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Production and characterization of a bioflocculant produced by microorganisms isolated from earthen pond sludge

Oluwafemi Adebayo Oyewole [a,b,*], Aliyu Jagaba [a], Abdullahi Afolabi Abdulhammed [a], Japhet Gaius Yakubu [a], Asmau Mohammed Maude [a], Olabisi Peter Abioye [a], Olalekan David Adeniyi [c], Evans Chidi Egwim [b,d]

[a] Department of Microbiology, Federal University of Technology, Minna, Nigeria
[b] African Center for Excellence for Mycotoxin and Food Safety, Federal University of Technology Minna, Nigeria
[c] Department of Chemical Engineering, Federal University of Technology, Minna, Nigeria
[d] Department of Biochemistry, Federal University of Technology, Minna, Nigeria

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ABSTRACT

Bioflocculants are biodegradable polymers produced by microorganisms. The aim of this study was to produce and characterize a bioflocculant by microorganisms isolated from earthen pond sludge. Sludge from the earthen pond was collected from 'Lapai Gwari' in Minna, Nigeria. The microorganisms were screened for the potential of producing bioflocculant using kaolin suspension and a selective medium. The isolate with the highest flocculating potential was identified based on its molecular characteristics. The bioflocculant produced was characterized using thermogravimetric analysis (TGA), Fourier-transform infrared (FTIR) spectroscopy and scanning electron microscopy (SEM). Four bacteria and three fungi were isolated from the sludge. *Priestia megaterium* (ON184360) was identified as the isolate with the highest flocculant production potential, with 10.67 g of bioflocculant produced from 500 mL of medium, which was confirmed by TGA, FTIR and SEM results. The findings of this study showed that *Priestia megaterium* is a more efficient alternative for flocculant production.

1. Introduction

Bioflocculation is a process during which flocculants mediation is achieved owing to microbial presence or the macromolecular biodegradable flocculants they release into their immediate environment (Das et al., 2022). Various microorganisms (i.e., actinomycetes, fungi, algae, and bacteria) have been reported to produce bioflocculants. For example, the biomass of *Arthrobacter* sp. (Salehizadeh and Shojaosadati, 2015), *Bacillus* sp. AEMREG7 (Okaiyeto et al., 2015) and *Pichia kudriavzevii* MH545928.1 (Tsilo et al., 2021).

Biopolymers of microbial origin have gained recognition as anti-algal, anti-viral and anti-bacterial agents. Also, they are recognized as inducers of biofilm formation and microbial aggregation. Furthermore, flocculants from microorganisms have been applied in numerous industries for several purposes, for example in the pharmaceutical and food industries, bioflocculants are utilized as stabilizing, emulsifying and viscosifying agents. They are also utilized in water purification and treatment of wastewater (Zhong et al., 2018). Bioflocculants are utilized as biosorbents in the removal of numerous types of metallic pollutants from wastes generated by manufacturing industries (Salehizadeh and Yan, 2014). In beverage primary treatment, biocoagulation and bioflocculation are cost-effective methods that are being utilized (Salehizadeh and Yan, 2014).

Although synthetic flocculants have been demonstrated to be efficient in causing flocculation of colloidal substances in a liquid medium. However, some synthetic flocculants are of concern as they pose a serious risk to the health of humans. For example, Alzheimer's disease has been related to aluminum salts (Liu et al., 2021). In addition, monomeric units can cause serious carcinogenic and neurotoxic effects. Due to the non-degradable nature of acrylamides, their use as flocculants has been banned or limited in several countries (Abu-Tawila et al., 2018). As such, bioflocculants that are efficient, safe and biodegradable with better flocculating activities are attracting the interest of researchers across the globe. In addition, microorganisms utilized in the production of bioflocculants can be characterized and the optimal conditions that will support their growth and metabolism can be determined

* Corresponding author at: Department of Microbiology, Federal University of Technology, Minna, Nigeria.
E-mail address: oa.oyewole@futminna.edu.ng (O.A. Oyewole).

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to enable their use for improved product yield (Tsilo et al., 2021; Agunbiade et al., 2022). Therefore, as the search for improved strains of bioflocculants-producing microorganisms increases, the screening of indigenous microorganisms in Minna, Nigeria may provide an isolate with a more efficient bioflocculants production potential. Thus, leading to affordable, safer, and readily available flocculants with environmental and industrial applications. The aim of this study was to produce and characterize a bioflocculant from microorganisms isolated from earthen pond sludge. The choice for exploring the earthen pond sludge for the presence of bioflocculant-producing microorganisms in this study was because the pond sludge is a pool deposit of naturally occurring organic materials that develop over time. In addition, the earthen pond sludge is rich in clay, which is key to water retention. Besides, kaolin clay has been used extensively to screen for bioflocculants-producing microorganisms by several authors (Harun et al., 2017; Abu-Tawila et al., 2018; Bisht and Lal, 2019; Nkosi et al., 2021; Tsilo et al., 2021).

2. Materials and methods

2.1. Collection of Samples

Sludge from the earthen pond was collected from 'Lapai Gwari' community in Bosso Local Government Area of Minna, Niger State, Nigeria located at longitude 6.5052 °E and latitude 9.5246 °N. The sludge samples were taken to the Microbiology laboratory at the Federal University of Technology, Minna Nigeria for analysis.

Waste oranges were collected from fruit vendors in Minna under aseptic conditions. The samples were stored in a sterile plastic container and immediately transported to the Microbiology laboratory of the Federal University of Technology, Minna. The oranges were washed with tap water, peeled and cut into smithereens using a sterile knife. The juice in the sliced fruits was extracted with the aid of a juice extractor. The fruit juice sample was then stored under refrigeration at 4 °C until required for further analysis.

2.2. Screening of bioflocculating microorganisms using selective medium and kaolin suspension

The sludge samples were serially diluted tenfold. The bacteria with bioflocculating ability were screened using a flocculation selective medium containing NaCl 0.1 g, $MgSO_47H_2O$ 0.2 g, urea 0.5 g, K_2HPO_4 5 g, yeast extract 0.5 g, KH_2PO_4 2 g, glucose 10 g, in 1 L of distilled water and the pH was maintained at 7.0. The medium was sterilized using the autoclave at 121 °C for 15 min, which was allowed to cool to 45 °C before pour plating it with 0.5 mL of 10^{-4} dilution factor. This was followed by incubation for 4 days at 37 °C (Chen et al., 2016).

The fungi with bioflocculating ability were screened using a flocculation selective medium containing $MgSO_47H_2O$ 0.2 g, urea 0.5 g, K_2HPO_4 5 g, yeast extract 0.5 g, KH_2PO_4 2 g, glucose 10 g in 1 L of distilled water. The medium was sterilized and was allowed to cool and pour plated with 0.5 mL of 10^{-4} dilution factor, before incubating at room temperature (28 °C (±2)) for 7 days (Zhang et al., 2007). The growths on the selective medium for both bacteria and fungi were subcultured repeatedly on nutrient agar (NA) and Sabouraud dextrose agar (SDA) respectively, to get pure isolates (Suryan et al., 2012).

