar



Fig. 1 Map of the sampling location of *A. digitata*

Table 1 Agar medium composition for isolating and screening micro-organisms for bioflocculant production

Ingredients	Amount
Glucose	20.0 g
Yeast extract powder	0.5 g
Urea	0.5 g
(NH ₄) ₂ SO ₄	0.2 g
K ₂ HPO ₄	5.0 g
KH ₂ PO ₄	2.0 g
NaCl	0.1 g
MgSO ₄ ·7H ₂ O	0.2 g
Agar	15 g
Autoclaved distilled water	1 L

and pestle were used to crush the disinfected roots in 50 mL of sterile 0.85% saline solution to release the endophytes. To reduce the microbial concentration, the root extract was serially diluted in a sterile 0.85% saline solution. About 100 µL of each diluted (10^{-1} , 10^{-2} and 10^{-3}) and undiluted sample were pipetted and spread-plated on the antifungal supplemented bioflocculation production media (BPM) agar plates using a sterile glass spreader. The plates were incubated at 30 °C for 7 days. After that, colonies were selected at random based on variations in morphology, size and colour. The selected isolates were purified twice on nutrient agar prior to the screening for bioflocculant production.

Screening for bioflocculant-producing bacteria

Pure bacterial isolates were inoculated into 250 mL conical flasks containing 50 mL sterile BPM broth. The bacteria were then incubated in a shaker (Lasec Being Incubator, BSI-21) at 30 °C for 3 days. Afterwards, the culture broth was centrifuged at 8000 rpm for 15 min to remove the bacterial cells. The cell-free supernatant was used to measure the flocculating activity. A non-flocculating strain, *Escherichia coli* (ATCC 10536), was included as a negative control (Chen et al. 2017).

Determination of bioflocculant activity

To ascertain the flocculating activity (FA) of the bioflocculant from the cell-free supernatant, the technique outlined by Maliehe (2018) was applied. Briefly, 2 mL of the cell-free supernatant was pipetted into 95 mL of the 0.3% kaolin clay suspension and 3 mL of 1% CaCl₂. The mixture was vigorously agitated at 200 rpm for 60 s to reduce repulsion forces between particles and was gently shaken at 40 rpm for 2 min to promote agglomeration. The mixture was then poured into a 100 mL measuring cylinder and allowed to stand for 5 min. The control was done similarly, except that the cell-free supernatant was replaced by 2 mL of deionised water. A UV/Vis spectrophotometer (Jenway, 7205) was used to measure the optical density (OD) of the transparent upper phase layer at 550 nm after 2 mL of the layer had been aseptically extracted. The percentage flocculation activity (%FA) was calculated using the formula below:

$$\%FA = \frac{A_0 - A_1}{A_0} \times 100 \quad (1)$$

whereby A_0 and A_1 are the ODs of the control and sample, respectively. The isolate that gave the highest bioflocculant production, expressed as FA (> 50%) was selected for identification and further studies.

Phenotypic identification and biochemical properties of the selected bioflocculant producers

The chosen bacterial isolate was phenotypically characterised by the Gram staining technique. Bacterial smears were prepared by heat-fixing on glass slides. The slide was placed on a tray and the smears flooded with crystal violet for 1–2 min, then rinsed with water. The smear was further soaked with Gram's iodine for 1 min and rinsed with water. Subsequently, the smear was rinsed with 95% ethanol for 10–15 s and rinsed again with water followed by soaking the smear with counter stain safranin for 1 min and washed thoroughly with water then air dried. The findings were examined with a compound Brightfield microscope (Leica, DM500) while submerged in oil. Furthermore, the biochemical properties (spore, citrate, indole, oxidase, Voges-Proskauer, nitrate reduction, catalase, and fermentation tests) of the selected isolate were determined.

Molecular identification of bioflocculant-producing bacteria

The bioflocculant-producing bacterial isolate was identified by 16S rRNA sequencing, outsourced to Inqaba Biotechnical Industries (Pty) Ltd., South Africa. Genomic DNA was extracted using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, D6005). The DNA purity and concentration were determined using a Thermo Scientific nanodrop OneC, Microvolume UV/Vis spectrophotometer. PCR amplification of the 16S rRNA gene was performed using universal primers 27F (5'-AGAGTTTGATCMTGG CTCAG-3') and 1492R (5'-CGGTTACCTTGTTCAGAC TT-3'), with NEB One Taq 2X Master Mix. The thermal cycling conditions included initial denaturation at 94 °C for 5 min, followed by 35 cycles (94 °C for 30 s, 50 °C for 30 s, 68 °C for 1 min), and a final extension at 68 °C for 10 min. PCR products were visualised on a 1% agarose gel

stained with EZ-vision® dye, and NEB Fast Ladder (N3238) was used as a size marker. Amplicons were purified (Zymo, D4050) and sequenced bidirectionally (Nimagen, BRD3-100/1000) using an ABI3730xl Genetic Analyzer. Chromatograms were viewed with Finch TV, and sequences assembled using CLC Bio Main Workbench. Identification was done via BLASTn on NCBI, with ≥ 99% query coverage and identity (Chigede et al. 2024).

Biosynthetic gene clusters responsible for bioflocculant production

The extracted DNA was also used to identify the biosynthetic gene clusters responsible for bioflocculant production. Briefly, a volume of 25 µL, consisting of 12.5 µL of NEB OneTaq 2X Master mix with a standard buffer, 2 × 1 µL of forward and reverse primers, 2 µL of the DNA sample (10–50 ng/mL) and 8.5 µL of nuclease-free water, was prepared. The following primers were used to amplify the extracted DNA: KSF (5'-GTSCCSGTSSCRTGSSHY-TCSA-3') and KSR (5'-TSGCSTGCTTGGAYGCSATC-3') targeting PKS-I KS and methyl malonyl transferase domains; KsaF (5'-TSGCSTGCTTGGAYGCSATC-3') and KsaR (5'-TGGAANCCGCCGAABCCGCT-3') targeting PKS-II KSa genes; A3F (5'-GCSTACSYSATSTACACSTCSGG-3') and A7R (5'-SASGTCVCCSGTSCGGTAS-3'), targeting NRPS A domain sequences. The DNA amplification was done using the thermocycler following the PCR protocol conditions outlined in Table 2. The PCR products were visualised on gel electrophoresis.

