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Screening, characterization, and production of *Bacillus cereus* (S55) bioflocculant isolated from soil for application in wastewater treatment

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ABSTRACT

This research evaluated a bacterial strain, *Bacillus cereus* (S55), isolated from the soil for its bioflocculant production capabilities. The strain was identified through morphological and 16s rRNA gene sequencing. The optimization of culture conditions, using One-factor-at-a-time method, significantly enhanced bioflocculant production with glucose and urea. FTIR analysis showed the presence of hydroxyl, amine and carboxylate functional groups, with polysaccharides as predominant components. Scanning electron microscopy and X-ray diffraction confirmed the crystalline nature of the bioflocculant. The strain studied showed potential in treating household wastewater and was effective at removing dyes, suggesting alternatives for wastewater management.

1. Introduction

Water, an indispensable resource for all life, is facing a crisis. With escalating population growth, urbanization, and agricultural practices, the demand for clean water rapidly increases, leading to a significant rise in wastewater generation.[1] Global estimates suggest that approximately 380 billion cubic meters of wastewater are generated annually. Projections suggest a 24 % increase by 2030 and a 51 % rise by 2050.[2] Only a mere 24 % of this wastewater undergoes treatment before being discharged into fluvial systems or reused in agricultural applications. This untreated wastewater, laden with various pollutants such as organic matter, microorganisms, salts, dyes, toxic compounds, and heavy metals,[3] poses severe threats to aquatic ecosystems and can adversely impact living beings.

Flocculation, a process that destabilizes and facilitates the removal of suspended solids contaminants through settling, is one of the most efficient and modest methodologies for purifying drinking water and wastewater. This technique employs flocculants-polymeric compounds, which can be utilized independently or in conjunction with various salts. Flocculants are categorized into organic, inorganic, and natural types. While organic and inorganic flocculants are effective, they pose environmental and health risks due to their toxicity and difficulty in degradation, potentially leading to diseases such as cancer, Alzheimer's disease and other neurological disorders.[4] Given these concerns, there is an urgent need for a significant shift towards research on bioflocculants, which promise a safer and more sustainable future for water treatment and motivates the exploration of alternatives for chemical treatment.

Bioflocculants, which are extracellular polymeric substances (EPS), represent a promising category of flocculants that are biodegradable and enhanced safety.[5] These biologically derived substances are produced by diverse microorganisms and crafted with complex compounds, namely glycoproteins, carbohydrates, lipids, polyaminoacids, nucleic acids, and functional compounds like hydroxyl, amine, and carboxyl groups.[4] The precise mechanism of bioflocculant action remains not fully elucidated; however, it is predominantly understood to involve bridging and charge neutralization processes.[6] Bacterial flocculants possess enhanced qualities; however, their commercialization is hindered by immoderate cost and nominal yield.[7] Consequently, researchers continue to seek novel bioflocculant-producing strains with enhanced yields and effectiveness, particularly in soil environments.[8]

Notably, bacterial classes like Actinobacteria, Bacilli, Proteobacteria, and Betaproteobacteria have been identified as prolific producers of bioflocculants.[9] Phylum Firmicutes includes numerous strains of *Bacillus* species and are characterized as rod-shaped, gram-positive organisms that appear either as obligate aerobes or facultative anaerobes; they have been particularly noted for their industrial applications due to their

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robust bioflocculant production capabilities.[10]

The objective of this study was to isolate and characterize a bacterial strain capable of producing bioflocculants from soil samples obtained from Coimbatore, Tamil Nadu, India. The isolated strain was identified as *Bacillus cereus* (S55) through morphological assessment and 16s rRNA sequencing. The study included the optimization of various culture parameters to enhance production, as well as purification and characterization of the bioflocculant. Additionally, the study evaluated the potential applications of the bioflocculant in dye removal and domestic wastewater treatment. This study investigates the potential applications of *B. cereus*, a subject that has been sparsely explored in the context of bioflocculant usage.