The isolated fungi and bacteria were further screened for bioflocculant production on a waste fruit juice. The cultures of all the isolated bacteria and fungi were prepared. The pure colonies grown on NA and SDA were transferred into 20 mL of sterilized nutrient broth (NB) and Sabouraud dextrose broth (SDB) in Erlenmeyer flasks. The flasks were incubated at 37 °C and 28 °C (±2) respectively, at 150 rpm until growth was observed. The broth from the incubated flask was used as inoculum for bioflocculant production in the waste fruit juice. The 5 % (5 % *v*/v) of inoculum was transferred into a 250 mL conical flask containing 100 mL of sterilized waste fruit juice. The flasks were incubated in triplicate at 37 °C and 28 °C (±2) and 150 rpm for 72 h to allow the formation of bioflocculant. Samples were taken from the culture after each 24 h to determine the amount of bioflocculant produced as well as the efficiency of the bioflocculant. The formation of floc in the waste fruit juice is an indication that the organisms had the potential to produce bioflocculant. The amount of bioflocculant produced was measured using spectrophotometry (UV–visible model 759 s & 756 s, China). The culture producing the highest bioflocculant quantity was selected for further screening and analysis (Agunbiade et al., 2019).

2.3. Determination of flocculating activity and bioflocculant production

A wastewater model (kaolin clay suspension) was used in determining the flocculating activity as well as the efficiency of the bioflocculants as reported by Kurane et al. (2015). Kaolin clay (2 g) was suspended in 250 mL of distilled water in 14 of 500 mL conical flasks, labelled $A_1 - A_7$ and B_1-B_7 where $A_1 - A_7$ were without isolate and B_1-B_7 were inoculated with 2 mL of fresh cultures of each isolate. Flasks A and B were mixed vigorously for a minute before allowing them to settle for 5 min at 28 ± 2 °C to allow floc collection to be possible (Kurane et al., 2015; Mohammed et al., 2023). A spectrophotometer (UV–visible model 759 s & 756 s, China) was used to determine the optical density (OD) of the upper liquid at 550 nm and the organism with the highest absorption was selected as the isolate with the highest ability to produce flocculants (Gao et al., 2016; Khiew et al., 2016).

The isolate was used to produce bioflocculant using 500 mL of the selective medium in a 1 L conical flask. The medium was incubated for 5 days at 37 °C and centrifuged at 5000 rpm for 15 min using high speed refrigerated centrifuge (LR10-2. 4A, India). The sediment was taken as the bioflocculant. The bioflocculant was then freeze-dried using a lyophilizer (LGJ18, China). The lyophilized flocculant was kept for further analysis.

2.4. Identification of isolates with flocculating activity

The bacterial isolates were identified based on their cultural, morphological and biochemical characteristics as described by Cheesbrough (2006). The identification of fungal isolates was based on their cultural, microscopic and morphological characteristics. The fungi were stained with lactophenol cotton blue and viewed on a bionocular microscope with x10 and x40 objectives noting features including hypha, septa, conidiophores, sporangia and spores (Kidd et al., 2016; Alsohaili and Bani-Hasan, 2018). In addition, the isolate that exhibited the highest flocculating potential was identified based on its molecular characteristics (Wawrik et al., 2005; Frank et al., 2008).

The extraction of DNA was guided using protocols of Wawrik et al. (2005) and Frank et al. (2008). The pure colony of the isolate was then transferred from the solid medium into liquid medium (1.5 mL) and grown in an incubator with a shaker at 28 °C for 48 h. At the end of the incubation period, the cultures were spun at 4600*g* for 5 min. The pellets obtained were then suspended in 520 μL of Tris-EDTA (TE) buffer (pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris-HCl) followed by the addition of protease K made from 20 mg/mL (3 μL) and 15 μL of 20 % sodium dodecyl sulfate-SDS. The mixture obtained was then incubated at 37 °C for 1 h, followed by the addition of 5 M NaCl (100 μL) and 10 % solution of 80 μL cetyltrimethylammonium bromide (CTAB) in 0.7 M of NaCl before vortexing for 5 min. The suspension was further incubated at 65 °C for 10 min and then kept on ice for 15 min. An equal volume of isoamyl alcohol: chloroform (1:24) was added before incubating for 5 min on ice followed by centrifugation for 20 min at 7200*g*. A new test tube was utilized in collecting the aqueous phase of the centrifuged mixture before adding isopropanol (1: 0.6) followed by DNA precipitation for 16 h at −20 °C. Further centrifugation was done to obtain the DNA at 13000 for 10 min. The DNA was then washed with 500 μL of ethanol (70 %) before air-drying at room temperature (28 °C ± 2) for 3 h and finally dissolved in 50 μL of TE buffer.

2.5. Polymerase chain reaction (PCR) and gel electrophoresis

The cocktail used in the preparation of PCR sequencing contains 5× GoTaq (10 μL) colourless reaction, 25 mM of $MgCl_2$ (3 μL), a mix of 10 mM dNTPs (1 μL), 1 μL of 10 pmol each from 1525 R and 27 F primers, Taq DNA polymerase (0.3 units) (Promega, USA) and made up to 42 μL with sterile distilled water to which 8 μL of DNA template was added. A GeneAmp Thermalcycler 9700 PCR system (Applied Biosystem Inc., USA) was utilized in carrying out the PCR; this consist of a 5 min initial denaturation at a temperature of 94 °C, 30 cycles immediately carried out consisting of 94 °C, 50 °C, and 72 °C for 30 s, 60 s, and 90 s respectively. The cycle was finally terminated at 72 °C for 10 min and cooled at 4 °C.

To confirm gene amplification, an agarose gel (1.5 %) was used to run an integrity check on the fragments of the amplified gene. The agarose gel (1.5 %) was prepared using –1× Tris-acetate-EDTA (TAE) buffer. A microwave was used to boil the suspension for 5 min before allowing the molten agarose to cool to a temperature of 60 °C. The molten agarose gel was stained using 3 μL of 0.5 g/mL ethidium bromide (this stain is responsible for the absorption of invisible UV lights, the energy absorbed is being transmitted as viable orange light). A comb, which contained the molten agarose gel was slotted into the casting tray before pouring molten agarose. The wells were formed by allowing the gel to solidify for 20 min. Onto the gel tank was poured 1xTAE buffer, which barely submerged the gel. A loading dye of 10× blue gel (2 μL) (which is responsible for giving colour and density as well as enhancing loading of the samples into the wells; and allows monitoring of the entire progress of the gel) was added to each PCR product (4 μL) before loading it into the wells. The 100 bp DNA ladder was also loaded into well 1. Electrophoresis of the gel was done at 120 V for 45 min followed by visualization before being photographed after UV transillumination was done. The size of the PCR products was estimated by comparing the movement of the PCR product side-by-side with the mobility of the 100 bp molecular weight ladder in the gel (Wawrik et al., 2005; Frank et al., 2008).