Bioflocculant extraction

The bioflocculant extraction and purification was done as described by Tsilo et al. (2022). The isolate was cultured in 1 L of optimised medium, and the broth was centrifuged (8000 rpm, 4 °C, 30 min) to obtain a cell-free supernatant. Equal volume of sterile water was added, re-centrifuged, then two volumes of ice-cold ethanol were added (2:1, v/v) and precipitated at 4 °C for 12 h. The crude bioflocculant was vacuum dried, re-dissolved in 100 mL sterile water, then mixed with butanol:chloroform (5:2, v/v). After standing 12 h at room temperature, the supernatant was discarded, and the purified precipitate was vacuum dried. Yield was expressed in g/L.

Table 2 PCR conditions for amplification of the PKS-I, PKS-II and NRPS genes

Primers	Initial denaturation	Cycles	Initial denaturation	Annealing	Extension	Final extension	Hold
KSF/KSR	94 °C, 4 min	32	94 °C, 1 min	56.9 °C, 30 s	72 °C, 1 min and 30 s	72 °C, 10 min	4 °C
KSAF/KSAR	94 °C, 4 min	32	94 °C, 1 min	61.1 °C, 30 s	72 °C, 1 min and 30 s	72 °C, 10 min	4 °C
A3F/A7R	94 °C, 4 min	35	94 °C, 1 min	56.9 °C, 30 s	72 °C, 1 min and 30 s	72 °C, 10 min	4 °C

Biofloculant characterisation

Total carbohydrate and protein contents

The total sugar content was determined by the phenol-sulfuric acid method using glucose as a standard (DuBois et al. 1956), with absorbance measured at 490 nm. Protein content was assessed using the BCA kit (Sigma-Aldrich, South Africa) following the manufacturer's instructions, with bovine serum albumin (BSA) as the standard and absorbance read at 562 nm using a Jenway 7205 UV/Vis spectrophotometer.

Surface morphology and elemental composition of the biofloculant

A scanning electron microscope (SEM-Sipma-VP03-67, Zeiss) equipped with an elemental analyser was used to examine the surface morphology and elemental composition of the biofloculants. Samples were rinsed with phosphate buffer saline (0.01 M, pH 7.4), fixed overnight in 3% glutaraldehyde, followed by osmium tetroxide. After dehydration in graded ethanol (20–100%), samples were CO₂-dried. Approximately 0.5 mg of each was mounted on a silicon-coated slide using a spin coater (1000 rpm, 1 min) before SEM imaging and elemental analysis (Li et al. 2022; Khadhraoui et al. 2019).

Analysis of functional groups of the biofloculant

The functional groups of the biofloculant were identified using Fourier transform infrared (FTIR) spectroscopy (Perkin Elmer System 2000, Cambridge, England) to evaluate the active groups responsible for bioflocculation. The pure biofloculant was ground to fine powder and placed on the sample holder and the sample spectrum collected. The spectra were recorded with a resolution of 4 cm⁻¹ which was conducted over a wavenumber range of 4000 to 350 cm⁻¹ (Hua et al. 2021). The functional groups were then assigned to the absorption bands using a spectral library by Bruker optics.

Crystallinity of the biofloculant

The crystallinity of the biofloculants was analysed using powder X-ray diffraction (XRD). The patterns were recorded with a Bruker D8 Advance diffractometer, which was equipped with Cu-K α radiation ($\lambda = 1.5406 \text{ \AA}$) and operated at 40 kV and 40 mA at room temperature. The biofloculant sample was ground and placed in an aluminium holder and placed into the machine sample assembly and scanned between 5 to 90° with a scan rate of 0.03°/s and a step size

of 0.00657°. The Bruker diffraction Eva program was used to evaluate and process the XRD scan (Muthulakshmi et al. 2023).

Pyrolysis property of the biofloculant

The thermogravimetric analysis (TGA) of the biofloculants was carried out with TGA instrument (Model: DTG-60; Shimadzu, Japan) to determine the degradation behaviour of the biofloculant during pyrolysis (Okaiyeto et al. 2016). The study was performed in an inert atmosphere (nitrogen) from 25 to 800 °C. The heating rate was uniform at 5 °C/min. The dry, powdered biofloculant samples were placed in the TGA, and the weight loss as a function of temperature was recorded. Thereafter, the pyrolysis parameters (onset temperature, peak temperature and residual weight) were calculated.

Application of the biofloculants in wastewater treatment

The biofloculant was used to treat brewery and sewage wastewater using the previously optimised operational conditions pH 10, (CaCl₂ (2%), settling time (10 min) and biofloculant dosage size (0.5 mg/mL)) (unpublished). The pH of both wastewaters was adjusted to pH 10 using 0.1 M NaOH and 0.1 M HCl. The aluminium sulphate and polyaluminium chloride were utilised as positive controls. The removal efficiencies of the flocculants on chemical oxygen demand (COD) and turbidity were measured by Spectroquant COD test kit and turbidimeter Hanna HI801-01. The removal efficiency (RE) was arithmetically calculated and expressed as percentage RE (%RE) using Eq. 2:

$$\%RE = (W_o - W) / W_o \times 100 \quad (2)$$

whereby W_o and W represented the parameter values obtained before and after treatment, respectively (Dlamini et al. 2019).