2. Materials and methods

2.1. Isolation of bacteria from soil samples

The soil samples were accumulated aseptically from Coimbatore, Tamil Nadu, India. One gram of soil was suspended in 9 ml of sterile distilled water, agitated for half an hour, and then allowed to settle for 10 min.[11] From the settled soil samples, 1 ml of the supernatant was transferred into a medium containing 10 g glucose, 1 g yeast extract, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g of KH_2PO_4, NaCl, and K_2HPO_4. This concoction faced incubation within an incubator shaker at a constant temperature of 30 °C at 160 rpm for 72 h. Following incubation, culture dilutions (up to 10^{-7}) were made and plated on a medium with 15 g glucose, 2 g yeast extract, 1.5 g KH_2PO_4, 0.5 g of $MgSO_47H_2O$, NaCl, 4.5 g K_2HPO_4, and 20 g agar per liter.[12] Once initial growth was observed, selected colonies were isolated and cultured on Luria-Bertani agar medium and then preserved in glycerol stocks at −20 °C for ensued procedures.

2.2. Screening and cultivation of organisms in growth medium

Selected colonies exhibiting ropy and mucoid phenotypes, indicative of potential exopolysaccharide production, were inoculated into Congo Red Agar (CRA) medium, supplemented with 5 % glucose, and incubated for 24–48 h at 30 °C.[13]

Selected colonies were propagated in the screening medium formulated with 10 g glucose, 10 g sucrose, 4.5 g K_2HPO_4, 1 g urea, 0.5 g of peptone, yeast extract and ammonium sulfate, 1.5 g KH_2PO_4, 0.1 g NaCl, 0.2 g $MgSO_4{\cdot}7H_2O$ per liter of distilled water.[14] Purified isolates were introduced into 30 ml of screening broth medium and shaking incubated for approximately 72 h at a shaking speed of 140 rpm, maintaining a temperature of 30 °C. The incubated broth was centrifuged to obtain a supernatant without cells, and the flocculation property was evaluated.

2.3. Flocculation activity assessment

Bioflocculant production efficiency was ascertained utilizing a jar test apparatus and a kaolin mixture. Kaolin was utilized to conduct an initial evaluation of the bioflocculant's potential before its application.[5] Approximately 4 g of kaolin disintegrated per liter of sterile water to prepare the suspension. To this, 100 ml of the kaolin suspension was added along with 2 ml of supernatant and 1 % (*w/v*) with volume of 3 ml calcium chloride solution, enhancing flocculant binding due to the ionic strength introduced by calcium ions.[15] The mixture was subjected to intensive stirring at varying speeds: 200 rpm/min for 2 min, 120 rpm/min for 3 min, and 80 rpm/min for 10 min; subsequently, a quiescence period of 10 min at ambient temperature to facilitate particle sedimentation was done.[16] Optical density was quantified at 550 nm by a Biospectrometer (UV/Visible and fluorescence). A control mixture of 2 ml of the production medium was similarly analyzed for optical density. A designated mathematical equation subsequently quantified the flocculation activity (FA):

$$FA(\%) = \frac{(A - B)}{A} X\,100$$

in this study, the absorbance values at 550 nm, denoted as A and B, correspond to the control and sample measurements, respectively. The kaolin powder and flocculated sediment were subjected to drying, followed by microscopic analysis for observation.