To remove PCR reagents used for the gel integrity test, the amplified fragments were purified with ethanol. A 3 M Na acetate (7.6 μL) and 240 μL (95 %) ethanol were added to the products of PCR that were amplified in a sterile Eppendorf tube (1.5 μL), the solution was then mixed thoroughly using a vortex mixer and kept for 30 min at 20 °C. The mixture was then centrifuged at 13,000*g* for 10 min at a temperature of 4 °C before the supernatant was removed. The pellets were washed via the addition of 150 μL ethanol (70 %), and mixed thoroughly before centrifuging at 7500*g* for 15 min at a temperature of 4 °C. All the supernatants were removed and the tube was inverted on tissue paper in the fume hood for 15 min. Sterile distilled water (20 μL) was used for the resuspension of the mixture and held at 20 °C before the time for sequencing. The purified fragment was subjected to agarose gel (1.5 %) and ran for an hour on a voltage of 110 V to confirm the purity of the PCR products, which was further quantified with a nanodrop (model 2000, Thermoscientific) (Wawrik et al., 2005; Frank et al., 2008). A genetic analyzer (3130xl sequencer, Applied Biosystems) was used in sequencing the amplified fragments; BigDye Terminator (v3.1 cycle kits for sequencing). MEGA 6 and Bio Edit software were utilized during all the genetic analyses (Wawrik et al., 2005; Frank et al., 2008). The gene sequences of the isolate were submitted to the NCBI Gene Bank and the accession number provided was ON184360.

2.6. Molecular identification of tyrosine-protein kinase gene using genomic specific primers

Molecular investigations of the species-specific tyrosine-protein kinase genomic regions responsible for bioflocculant production were achieved by conducting PCR utilizing a genomic-specific primer on the extracted DNA. For all the primer sets per PCR used, the reaction cocktail consisted of 0.25 μL of 10 pM DNTP, 0.75 μL $MgCl_2$, 2.5 μL 5× PCR SYBR green buffer, and 0.25 μL of 10 pM each for forward GGTGCCGATGTATACCAACA and backward ACCCAAACATCCA-TACTCCTAAC primer, 0.06 μL of 8000U Taq DNA polymerase, which was further made up to 10.5 μL using distilled sterile water into which template of 2 μL was added. PCR profile included a 5 min initial denaturation at a temperature of 94 °C. A 30 cycle was immediately carried out consisting of 94 °C, 50 °C, and 72 °C for 30 s, 60 s, and 90 s respectively; the cycle was finally terminated at 72 °C for 10 min utilizing the recommendable profile designed for each pair of primer (Wawrik et al., 2005; Frank et al., 2008).

2.7. Analysis of the bioflocculant

The thermogravimetric property of the bioflocculant was determined using a TGA analyzer (PerkinElmer, Inc., Waltham, MA, USA) at 10 °C per minute with a heating rate of 23–884.2 °C. Nitrogen gas flow was used to keep the condition constant during the process (Damini et al., 2020).

The detection of functional groups responsible for flocculating activities of the flocculant was determined using the FTIR analysis. This analysis utilizes a Bruker FTIR spectrophotometer (Tensor 27) at 3500–1000 cm^{-1} on a resolution of 4 cm^{-1}. Bioflocculant (5 mg) was mixed with a powder of potassium bromide before pressing it into pellets (Luo et al., 2014).

The structural component of the bioflocculant was determined using scanning electron microscopy (SEM) facilitated by an elemental analyzer (JEOL, Peabody Inc., MA, USA). Five milligrams (5 mg) of the flocculant were added to a slide and silicon-coated. A spin coater was used in fixing the silicon-coated slide and spun for 60 s at 1000 rpm before inserting it into the analyzer for analysis (Rasulov et al., 2017).

3. Results

3.1. Microorganisms Isolated from Earthen Pond

Four bacteria (*Acinetobacter* spp., *Priestia megaterium, Achromobacter* spp. and *Bacillus subtilis*) and three fungi (*Aspergillus terreus, Aspergillus flavus* and *Aspergillus fumigatus*) were isolated from the earthen pond sludge (Tables 1 and 2). One of the microbial isolates produced the highest optical density of 0.108 and flocculating activity of 30 % in the suspension of kaolin clay (Table 3). This isolate on the NA plate appeared smooth, pale white and circular with a diameter of 1 μm. The result from Gram's reaction showed that the isolate was a Gram-positive rod with cream-coloured colonies.

3.1.1. The molecular identity of bacterial isolate

Fig. 1a shows the agarose gel image of the isolate and it shows that the size of the isolate is 1500 bp. NCBI blast results showing sequence identity as compared to established sequences in the NCBI database along the 16 s rRNA are presented in Table 4. The sequence of the isolate confirmed that it is 99.58 % similar to *Priestia megaterium*. The gel image of *P. megaterium* shows a positive amplification along the 16s rRNA to gene tyrosine-protein kinase, the gene that codes for the production of bioflocculant (Fig. 1b, Table 5).

3.2. Thermogravimetric property of the bioflocculant produced by *P. megaterium*

Priestia megaterium produced 10.67 g of bioflocculant from 500 mL of the selective medium. At 300 °C, the weight decrease by 5 % with a further decrease of 30 % at 350 °C (Fig. 2). Loss in weight was observed at temperatures ranging from 40 to 230 °C. At 300 °C, the weight loss of the bioflocculant decreased much more, up to 400 °C. Between 400 and 500 °C, there was a 90 % decrease in weight, until 800 °C, at which point, a total loss in weight was observed.

Table 1
Morphological and biochemical characteristics of the bacterial isolates.

Isolates Code	Gram's Reaction	Catalase	Coagulase	Starch hydrolysis	Indole	Oxidase	Urease	Citrate	Methyl Red	Voges Proskauer	Probable isolate
BIA	- rod	+	−	+	−	−	+	+	−	+	*Acinetobacter* spp
BIB	+ rod	+	−	−	−	−	−	−	−	−	*Priestia megaterium*
BOA	- rod	+	−	−	−	+	+	+	−	+	*Achromobacter* spp.
BOB	+ rod	+	−	+	−	−	+	+	−	+	*Bacillus subtilis*

NB: BIA, BIB were bacteria isolated from sludge inside the earthen pond. BOA and BOB were bacteria isolated from earthen sludge after overnight excavation.

Table 2
Morphological features of fungi identified from the sampling sites.