Data analysis

All experiments were conducted in triplicate, and the results were presented as mean values with their corresponding standard deviations. Statistical analysis was performed using one-way analysis of variance (ANOVA) with GraphPad Prism™ 9, considering a p value less than 0.05 as the threshold for statistical significance. Tukey's honest significant difference test was applied to compare group means for significant differences.

Results

Isolation of bioflocculant producer

Eighteen bacterial colonies were isolated from *A. digitata* roots. Amongst the 18 isolates, Gt18 had the highest flocculating activity of 99%. Therefore, the isolate Gt18 was selected and utilised for further experiments due to its consistent and robust flocculation performance across repeated tests.

Biochemical characteristics of Isolate Gt18

The selected bacterial isolate had a smooth, round, and cream-white appearance as shown in Fig. 2A. The bacterial strain was rod-shaped and Gram-positive, as shown in Fig. 2B.

Biochemical tests were further conducted on the selected bacterial strain, and the results are presented in Table 3. The fermentation studies displayed that the chosen bacterial strain was able to ferment glucose, lactose, and maltose as carbon sources.

Molecular identification of of isolate Gt18

The 16S rRNA gene sequencing was used to confirm the identity of the selected isolate. Based on the BLAST results, the selected bacterial isolate was shown to have 99% similarity to *Bacillus albus* with accession number PX048744.

Cluster genes responsible for bioflocculant production

The presence of the bioflocculant-producing genes was detected using appropriate primers targeting PKS-I KS, methyl malonyl transferase domains, PKS-II KSa genes and NRPS A domain sequences, and the results are shown in

Table 3 Biochemical characterisation of the bioflocculant-producing endophytic bacterium isolated from *A. digitata* roots

Biochemical test	Isolate Gt18
Spore test	+
Citrate	+
Indole	–
Oxidase	–
Voges-Proskauer	+
Nitrate reduction	+
Catalase	+
Glucose	+
Lactose	+
Mannitol	–
Raffinose	–
Maltose	+
Xylose	–

Key: + : positive, - : negative

Fig. 3. The selected bacterial strain was found to be positive for one biosynthetic gene cluster PKS-II. The presence of this amplified gene was indicated by the two bands observed in lane 2 and 4, as displayed in Fig. 3C. The other two gene clusters were not detected, and only gene dimers were observed (Fig. 3A, B).

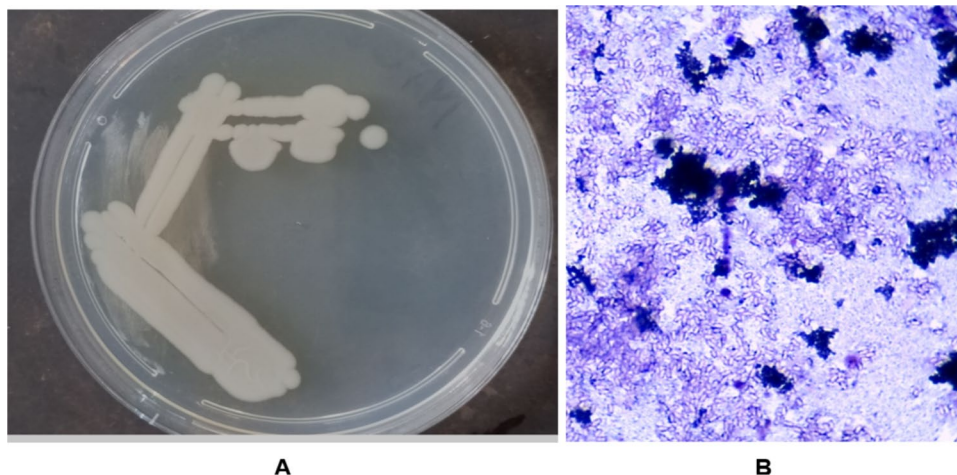
Extraction of the bioflocculant

The bioflocculant was extracted from *B. albus* using ethanol extraction. The extraction process of the crude and purified bioflocculants is illustrated in Fig. 4. Figure 4A, B shows the dried crude and purified bioflocculant, respectively. The yield of the purified bioflocculant was 3.41 g/L.

Chemical composition of the bioflocculants

The chemical composition of the purified bioflocculant was assessed. The chemical analysis revealed that the

Fig. 2 The morphological (A) and Gram stain reaction (B) of the selected highest bioflocculant-producing isolate