2.4. Morphological and biochemical identification of bioflocculant-producing bacteria

The isolates exhibiting high bioflocculant potential underwent macroscopic identification and Gram staining to elucidate their morphological characteristics, including cellular shape and cell wall structure.[17,18]

Biochemical tests encompassed indole production, the Voges-Proskauer reaction, and hydrolysis of casein, gelatin and starch. Carbohydrate fermentation was evaluated using Durham tubes, while catalase and oxidase tests were also performed. These analyses adhered to the standardized methodologies detailed by Cappuccino and Sherman.[19]

2.5. Molecular identification of bacteria producing bioflocculant

Isolates exhibiting elevated activity were discerned molecularly through 16S rRNA sequencing. The gDNA was extracted from isolate S55, and the phenol-chloroform classic extraction was utilized. Its integrity was confirmed by electrophoresis on 1.0 % agarose gel. Universal primers 27F and 1492R were used by PCR to amplify the 16S rDNA gene. The PCR amplified templates were purified and sequenced bidirectionally utilizing BigDye™ Terminator v3.1 Cycle Sequencing Kit. Sequencing was conducted on an ABI 3730xL Genetic Analyzer, and sequences obtained were compared with the NCBI database known sequence using the BLAST tool to confirm the taxonomic identities of the isolates.

2.6. Insights from evolutionary traces

2.6.1. Sequence submission and dataset preparation

The sequences of the target isolate were initially examined for any uncovered or chimeric regions and subsequently trimmed using BioEdit v7.2.[20] The trimmed sequence was then deposited in the NCBI repository,[21] tagged with a specific accession number (**OQ359411**: *B. cereus* SS2 (S55)). The homologous/similar sequence with high query coverage and identity of target isolate was retrieved from NCBI--BLAST.[22] The top hit sequences, most like the isolate under study, were represented in **Supplementary Excel 1**. The nucleotide composition of the target and template sequence was computed using the BioEdit software.

2.6.2. Analysis of evolutionary footprints

Multiple sequence alignment of the selected dataset was conducted using the ClustalW algorithm.[23] A sequence-based phylogeny tree was designed using MEGA 11 software.[24] The neighbour-joining method was used to build a tree,[25] with the Kimura 2-parameter[26] as the evolutionary model. Additionally, the pairwise genetic diversity between the isolate and its closest phylogenetic neighbors (as determined by sequence similarity) was calculated using MEGA 11.

2.7. Optimizing culture growth criteria for producing bioflocculant

1 % of carbon compounds like glucose, sucrose, mannitol, fructose, lactose, maltose, citric acid and starch, as well as nitrogen sources including ammonium sulfate, yeast extract, peptone, tryptone and urea, were independently assessed.[27] pH of the medium was systematically

altered from 3 to 11.[28] The culture media were subjected to a range of discrete incubation temperatures: 20 °C, 30 °C, 40 °C and 50 °C to pinpoint the thermal parameter optimal for flocculant efficacy.[29] The impact of various cation valence and flocculation activity was rigorously investigated, considering monovalent (K^+, Na^+, Li^+), divalent (Mn^{2+}, Ba^{2+}, Ca^{2+}), trivalent (Al^{3+}, Fe^{3+}) and control.[30] The impact of shaking speed on bioflocculant production was evaluated by incubating isolates at specific speeds from 100 to 200 rpm. The optimal inoculum dosage for efficient bioflocculant mass production was determined by differing inoculum concentrations in the production broth medium, ranging from 0.1 % to 10 % (*v/v*).[31]

2.8. Time course assay of B. cereus

The optimized production media was inoculated and incubated for about 120 h with a shaking speed of 140 rpm at a temperature of 30 °C. Every 12 h, the culture broth was drawn, and the pH and culture broth density (OD_{660}) were evaluated.[7] About 5 ml broth was centrifuged at 10000 rpm for 30 min, and flocculation activity was assessed.

2.9. Production and purification of bioflocculant

Bioflocculant obtained from *B. cereus* was harvested under conditions optimized to enhance yield efficiency. For *B. cereus*, carbon sources, such as glucose and nitrogen sources, such as urea, were utilized. Media were adjusted to a pH 7 and incubated at 30 °C for 72 h. The inoculum dosage was set at 2 % for *B. cereus,* with incubation performed in a shaking incubator at 140 rpm.