Isolate code	Macroscopy	Microscopy	Probable isolates
FIA	White and woolly appearance (surface view)	Thread-like septate hyphae	*Aspergillus terreus*
FIB	Pale yellow with a brick appearance	Unbrached, non-septate conidiophore	*Aspergillus flavus*
FOA	Dark greenish	Narrow hyphae intertwined together	*Aspergillus fumigatus*

NB: FIA, and FIB were fungi isolated from sludge inside the earthen pond. FOA was fungi isolated from earthen sludge after overnight excavation.

Table 3
Flocculating activities of bacterial and fungi isolates.

Isolates	Optical density	Flocculating activities
BIA	0.082	23 %
BIB	0.108	30 %
BOA	0.079	21 %
BOB	0.017	5 %
FIA	0.031	9 %
FIB	0.006	2 %
FOA	0.044	12 %

NB: Where BIA, and BIB were bacteria isolated from sludge inside the earthen pond. BOA and BOB were bacteria isolated from earthen sludge after overnight excavation. FIA were fungi isolated from sludge inside the earthen pond FOB fungi were isolated from earthen sludge after overnight excavation.

3.3. Functional groups determination by Fourier-transform infrared (FTIR)

Different microorganisms produced varying types of bioflocculants, which are different in their composition. A bioflocculant activity is solely dependent on the types of chemical structure, which is the responsibility of the functional groups present in the molecule. Fig. 3 shows the FTIR spectra, which reveal the different functional groups present in the bioflocculant. The FTIR contains a characteristic functional group of a bioflocculant, with the band at 3213 cm^{-1} describing the presence of a C—O group (i.e., carboxyl group), the peak (1397.8 cm^{-1}) band exhibits the characteristics of the hydroxyl bending group and peak 1118.2 cm^{-1} is an amine with N—H bending group. Sugar derivatives are indicated in the purified flocculant based on 928 and 849 cm^{-1} adsorption bands. The presence of sugar monomers and β-glycosidic linkages are indicated by small absorption peaks.

3.4. Scanning electron micrograph of the bioflocculant

The scanning electron micrographs of the bioflocculant at different magnifications (x300, x500, x1000 and x1500) are shown in Fig. 4. The micrographs show the white flakes characteristic of flocculants.

4. Discussion

Microorganisms were isolated from earthen pond sludge and were used to produce flocculants in this study. Various attempts have been made to reduce the cost of producing bioflocculants. Despite being effective, they have been insufficient in meeting the global demand for flocculating substances. This has made researchers explore various environments for isolating more efficient bioflocculant-producing microorganisms. In this study, bioflocculating microorganisms were screened from the sludge of an earthen pond. At the end of the screening process, 7 isolates were obtained, 3 fungi and 4 bacteria. However, *Priestia megaterium* was finally selected for flocculant production because it gave the highest flocculating activity (30 %) in the kaolin solution.

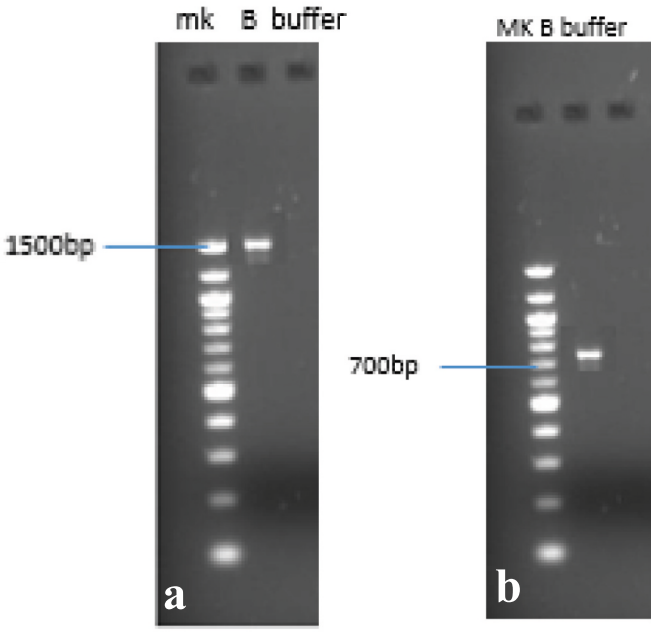

Fig. 1. (a) Gel image showing the size of isolate (b) Agarose gel showing the positive amplification of the tyrosine-protein kinase gene amplified from DNA isolated from cultured bacteria using gene-specific primers. The presence of 700 bp indicates a positive amplification in extracted DNA.

Several authors have reported the microbial communities of earthen fish ponds with the purpose of determining the microbiological quality of the ponds and tracing it to the presence of pathogenic organisms in fish. However, few researchers have reported on the microbiological contents of earthen pond sludge and their possible role in bioflocculants production. The earthen pond is a rich sediment comprising nutrients from leftover feeds or dead fishes as well as clay, which provides a suitable environment for the growth of varying microbial communities with potential application in numerous industrial processes. The microbial species (*Bacillus subtilis Priestia megaterium, Aspergillus terreus, A. flavus, A. fumigatus* and species of *Acinetobacter* and *Achromobacter*) isolated in this study affirm that microorganisms are ubiquitous with adaptive features that allow them to survive varying environmental conditions such as the one found in the earthen pond sludge. This also signifies that the earthen pond sludge contains essential nutrients that

Table 4
NCBI blast results showing sequence identity along the 16 s rRNA.

SAMPLE CODE	Scientific Name	Max Score	Total Score	Query Cover	Error value	% Identity	Accession
B	*P. megaterium*	2588	2588	99 %	0	99.58 %	ON184360

Table 5
NCBI blast results showing sequence specific to tyrosine-protein kinase gene.

Scientific Name	Max Score	Total Score	Query Cover	Error value	% Identity	length of sequence	Accession
P. megaterium tyrosine-protein kinase gene	1375	1435	99 %	0	99.34 %	760	CP058262.1