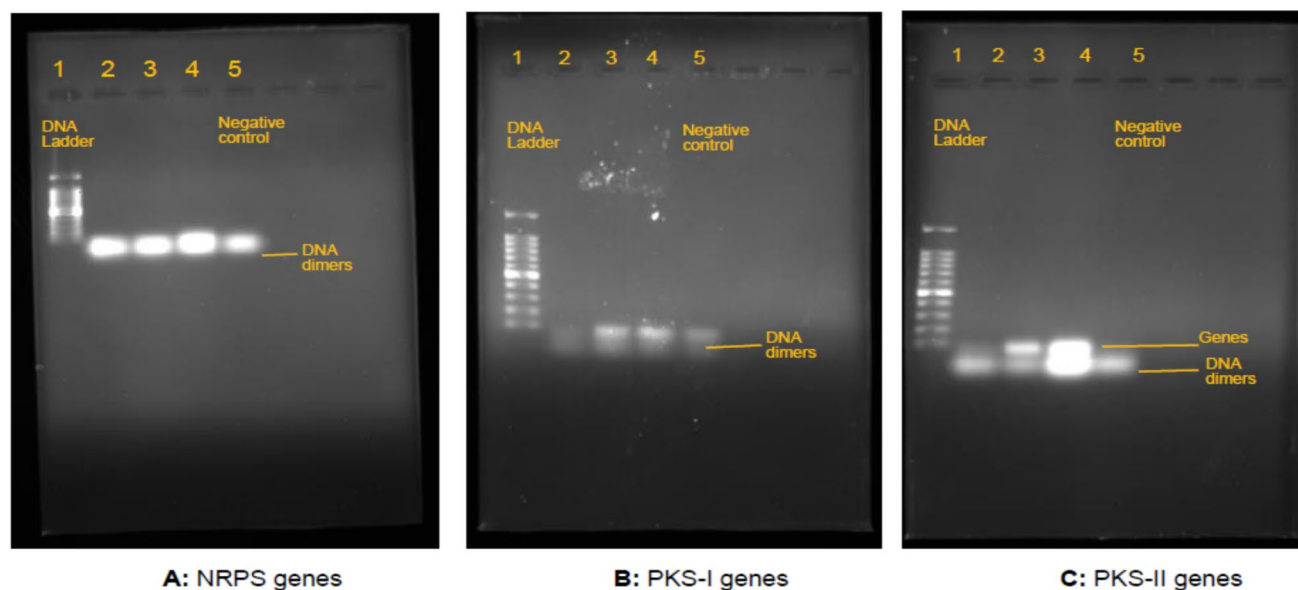
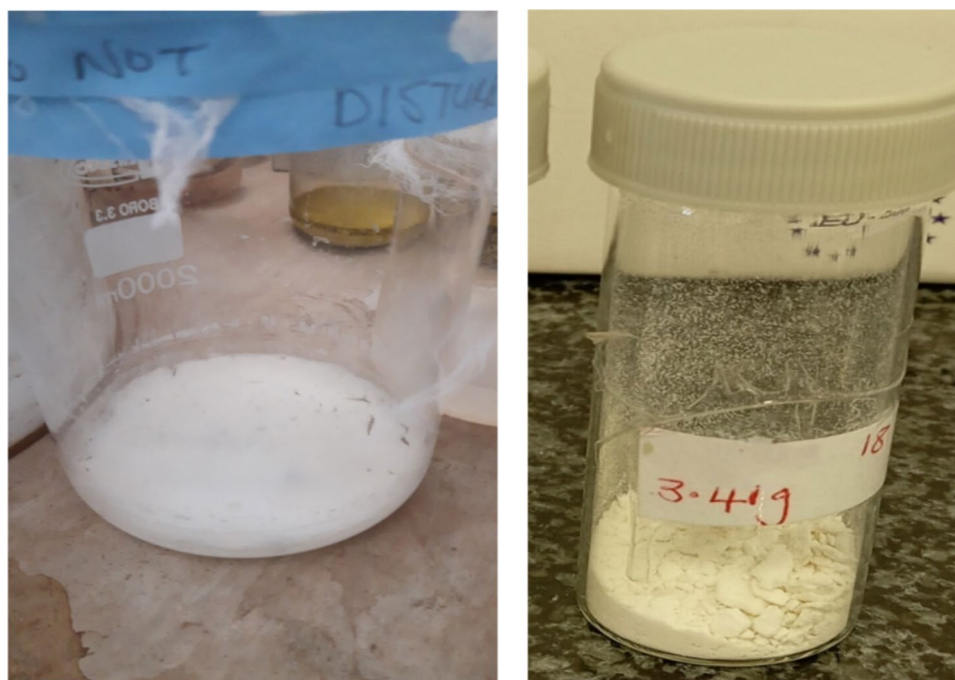


Fig. 3 Gel electrophoresis images of PCR gene products of *B. albus* run in triplicate for each primer pair. Lane 1: 1000 bp DNA ladder; Lane 2–4, PCR products of **A** NRPS gene cluster, **B** PKS-I gene cluster, and **C** PKS-II gene cluster. Lane 5: negative control (without template DNA)

Fig. 4 The crude (**A**) and purified (**B**) bioflocculant



bioflocculant of *B. albus* contained 80% sugar and 6% proteins.

Functional groups of the bioflocculants

The FTIR spectra of the bioflocculants produced by *B. albus* are displayed in Fig. 5. The broad stretching absorption peak at around 3400 cm^{-1} indicates the presence of

hydroxyl and amino groups. The small peak observed around 2300 cm^{-1} suggested the presence of aliphatic C-H stretch. A distinctive peak at 1650 cm^{-1} corresponds to the carbonyl group, which may arise from carboxylic acids or amide linkages. Additionally, the weak absorption peaks observed around 1200 cm^{-1} confirmed the presence of aromatic and aliphatic amide groups, respectively. The peak at 1020 cm^{-1} indicated the C–N stretch.

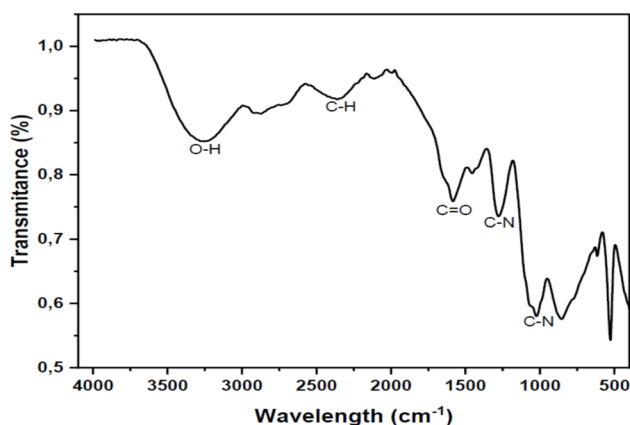


Fig. 5 FTIR spectrum of bioflocculant derived from *B. albus*

Morphological analysis of the bioflocculant

The surface morphology structure of the purified bioflocculant is presented in Fig. 6. The SEM images indicated that the purified bioflocculant of *B. albus* consisted of square-like shapes (Fig. 6A) made of plate-like rods of regular pattern (Fig. 6B).