The incubated broth was centrifuged at 10,000 rpm at 4 °C for 15 min to obtain the supernatant. Two-fold 95 % ethanol was added to the supernatant and kept for 12 h at a temperature of 4 °C to precipitate the bioflocculant. The resulting precipitate was collected, dissolved in distilled water and then purified using chloroform: butanol (5:2 *v/v*) and two-fold ethanol. The purified precipitate was subsequently lyophilized to yield the purified bioflocculant, which was ready for further characterization and application.

2.10. Characterization studies of purified bioflocculant

The comprehensive study about total sugar in bioflocculant was quantified by the phenol-sulfuric acid assay with standard (glucose).[32] The amount of protein in bioflocculant was determined by the Lowry method with standard (BSA).[33] To further elucidate the chemical composition, the functional groups in the purified bioflocculant were analyzed by Fourier Transform Infrared Spectroscopy (FTIR) within a spectral order of 400–4000 cm^{-1} (Shimadzu, ATR-MIRacle 10, Japan). Additionally, Scanning Electron Microscopy (SEM) (Zeiss Sigma VP, Germany) and an elemental analyser characterised the morphological and elemental composition. The experiment utilized kaolin clay powder as control. Moreover, X-ray diffraction (XRD) with a copper K alpha radiation with a scattering angle of 10° to 80° (2θ) on an X' Pert Pro analytical diffractometer was used for analysis.[34]

2.11. Safety assessment of isolated strains

2.11.1. Antibiotic susceptibility test

Utilizing the disc diffusion method on Luria-Bertani agar plates, cultures with a density of 10^8 CFU ml^{-1} in their exponential phase were uniformly inoculated on agar surfaces using sterile swabs.[35] A diverse set of antibiotic discs was individually and independently placed on inoculated plates using sterile forceps. The antibiotics tested included amikacin, amoxicillin, ampicillin, chloramphenicol, gentamycin, imipenem, streptomycin and tetracycline (each at 10 μg). Additionally, ciprofloxacin and rifampicin at 5 μg, penicillin at 10 units, erythromycin at 15 μg, kanamycin, nalidixic acid and vancomycin, each at 30 μg. The inoculated plates containing the discs were incubated at optimal growth temperature for 24–48 h. After the incubation period, the inhibition zone was measured and categorized as Sensitive (S) or Resistance (R) by the standards of the National Committee for Clinical Laboratory Standards (NCCLS).

2.12. Flocculation properties of a purified bioflocculant

2.12.1. Effect of dosage concentration of the purified bioflocculant

A series of purified bioflocculant concentrations ranging from 0.1 mg/ml to 1.0 mg/ml were prepared for the study and evaluated using the Jar test method. From this preparation, approximately 2 ml of the bioflocculant was combined with 100 ml of kaolin suspension and 3 ml of $CaCl_2$. Stirring process was conducted according to the procedures outlined in section 2.3, after which the concoction was allowed to settle for 10 min. The supernatant was spectroscopically analyzed to measure flocculation activity.[18]

2.12.2. Effect of pH and heating stability of the purified bioflocculant

A kaolin solution with a concentration of 4 g/L was prepared and its pH was adjusted to a range between 3 and 11. Following this, 2 ml of the purified bioflocculant was incorporated into the concoction, and subsequent experimental procedures were performed. To evaluate the thermal stability of the purified bioflocculant, it was subjected to heating at temperatures varying from 20 °C to 120 °C for 30 min. Flocculation activity was subsequently assessed at room temperature and analyzed.[18]

2.12.3. Effect of divalent cation on the purified bioflocculant

Calcium chloride solutions were prepared at concentrations ranging from 0.5 % to 3 %. A volume of 3 ml of each calcium chloride solution was combined with 2 ml of the bioflocculant solution within a kaolin mixture, followed the subsequent procedures. The study evaluated the flocculation activity and efficacy of divalent cation.