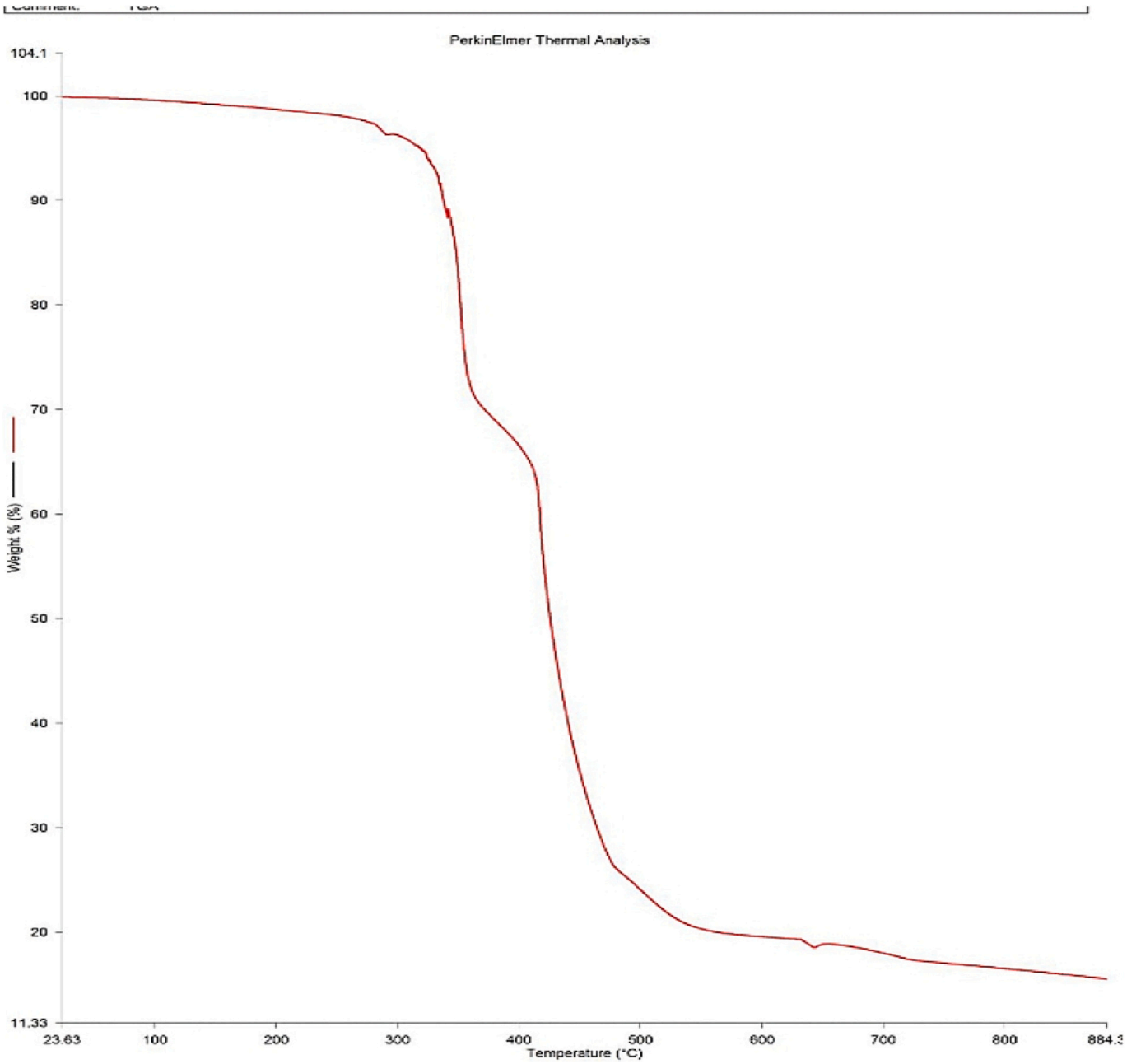

Fig. 2. Thermogravimetric property of the bioflocculant.

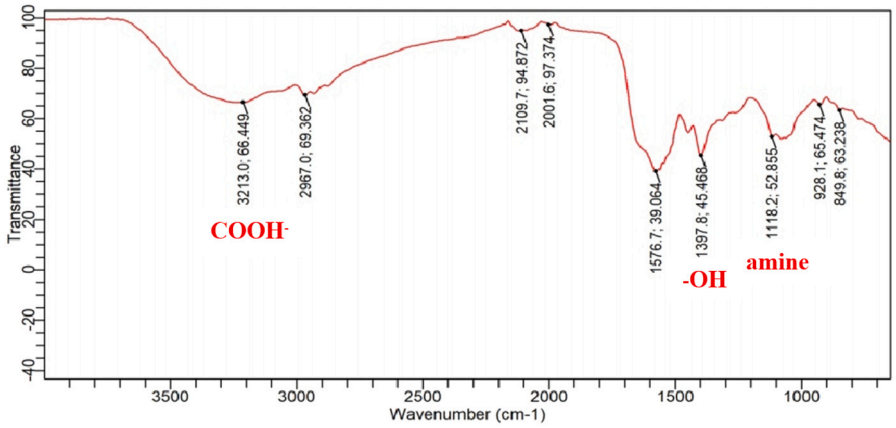

Fig. 3. Functional groups in the bioflocculant determined by FTIR.

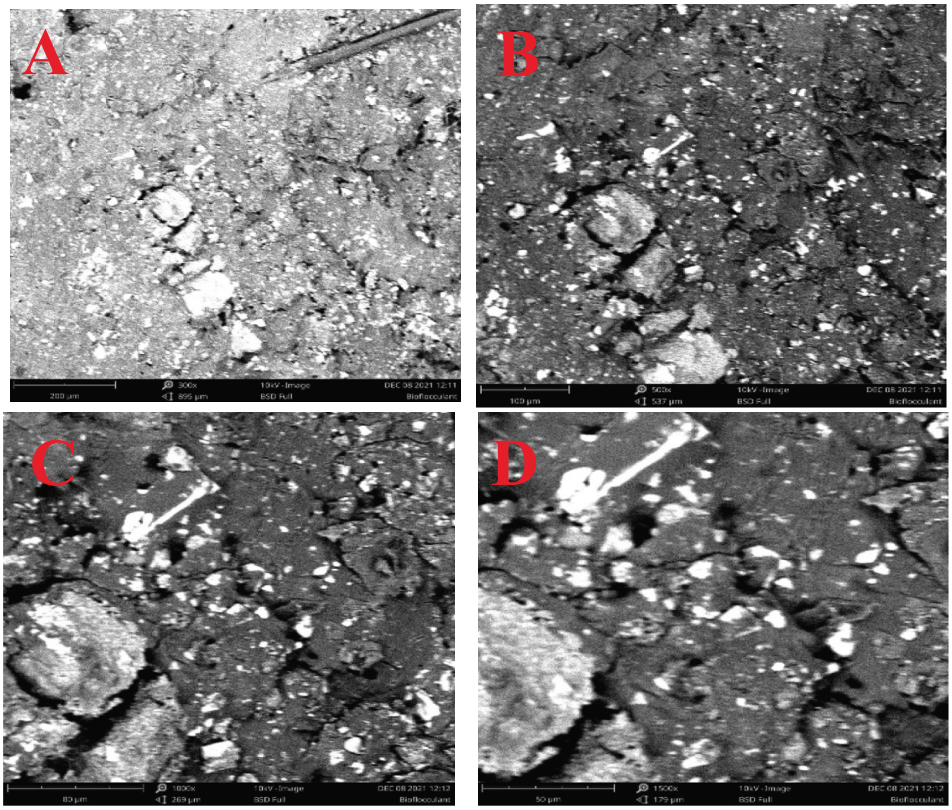

Fig. 4. Scanning electron micrograph of bioflocculant, A – x300, B – x500, C - x1000 and D – x1500.

support microbial growth and metabolism. The ability to trap nutrients such as phosphorus and nitrogen has been demonstrated by the earthen pond. These essential nutrients are concentrated in the sediments. Pouil et al. (2019) reported that on average, phosphorus (77 %) and nitrogen (61 %) inputs are trapped in the accumulated sediments, giving credence to the fact that trapping by accumulated sediments was the main sink of nutrients in earthen pond. Njoku et al. (2015) reported the presence of *Aspergillus* sp. in some earthen ponds in the Niger Delta area of Nigeria but did not report the presence of *Bacillus, Acinetobacter* and *Achromobacter* sp. probably because their investigation was on fish pond water and not the sludge.