Elemental composition of the bioflocculant

The EDX analysis of the bioflocculant of *B. albus* is presented in Fig. 7. The EDX analysis revealed the elemental composition in mass proportion (% w/w). The peaks indicate the presence of elements and their intensities (height) relate to their abundance. The elemental spectrum shows the absorption peaks at a mass fraction of C:O:P:K detected in 1.48:40.47:17.44:28.58 (% w/w), respectively.

Crystallinity of the bioflocculants

The XRD analysis of the purified bioflocculant is presented in Fig. 8. The three prominent diffraction peaks at $2\theta = 24^\circ$, 31° , and 47° , corresponding to (111), (200),

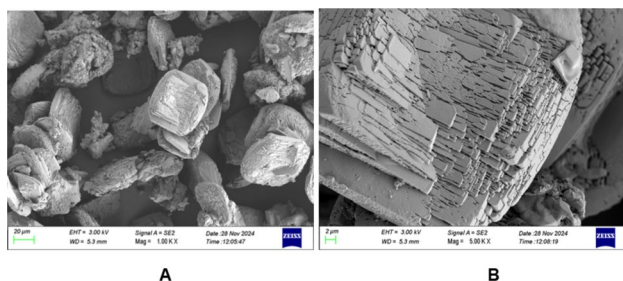


Fig. 6 SEM images for the purified bioflocculant of *B. albus*

and (220) crystal planes, respectively, suggested the presence of a crystalline lattice within the bioflocculant. Overall, the crystallinity index (CI) was estimated to be about 83.33%, signifying a crystalline nature bioflocculant.

Pyrolysis profile of the bioflocculant

The pyrolysis profile of the bioflocculant is shown in Fig. 9. The thermograph displays different stages of weight loss. The bioflocculant exhibited an initial weight loss of 2% at temperatures between 30 and 180 °C. The first significant weight loss of 24% occurred at a temperature between 180 and 250 °C. The second gradual decomposition phase was observed at 250–600 °C, accounting for 14% mass loss. The final steady decline in weight loss was observed between 600 and 998 °C, resulting in about 50% final residue.

Efficiency of bioflocculant in wastewater treatment

The removal efficiencies of the flocculants on COD and turbidity were assessed, and the results are displayed in Table 4. The bioflocculant performed significantly better in COD removal (p value < 0.05) in comparison to aluminium sulphate and polyaluminium chloride in both wastewaters. It had the highest COD removal efficiencies of 70 and 57% on sewage and brewery wastewater, respectively. Moreover, it performed relatively the same (p value > 0.05) with the other tested flocculants on turbidity reduction of sewage water, giving 99.8% activity. However, it surpassed aluminium sulphate on turbidity reduction on brewery wastewater and illustrated the efficiency of 75%.

Discussion

In this study, out of 18 bacterial endophytic isolates, Isolate Gt18 illustrated the highest bioflocculant production, confirming *A. digitata* as a reservoir of potent bioflocculant producers. This aligned with the study by Saha et al. (2024), who reported endophytic microbial bioflocculant from *Globba marantina*, further underscoring plants as potent sources of endophytic bioflocculant producers.

Isolate Gt18 was identified as the member of the *Bacillus* species, namely *Bacillus albus*. Thus, *B. albus* belongs to the phylum Firmicutes, which primarily consists of Gram-positive, rod-shaped, spore-forming and facultatively anaerobic microorganisms (Abu Tawila et al. 2018). The genus *Bacillus* consists of a diversity of bacterial species that have shown high capabilities of secreting effective bioflocculants (El-Rouby et al. 2015). For instance, *Bacillus clausii* NB2 (Bukola and Gboyega 2014), *Bacillus subtilis* ZHX3 (Xia et al. 2022) and *Bacillus pumilus*