2.13. Application of bioflocculant in dye removal

The efficacy of the purified bioflocculant for dye removal was assessed using a range of dye solutions, encompassing Nigrosin, Congo red, Safranin, and Methylene blue, at a concentration of 4 g/L. Gradient concentrations of bioflocculant solution ranging from 250 to 1000 mg/L were prepared and subsequently introduced into each dye solution, accompanied by the addition of 3 ml of $CaCl_2$. The concoction was then agitated at different speeds and let to settle for 10 min. A control solution without the bioflocculant served as a basis for comparison. The efficiency of dye removal was subsequently determined to evaluate the effectiveness of the bioflocculant in eliminating dyes from solution.[36]

2.14. Application of B. cereus bioflocculant in domestic wastewater

Domestic wastewater collected from Coimbatore city was treated with a bioflocculant to enhance the purification process. Initially, the pH of the wastewater was measured, and then the sample was refrigerated at 4 °C for subsequent analysis. Following this, a treatment involving the application of bioflocculant, and cation was administered to augment the flocculation efficiency. Subsequently, the efficacy of the bioflocculant in removing biological oxygen demand, chemical oxygen demand, and other relevant parameters was systematically evaluated.

2.15. Statistics and software

All the experiments were conducted in triplicate. The mean and standard deviation were computed using Graph Pad Prism version 10. Statistical significance was deduced at p values < 0.05.

3. Results and discussion

3.1. Isolation and screening of isolates

The selection process concentrated on isolates exhibiting ropy and mucoid characteristics, leading to the identification of approximately 225 isolates. This suggests a potential for flocculation, attributed to their production of exopolysaccharides, which play a pivotal role in effective flocculation. To further refine the phenotypic screening, a Congo Red medium plate was employed to identify exopolysaccharide producers, which were distinguished by their distinctive black colouration. From this refined selection, 63 isolates were chosen. Among these, 9 isolates displayed significantly enhanced flocculation activity, which is described in Table 1. Notably, isolate S55 (Fig. 1a) was prioritized for in-depth characterization to assess its effectiveness in bioflocculation applications.

3.2. Flocculation activity assessment

The kaolin assay assessed the flocculation capabilities in percentage, which exhibited flocculation activities ranging from average to high. Among those, the S55 isolate displayed the highest flocculation activity and was selected for further cultivation and analysis to assess their flocculating stability. Ultimately, S55 was identified based on its stable and superior flocculation performance.

Kaolin powder served as control in the study, revealing a dispersed particle morphology when examined microscopically. In contrast, kaolin that had been flocculated with a bioflocculant displayed a distinct morphology characterized by agglomerated particles, as illustrated in Fig. 1b–c.

3.3. Morphological and biochemical characterization of potential bacteria

The colonies of S55 exhibited a white-to-pale colour with wavy edges. Gram staining of S55 confirmed their classification as Gram-positive rods; microscopic examination further revealed the bacilli formed elongated chains. The results of biochemical characterization of this isolate were presented in Table 2. The strain tested positive for catalase assay and demonstrated the ability to hydrolyze starch, casein and gelatin, distinguishing it from other microorganisms. López and Alippi[37] state that gram-positive organisms typically exhibit catalase positivity and a positive Voges-Proskauer (VP) reaction. The strain utilized glucose, sucrose, fructose and maltose as carbon sources and variable assimilation was observed with arabinose and xylose.

3.4. Molecular identification of bioflocculant-producing bacteria

BLAST analysis revealed a 99 % similarity for isolate S55, confirming its identification as *B. cereus*. The strain has been classified within the Bacillaceae family and submitted to GenBank. QC data for strain S55 are presented in Fig. 2.

Table 1
The Percentage of Flocculation for the selected isolates is presented as mean numbers ± Standard deviation (n = 3).