Priestia megaterium isolated from the earthen sludge had the highest production of bioflocculant with 10.67 g of bioflocculant in 500 mL of the selective media. This could be due to the presence of high extracellular polymeric substances (EPS), a complex high-molecular-weight mixture of polymers excreted by *P. megaterium* when compared with the other isolates. The EPS plays an important role during aggregate formation (Guo et al., 2016). According to Yuan et al. (2011), EPS is produced from cell lysis. Structurally, *P. megaterium*, initially called *Bacillus megaterium* is a Gram-positive, rod-shaped, aerobic, spore-forming bacterium widely found in habitats with adverse conditions with a cell length of up to 4 μm and a diameter of 1.5 μm. These characteristics suggest the reason it was found in earthen pond sludge. *P. megaterium* is amongst the biggest known bacteria where the cells often occur in pairs and chains, which are joined together by polysaccharides on the cell walls. The presence of thick polysaccharides in the cell walls may be responsible for the massive production of bioflocculant during screening, as reported in this study. This attribute made it possible for the wide applicability of the bacterium. *Priestia megaterium* has been an important industrial organism for decades

(Gupta et al., 2020). According to Jianzhong (2019), *P. megaterium* can grow on many carbon sources, thus the earthen pond is a suitable place for the growth of *P. megaterium* due to the presence of ideal temperature, food remains from the feeding of fish, as well as clay or silt-clay that is excellent for water retention.

The production of 10.67 g/500 mL of bioflocculant by *P. megaterium* in this study is highly substantial compared to 65.6 mg bioflocculant recovered from 1 L of fermentation by the strain of *Enterobacter* sp. isolated from activated sludge (Tang et al., 2014). In addition, Tsilo et al. (2021) obtained 2.836 g/L of bioflocculant from *Pichia kudriavzevii* MH545928.1 previously isolated from Kombucha tea and Okaiyeto et al. (2015) recovered 1.6 g/1 L of bioflocculant from the fermentation broth of *Bacillus* sp. AEMREG7. Moreover, tyrosine-protein kinase found in *P. megaterium* has been documented by Liu et al. (2017) as the gene responsible for bioflocculant production.

The scanning electron micrographs showed aggregates and highly compacted flocs at various magnifications considered. The white flocs appeared clumped together after flocculation with bioflocculant. The floc allowed particles to be absorbed onto the bioflocculant, resulting in larger flocs observed at higher magnification (Tsilo et al., 2021). The bioflocculant appeared to be amorphous in structure. The aggregates formed by the bioflocculant are similar to the large flocs typical of bioflocculant produced by *Bacillus aryabhattai* strain PSK1 (Abd El-Salam et al., 2017) and the amorphous structure of bioflocculant reported by Nkosi et al. (2021).

The thermogravimetric analyzer revealed that the bioflocculant produced by *P. megaterium* had a 15 % loss in weight between temperatures of 23–300 °C, which could be attributed to a loss in the moisture content (Okaiyeto et al., 2016). Another weight loss of 30 % was obtained at around a temperature of 400 °C, which has been suggested to be an attribute of biopolymer decomposition. An increase in temperature above the aforementioned led to a further increase in weight loss of the bioflocculant. From the result obtained, it can be deduced that the bioflocculant produced by *P. megaterium* is thermostable. This attribute has been reported to be an important characteristic of a bioflocculant from sludge (Xiong et al., 2010; Tang et al., 2014; Li et al., 2018).

The Fourier transform infrared (FTIR) spectra of the bioflocculant showed characteristics of a carboxyl group with a band of 3213 cm^{-1}. Also, at a band of 2109 cm^{-1}, the bioflocculant can be said to contain uronate owing to symmetrical stretching observed in the carboxylate (Okaiyeto et al., 2016). The peak band at 1397.8 cm^{-1} is attributed to the presence of the OH group and the peak band at 1118.2 cm^{-1} is ascribed to amine with N—H bending group in the bioflocculant. Sugar derivatives were also suspected to be present owing to the absorption bands of 849 and 928 cm^{-1} that were recorded. Reports have been made by Agunbiade et al. (2018) that the small absorption peak recorded is associated with monomers of sugar and β-glycosidic linkages. The presence of hydroxyl and carboxyl functional groups suggests a unique position for the adsorption of suspended particles, underpinning it as the preferred choice of functional groups required for flocculating processes. The functional groups reported in this study have a great contribution to the remarkable flocculating activities observed. Okaiyeto et al. (2016) reported the presence of functional groups including amino, hydroxyl and carboxyl groups in bioflocculants produced by a strain of *Bacillus toyonensis* AEMREG6. This is also similar to the report of Nkosi et al. (2021) on bioflocculants produced by *Providencia rettgeri* KF534469.

According to Hua et al. (2021), strong interactions with particles may have been caused by the hydroxyl, carboxyl, and amine groups present on the surface of the bioflocculant. In addition, Okaiyeto et al. (2015) attributed the presence of hydroxyl, carboxyl and amine functional moieties in bioflocculant as the flocculation absorbents and binders. Interestingly, the biochemical analysis of the bioflocculants revealed the presence of polysaccharides (82 %) only. Although this result is remarkable, it only confirmed that polysaccharides are the major backbone of bioflocculants. Thus, supporting the flocculating activities recorded in this study.

The interaction of the polymer-floc of the flocculant produced by *P. megaterium* in this study was attributable to the exceptional removal of a high quantity of dissolved solids, owing to an increase in dissolved particle conglomeration. Nonetheless, the functional groups reported in the bioflocculant could also be attributed to the high flocculating activities observed, which resulted in the high forces of adsorption they possess in enhancing the aggregation process during the formation of flocs (Agunbiade et al., 2019). Findings from this study are similar to the characteristics of bioflocculants produced by *Bacillus licheniformis* and *Serratia ficaria* when compared with conventional flocculants reported by Gong et al. (2008).