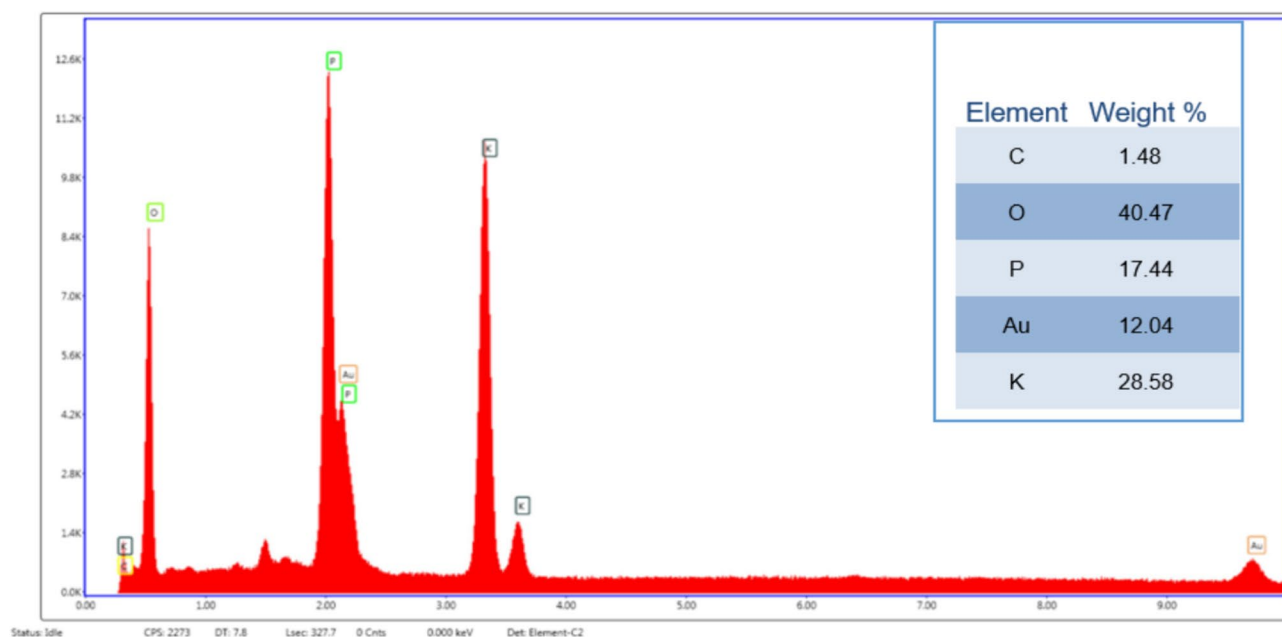


Fig. 7 Elemental analysis for biofloculant of *B. albus*

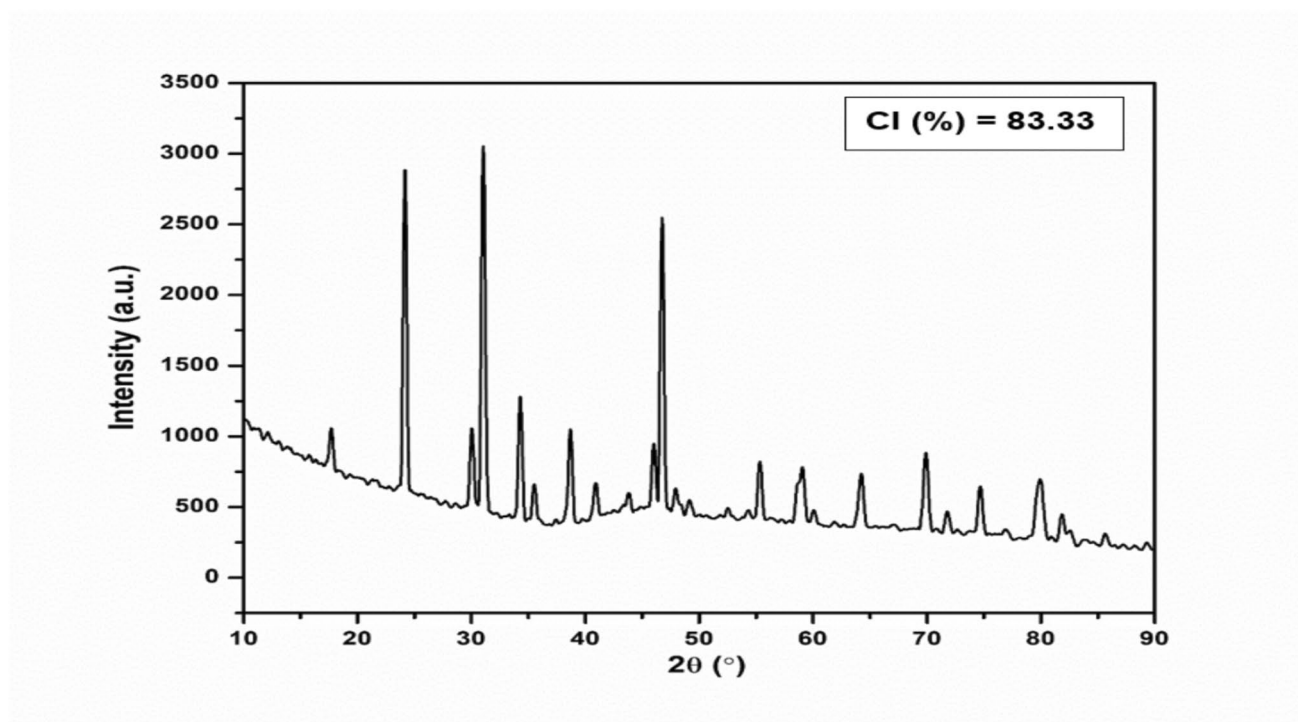


Fig. 8 X-ray diffraction patterns of the purified biofloculant of *B. albus*

(Maliehe et al. 2020), *Bacillus cereus* S55 (Harinisri et al. 2024), *Bacillus licheniformis* (Ramadhani et al. 2022) and *Bacillus megaterium* (Pu et al. 2020) have been reported to

be potent biofloculant producers. Nevertheless, according to our knowledge, *B. albus* has not been reported to produce biofloculant.

Fig. 9 The TGA spectrum of the purified bioflocculant of *B. albus*

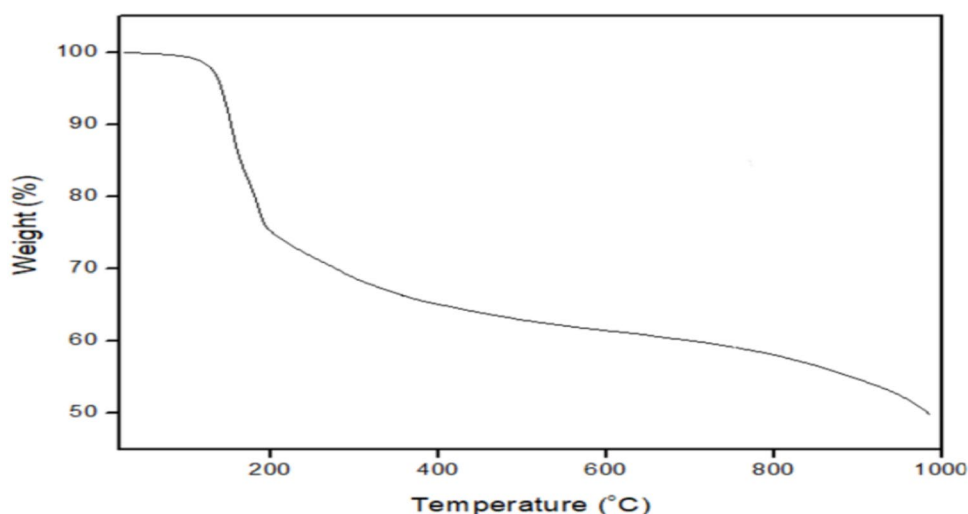


Table 4 The removal efficiency of the bioflocculant from *B. albus* in comparison to chemical flocculants