5. Conclusion

Four bacteria (*Bacillus subtilis, Acinetobacter* spp., *Achromobacter* spp. and *Priestia megaterium* (ON184360) and three fungi (*Aspergillus terreus, A. fumigatus* and *A flavus*) were isolated from sludge of earthen pond in 'Lapai Gwari' community, Minna Nigeria. The results of this study showed that *Priestia megaterium* (ON184360) isolated from earthen pond sludge is a more efficient alternative for the production of a thermostable flocculant having produced 10.67 g of bioflocculant from 500 mL of selective medium using the tyrosine-protein kinase gene. However, the conditions for bioflocculant production can be optimized to improve yield and alternative low-cost substrates can be used to reduce production costs. In addition, the bioflocculant can be produced on a large scale in a bioreactor and applied in municipal water and wastewater treatment and other industrial applications where bioflocculant will be required.

CRediT authorship contribution statement

Oluwafemi Adebayo Oyewole: Conceptualization, Supervision, Writing – Review & Editing. **Aliyu Jagaba:** Methodology, Supervision. **Abdullahi Afolabi Abdulhammed:** Investigation, Writing – Review & Editing. **Japhet Gaius Yakubu**: Investigation, Writing – Review & Editing. **Asmau Maude Mohammed**: Investigation. **Olabisi Peter Abioye**: Experimental design, **Olalekan David Adeniyi**: Writing – Review & Editing. **Evans Chidi Egwim**: Methodology, Supervision.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data used have been included in the manuscript

References

Abd El-Salam, A.E., Abd-El-Haleem, D., Youssef, A.S., Zaki, S., Abu-Elreesh, G., El-Assar, S.A., 2017. Isolation, characterization, optimization, immobilization and batch fermentation of bioflocculant produced by *Bacillus aryabhattai* strain PSK1. J. Genet. Eng. Biotechnol. 15, 335–344.

Abu-Tawila, Z.M., Ismail, S., Dadrasnia, A., Usman, M.M., 2018. Production and characterization of a bioflocculant produced by *Bacillus salmalaya* 139SI-7 and its applications in wastewater treatment. Molecules 23. https://doi.org/10.3390/molecules23102689.

Agunbiade, M., Pohl, C., Ashafa, O., 2018. Bioflocculant production from *Streptomyces platensis* and its potential for river and waste water treatment. Braz. J. Microbiol. 49, 731–741. https://doi.org/10.1016/j.bjm.2017.02.013.

Agunbiade, M.O., Pohl, C., Heerden, E., Oyekola, O., Ashafa, A., 2019. Evaluation of fresh water actinomycete bioflocculant and its biotechnological applications in wastewaters treatment and removal of heavy metals. Int. J. Environ. Res. Public Health 16, 3337.

Agunbiade, M., Oladipo, B., Ademakinwa, A.N.A., O., A., I.M., O., Ololade .O., O., Ojo, A., 2022. Bioflocculant produced by *Bacillus velezensis* and its potential application in brewery wastewater treatment. Sci. Rep. 12, 10945. https://doi.org/10.1038/s41598-022-15193-8.

Alsohaili, S.A., Bani-Hasan, B.M., 2018. Sciences morphological and molecular identification of fungi isolated from different environmental sources in the northern eastern desert of Jordan. Jordan J. Biol. Sci. 11 (3), 329–337.

Bisht, V., Lal, B., 2019. Exploration of performance kinetics and mechanism of action of a potential novel bioflocculant BF-VB2 on clay and dye wastewater flocculation. Front. Microbiol. https://doi.org/10.3389/fmicb.2019.01288.

Cheesbrough, M., 2006. District laboratory practice in tropical countries. Part 2, 1–434.

Chen, H., Zhong, C., Berkhouse, H., Zhang, Y., Lv, Y., Lu, W., Yang, Y., Zhou, J., 2016. Removal of cadmium by bioflocculant produced by *Stenotrophomonas maltophilia* using phenol-containing wastewater. Chemosphere 155, 163–169.

Damini, N.G., Basson, A.K., Pullabhotla, R.V., 2020. Green synthesis of iron nanoparticles by a polysaccharide bioflocculant from marine *Alcaligenes faecalis* HCB2 and characterization. Adv. Sci. Eng. Med. 12, 1034–1039.

Das, N., Shende, A.P., Keerthana, G., Mandal, K.S., 2022. Applications of microbial bioflocculants for environmental remediation: an overview. Res. J. Pharm. Technol. 15 (1883–0) https://doi.org/10.52711/0974-360X.2022.00315.

Frank, J.A.R., I., C., Sharma, S., Weisbaum, J.S., Wilson, B.A., Olsen, G.J., 2008. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. Appl. Environ. Microbiol. 74, 2461–2470.

Gao, Q., Zhu, X.-H., Mu, J., Zhang, Y., Dong, X.-W., 2016. Using *Ruditapes philippinarum* conglutination mud to produce bioflocculant and its applications in wastewater treatment. Bioresour. Technol. 100, 4996–5001.

Gong, W.X., Wang, S.G., Sun, X.F., Liu, X.W., Yue, Q.Y., Gao, B.Y., 2008. Bioflocculant production by culture of Serratia ficaria and its application in wastewater treatment Bioresour. Technol 99, 4668–4674.

Guo, X., Wang, X., Liu, J., 2016. Composition analysis of fractions of extracellular polymeric substances from an activated sludge culture and identification of dominant forces affecting microbial aggregation. Sci. Rep. 6, 28391. https://doi.org/10.1038/srep28391.

Gupta, R.S., Patel, S., Saini, N., Chen, S., 2020. Robust demarcation of 17 distinct Bacillus species clades, proposed as novel Bacillaceae genera, by phylogenomics and comparative genomic analyses: description of Robertmurraya kyonggiensis sp. nov. and proposal for an emended genus Bacillus limiting it only to the members of the Subtilis and Cereus clades of species. Int. J. Syst. Evol. Microbiol. 70, 5753–5798. https://doi.org/10.1099/ijsem.0.004475.

Harun, A.A.C., Mohammad, N.A.H., Ikhwanuddin, M., Ismail, N., Ibrahim, Z., Nor Kasan, A., 2017. Consortium of bioflocculant-producing bacteria as inoculum on flocculation process for sustainable production of pacific whiteleg shrimp, *Penaeus vannamei*. J. Fish. Aquat. Sci. 12, 197–206.

Hua, J., Zhang, R., Chen, R., Liu, G., Yin, K., Yu, L., 2021. Energy-saving preparation of a bioflocculant under high-salt csssssssss by using strain *Bacillus* sp. and the interaction mechanism towards heavy metals. Chemosphere 267, 129324. https://doi.org/10.1016/j.chemosphere.2020.129324.

Jianzhong, Q., 2019. A Kind of Bioactivation Enzymatic Hydrolysis Phosphorus Agent and Preparation Method Thereof Containing *Bacillus megaterium*. CN109134083, China Patent.

Khiew, S.-K., Teng, T.-T., Wong, Y.-S., Ong, S.-A., Ismail, N., Alkarkhi, A., 2016. Effects of cationization hybridized biopolymer from *Bacillus subtilis* on flocculating properties. Desalin. Water Treat. 57, 16086–16095.

Kidd, S., Halliday, C., Alexiou, H., Ellis, D., 2016. Descriptions of Medical Fungi, third edition. Printed in Adelaide by Newstyle Printing, Manchester Street Mile End, South Australia.

Kurane, R., Hatamochi, K., Kiyohara, T., Hirao, M., Taniguchi, Y., 2015. Production of a bioflocculant by *Rhodococcus erythropolis* S-1 grown on alcohols. Biosci. Biotechnol. Biochem. 58, 428–429.

Li, Y.X., Xu, Y., Song, R., Tian, C., Liu, L., Zheng, T.Wang, 2018. Flocculation Characteristics of a Bioflocculant Produced by the Actinomycete *Streptomyces* sp. https://doi.org/10.1186/s12896-018-0471-9.

Liu, P., Chen, Z., Yang, L., Li, Q., He, N., 2017. Increasing the bioflocculant production and identifying the effect of overexpressing epsB on the synthesis of polysaccharide and γ-PGA in *Bacillus licheniformis*. Microb. Cell Factories 16, 163. https://doi.org/10.1186/s12934-017-0775-9.

Liu, C., Sun, D., Liu, J., Zhu, J., Liu, W., 2021. Recent advances and perspectives in efforts to reduce the production and application cost of microbial flocculants. Bioresour. Bioprocess 8, 51. https://doi.org/10.1186/s40643-021-00405-2.

Luo, Z., Chen, L., Chen, C., Zhang, W., Liu, M., Han, Y., Zhou, J., 2014. Production and characteristics of a bioflocculant by *Klebsiella pneumoniae* YZ-6 isolated from human saliva. Appl. Biochem. Biotechnol. 172, 1282–1292.

Mohammed, J.N., Majiya, H., Adisa, J.M., Kassim, Z.J., 2023. Production of bioflocculant through fermentation of spoilt orange juice with *Bacillus* spp isolated from sediment of local clay pot. J. Appl. Sci. Environ. Manage 27, 169–175.

Njoku, O.E., Agwa, O.K., Ibiene, A.A., 2015. An investigation of the microbiological and physicochemical profile of some fish pond water within the Niger Delta region of Nigeria. Eur. J. Food Sci. Technol. 3, 20–31.

Nkosi, N.C., Basson, A.K., Ntombela, Z.G., Maliehe, T.S., Pullabhotla, R.V.S.R., 2021. Isolation, identification and characterization of bioflocculant-producing bacteria from activated sludge of vulindlela wastewater treatment plant. Appl. Microbiol. 1, 586–606.

Okaiyeto, K., Nwodo, U.U., Mabinya, L.V., Okoli, A.S., Okoh, A.I., 2015. Characterization of a bioflocculant (MBF-UFH) produced by *Bacillus* sp. AEMREG7. Int. J. Mol. Sci. 16, 12986–13003. https://doi.org/10.3390/ijms160612986.

Okaiyeto, K., Nwodo, U.U., Okoli, S.A., Mabinya, L.V., Okoh, A.I., 2016. Implications for public health demands alternatives to inorganic and synthetic flocculants: bioflocculants as important candidates. Microbiologyopen 5, 177–211. https://doi.org/10.1002/mbo3.334.

Pouil, S., Samsudin, R., Slembrouck, J., Sihabuddin, A., Sundari, G., Khazaidan, K., Kristanto, A.H., Pantjara, P., Caruso, D., 2019. Nutrient budgets in a small-scale freshwater fish pond system in Indonesia. Aquaculture 504, 267–274.

Rasulov, B.A., Li, L., Liu, Y.-H., Mohamad, O.A., Xiao, M., Ma, J.-B., Li, W.-J., 2017. Production, Characterization and Structural Modification of Exopolysaccharide-based Bioflocculant by *Rhizobium radiobacter* SZ4S7S14 and Media Optimization.

Salehizadeh, H., Shojaosadati, S.A., 2015. Removal of metal ions from aqueous solution by polysaccharide produced from *Bacillus firmus*. Water Res. 37, 4231–4235.

Salehizadeh, H., Yan, N., 2014. Recent advances in extracellular biopolymer flocculants. Biotechnol. Adv. 32, 1506–1522.

Suryan, J., Zhang, X., Miao, X., Zhou, J., 2012. Preparation and characteristics of bioflocculants from excess biological sludge. Bioresour. Technol. 126, 362–366.

Tang, W., Song, L., Li, D., Qiao, J., T., Z., Zhao, H., 2014. Production, characterization, and flocculation mechanism of cation independent, ph tolerant, and thermally stable bioflocculant from *Enterobacter* sp. ETH-2. PLoS One 9, 114591. https://doi.org/10.1371/journal.pone.0114591.

Tsilo, P.H., Basson, A.K., Ntombela, Z.G., Maliehe, T.S., Pullabhotla, R.V.S.R., 2021. Isolation and optimization of culture conditions of a bioflocculant-producing fungi from kombucha tea SCOBY. Microbiol. Res. 12, 950–966.

Wawrik, B., Kerkhof, L., Zylstra, G.J., Kukor, J.J., 2005. Identification of unique type II polyketide synthase genes in soil. Appl. Environ. Microbiol. 71 (5), 2232–2238.

Xiong, Y., Wang, Y., Yu, Y., Li, Q., Wang, H., Chen, R., He, N., 2010. Production and characterization of a novel bioflocculant from *Bacillus licheniformis*. Appl. Environ. Microbiol. 76, 2778–2782. https://doi.org/10.1128/AEM.02558-09.

Yuan, S.J., Sun, M., Sheng, G.P., Li, Y., Li, W.-W., Yao, R.-S., H-Q, Y., 2011. Identification of key constituents and structure of the extracellular polymeric substances excreted by *Bacillus megaterium* TF10 for their flocculation capacity. Environ. Sci. Technol. 45, 1152–1157.

Zhang, Z.Q., Lin, B., Xia, S.Q., Wang, X.J., Yang, A.M., 2007. Production and application of a novel bioflocculant by multi-microorganism consortia using brewery wastewater as a carbon source. J. Environ. Sci. 19, 667–673. https://doi.org/10.1016/S1001-0742(07)60112-0.

Zhong, C., Xu, A., Chen, L., Yang, X., Yang, B., Hong, W., Mao, K., Wang, B., Zhou, J., 2018. Production of a bioflocculant from chromotropic acid waste water and its application in steroid estrogen removal. Colloids Surf. B: Biointerfaces 122, 729–737.