Flocculant	RE $\pm$ SD (%)			
	Sewage wastewater		Brewery wastewater	
	COD	Turbidity	COD	Turbidity
Bioflocculant	70 $\pm$ 0.21 ^a	99.8 $\pm$ 0.09 ^a	57 $\pm$ 0.38 ^a	75 $\pm$ 0.08 ^a
Aluminium sulphate	36 $\pm$ 0.06 ^b	97.8 $\pm$ 0.11 ^a	38 $\pm$ 0.07 ^b	47 $\pm$ 0.13 ^b
Polyaluminium chloride	47 $\pm$ 0.33 ^b	99.1 $\pm$ 0.05 ^a	38 $\pm$ 0.41 ^b	72 $\pm$ 0.04 ^a

The superscripts signify statistical significance at $p < 0.05$ where values sharing the same letter are not significantly different ($p > 0.05$)

The biosynthesis of microbial flocculants is a complex process that involves multistage with many biosynthetic genes and enzymes such as NRPSs and PKSs. Therefore, in this study, the gene clusters responsible for the synthesis of bioflocculants were investigated to understand the mechanisms behind bioflocculant production. *B. albus* was found to be positive for the gene cluster PKS-II, clustered on a 10 kb DNA segment. The PKS-II gene cluster mediates the biosynthesis of polyketide secondary metabolites such as bioflocculants by combinatorial functioning of various domains (Selvin et al. 2016; McBride et al. 2023). Therefore, its detection in this study implied that this cluster of genes might have been expressed during bioflocculant production by *B. albus*. However, further confirmatory studies are needed to support this claim. To our surprise, PKS-I and NRPS gene clusters were not detected, implying that they were not responsible for bioflocculant production by *B. albus*.

Bioflocculant productivity and yields are good qualitative indicators of how bacterial cells effectively convert culture medium components into bioflocculants (Maliehe 2018). In this study, *B. albus* yielded 3.41 g/L after 72 h of

fermentation. This was considered a good yield as most bioflocculants produced by bacterial strains are often less than 2 g/L. For instance, the *B. albus*' bioflocculant yield was significantly higher than the 1.15 g/L obtained from *Bacillus firmus* (Salehizadeh and Shojaosadati 2002), 1.36 g/L yield derived from *Proteus mirabilis* (Arafa et al. 2014) and 2.0 g/L derived from *Bacillus velezensis* (Hassimi et al. 2020). The high yield might be attributed to *B. albus*' ability to optimally survive in the set culture conditions and the expressed flocculation-related gene cluster as well as the polarity of the solvents that were used during extraction and purification processes.

The chemical composition revealed the bioflocculant to have a high content of total carbohydrates and low total protein content, suggesting it to be a glycoprotein biopolymer (Cherian et al. 2025). The carbohydrates are primarily composed of polysaccharides, which play a significant role in the flocculating activity of the bioflocculant due to their sticky nature and bridging mechanism which enhances effective particle aggregation (Wang et al. 2024). Furthermore, the high carbohydrate content suggested that the bioflocculant is a polysaccharide-rich biopolymer and is presumed to be the most responsible component for the high flocculating activity. Furthermore, the high carbohydrate content might attribute to the high thermal stability of this bioflocculant (Agunbiade et al. 2017). The proteins likely contribute to the structural stability and the interactions of the functional groups during the flocculation process. The other 14% may consist of other constituents such as uronic acids, nucleic acids, lipids and inorganic ions, which may assist in charge neutralisation, metal ion binding and enhancing flocculation efficiency (Mohammed and Dagang 2019). The findings aligned with the studies by Ntsangani et al. (2017) and Agunbiade et al. (2022), who both reported the bioflocculants obtained from *Bacillus* sp. AEMREG4 and *Bacillus velezensis* to mainly compose of carbohydrates.

The various functional groups found on molecular chains of biofloculants are responsible for the flocculation performance, especially in binding and aggregation of the suspended particles (Kurniawan et al. 2022). The observed functional groups (hydroxyl (-OH), carbonyl (C=O) and amino groups (C-N)) were presumed to have contributed to the flocculating activity and enhanced the stability of the biofloculant from *B. albus*. The presence of carbonyl and C-N stretch indicated the occurrence of polysaccharide backbone (glycosidic linkage). Moreover, the amide and amino groups affirmed the presence of proteinaceous nature of the biofloculant. Thus, the observed functional groups suggested the biofloculant to be a glycoprotein (composing of polysaccharides and proteins) molecule (Maliehe 2018). The findings were similar to those of Wu et al. (2023) and Joshi et al. (2019), who found hydroxyl and amino groups from biofloculants of *Bacillus thuringiensis* and *Bacillus licheniformis* NJ3, further confirming these groups as crucial in the bioflocculation process. Dai et al. (2024) also reported the presence of carbonyl groups which enhanced the adsorption properties of *Bacillus subtilis*' biofloculant in dye removal.

The surface morphology plays a significant role in the effectiveness or poor flocculating activities of the biofloculant (Li et al. 2021). The plate-like shape of the biofloculant from *B. albus* provided a broad surface area for the adsorption of suspended particles. These characteristics influenced its ability to bridge particles, stabilise flocs and ultimately improve the efficiency of the flocculation process (Srivastava et al. 2018). A similar morphology was observed in a biofloculant produced by *Bacillus velezensis* (Binmad et al. 2022).

The elemental composition of the biofloculants determines their flexibility or stability (Selepe and Maliehe 2024). Carbon was detected at a low energy range; however, its presence confirmed the organic nature of the biofloculant. Carbon is a primary element in polysaccharides and proteins, and it is responsible for forming the backbone of the biopolymeric structure (Su et al. 2021). Its presence implied that the biofloculant consisted of carbon-rich macromolecules such as exopolysaccharides (Atmakuri et al. 2024). For instance, Botirova et al. (2023) reported *Bacillus simplex* from soil as an exopolysaccharide-based biofloculant, which played a role in the biofloculant's stability and flocculation efficiency. The high presence of oxygen suggested a high content of oxygenated functional groups, which in this study included the hydroxyl and carbonyl groups, which are preferred for flocculation (Vimala et al. 2020). The finding corroborated the report of Giri et al. (2015), in which the elemental analysis of the biofloculant of *Bacillus subtilis* revealed oxygen in a high weight fraction of 54.7%. The detection of phosphorus implied the presence of phosphate groups, which may originate from nucleic acids within the biofloculant (Kaur et al. 2019). The potassium

peaks observed may be due to the residual ions from the culture medium used during biofloculant production and may not play any significant role in the structure of the biofloculant (Dlamini et al. 2020). Gold was also detected; however, it is not naturally present in biological materials. Its presence may be attributed to the gold coating process used during SEM/EDX sample preparation to enhance conductivity; therefore, it is not considered part of the biofloculant composition.

The XRD analysis of the *B. albus*' biofloculant provided an insight into the degree of crystallinity, molecular arrangement and possible presence of inorganic residues. The XRD pattern showed prominent sharp peaks, indicating that the biofloculant exhibits a crystalline nature. The sharp peaks may be due to the highly ordered polysaccharide chains, mineral residues and proteinaceous crystallites, possibly due to hydrogen bonding amongst functional groups (Song et al. 2021), which play a crucial role in improving mechanical strength and stability (Okaiyeto et al. 2015). This correlated with a study by Harinisri et al. (2024), where *Bacillus cereus* (S55) produced a crystalline nature biofloculant.

The different thermal profiles of the biofloculant were assessed to ascertain its thermal stability. The initial 2% weight loss observed was due to loss of moisture and loosely bound volatile components such as the hydroxyl group (Okaiyeto et al. 2016). Similarly, Mohite et al. (2019) and Artifon et al. (2023) reported the weight loss due to loss of moisture from biofloculants. Additionally, the weight loss at less than 180 °C indicated that the biofloculant can withstand moderate heat before starting to degrade. The first major decomposition of 24% was likely attributed to the thermal degradation of low molecular weight polysaccharides or proteins. This phase represented the decomposition of the biofloculant's less stable organic fractions. The second decomposition phase was attributed to the breakdown of more thermally stable functional groups such as the carboxylic acid, reflecting the degradation of the primary polysaccharide or glycoprotein backbone. After 998 °C, about 50% of the biofloculant was obtained, confirming the moderate thermal stability of the biofloculant, which was greatly attributed to the high carbohydrate composition (Sun et al. 2012). However, no true plateau or complete stabilisation was observed.

The removal efficiency of biofloculant in comparison to used conventional chemical flocculants (aluminium sulphate and polyaluminium chloride) was assessed. The comparatively higher COD and turbidity removal efficiencies demonstrated by the biofloculant in comparison to the tested conventional flocculants implied that the biofloculant from *B. albus* has potential to be used interchangeably with the conventional flocculants in wastewater treatment. This is the good news as this can mitigate the adverse effect implicated with the predominant utilisation of the chemical flocculant.

The findings agreed with those reported by Mburu et al. (2024) and Agunbiade et al. (2022), where biofloculants performed significantly better than conventional flocculants in reduction of COD and turbidity in wastewater. However, the lower efficiencies shown by conventional flocculants used in this study might be due to the fact that their operational conditions were not previously optimised but only for the biofloculant.

Conclusion

This study confirmed that endophytic bacteria are excellent biofloculant producers. Out of 18 selected isolates, Isolate Gt18, which was identified as *B. albus*, demonstrated the flocculating activity of 99% against kaolin clay solution. The biofloculant production was attributed to the presence of a type II PKS biosynthetic gene cluster. *B. albus* yielded promising biofloculant of 3.41 g/L, which was a glycoprotein with predominant carbohydrate composition. It had a plate-like structure and was composed of hydroxyl, amine, and carbonyl groups as the main functional groups. The biofloculant was found to have a moderate heat-stable and semicrystalline profile. The biofloculant illustrated high reduction efficiencies on COD and turbidity of the tested sewage and brewery wastewater. For future studies, the mechanism of action of the biofloculant from *B. albus* in the wastewater treatment process ought to be assessed.

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Author contribution The study was conceived and designed by K.M., T.S.M., and T.N.S. The experiments were conducted by M.G.R. and N.I.M. C.T.S., K.M., T.S.M., and T.N.S. contributed to data analysis. M.G.R. wrote the original draft. K.M. and T.N.S. reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Data availability The bacterium identified in this study is available from GenBank [<https://submit.ncbi.nlm.nih.gov/subs/?search=SUB15505987>]. Its accession number is provided in this manuscript. The other datasets used during this study are available from the corresponding author on request.

Declarations

Ethical approval This article does not contain any studies with animals or human participants performed by any of the authors.

Consent for publication All authors consent to this manuscript being published.

Competing interests The authors declare no competing interests.

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