Isolates	Flocculation Rate (%)
S14	84.82 ± 1.89
S35	70.51 ± 3.06
S36	73.90 ± 2.80
S37	80.55 ± 4.15
S46	69.32 ± 4.77
S47	67.84 ± 1.10
S51	74.19 ± 0.45
S55	**94.65 ± 0.72**
S62	85.36 ± 2.45

3.5. Analysis of phylogenetic imprints

The tree of phylogeny was constructed using 1000 bootstrap iterations to elucidate the evolutionary relationships among the taxa studied.[38–40] Ambiguous positions in the base pairs were excluded using the pairwise deletion option. Each phylogenetic analysis involved 15 sequences, including one from the target isolate and the rest being similar isolates obtained from NCBI-BLAST. The phylogenetic analysis for *B. cereus* SS2 showed close relationships with *B. paramycoides* and *B. proteolyticus*, as illustrated in Fig. 3.

Pairwise genetic diversity was calculated to assess the extent of base substitutions per site within the samples.[35,38,41] The computed statistical pairwise genetic diversity between the target and selected phylogenetic neighbors corroborated our findings, represented in **Supplementary Excel 2**. Furthermore, Tajima's Neutrality Test[42] revealed a nucleotide diversity (π) value of 0.004, indicating low divergence within the genus level and represented in **Supplementary Excel 3**. This was supported by the nucleotide composition analysis, which showed a G + C content greater than 52 %, significantly higher than the A + T content, as represented in Fig. 4; Supplementary Excel 4.

3.6. Optimizing culture growth conditions for bioflocculant production

This study meticulously evaluated the substantial impact of different carbon compounds on growth and bioflocculant mass production.[43] A standard of 1 % of the mentioned carbon sources was used for the analysis. Fig. 5a delineates the flocculation efficiency when different carbon sources were incorporated into the production medium. Glucose (91.5 %), sucrose (81.9 %), mannitol (80.8 %), and starch (87.3 %) were identified as suitable carbon sources, with glucose demonstrating the highest bioflocculant production and activity. Parallel studies have reinforced the significant role of carbon sources in bioflocculant production. Glucose serves as the principal carbon source for microorganisms and plays a vital role in bacterial growth, metabolism and biomass production.[44] This is particularly important for the effective production of bioflocculants. *Bacillus* species (BF9) isolated from shrimp pond water were studied by Gosai and Narolkar,[45] revealing high bioflocculant production and flocculation activity with glucose as the optimal carbon source. Correspondingly, *Streptomyces platensis*, as reported by Agunbiade et al. achieved the highest flocculating activity and cell growth when utilizing glucose as a carbon source.[46] *Virgibacillus* sp. exhibits the most significant flocculation activity when glucose is utilized as the carbon source.[47]

A standard of 1 % of the mentioned nitrogen sources was used for the analysis. Fig. 5b illustrates the impact of numerous nitrogen sources on *B. cereus* (S55) and showed significant growth with tryptone (81.03 %) and urea (92.8 %), with urea being chosen as the primary nitrogen source in the production medium. Moreover, experimental results indicated that peptone was less effective and exhibited significantly lower flocculating activity. In parallel studies, Bisht and Lal[48] reported *Bacillus* sp. TERI VB2 from soil and *Corynebacterium glutamicum* Cg1-P30 Liu et al. achieved maximal flocculating activity and optimal growth with urea in the production medium.[49] Additionally, Chen et al. observed synergistic enhancements in bioflocculant production resulting from the combined use of the organic source yeast extract and urea.[50] This finding indicates that mixed organic nitrogen substrates can significantly enhance bioflocculant production. Nitrogen plays a crucial role in cell growth, synthesis of cell walls,[51] and the production of proteins. These functions were fundamental to the effective production of bioflocculant. Among various nitrogen sources, urea, an inorganic compound, has been shown to be particularly effective in the production of bioflocculants and in enhancing their flocculating activity.

The pH value plays a vital role in modulating flocculation activity by influencing the ionization degrees of the bioflocculant.[4] This effect is prominently depicted in Fig. 5c. *B. cereus* (S55) showed an increase in flocculation activity from 56.7 % at pH 3–93.6 % at pH 7, followed by a

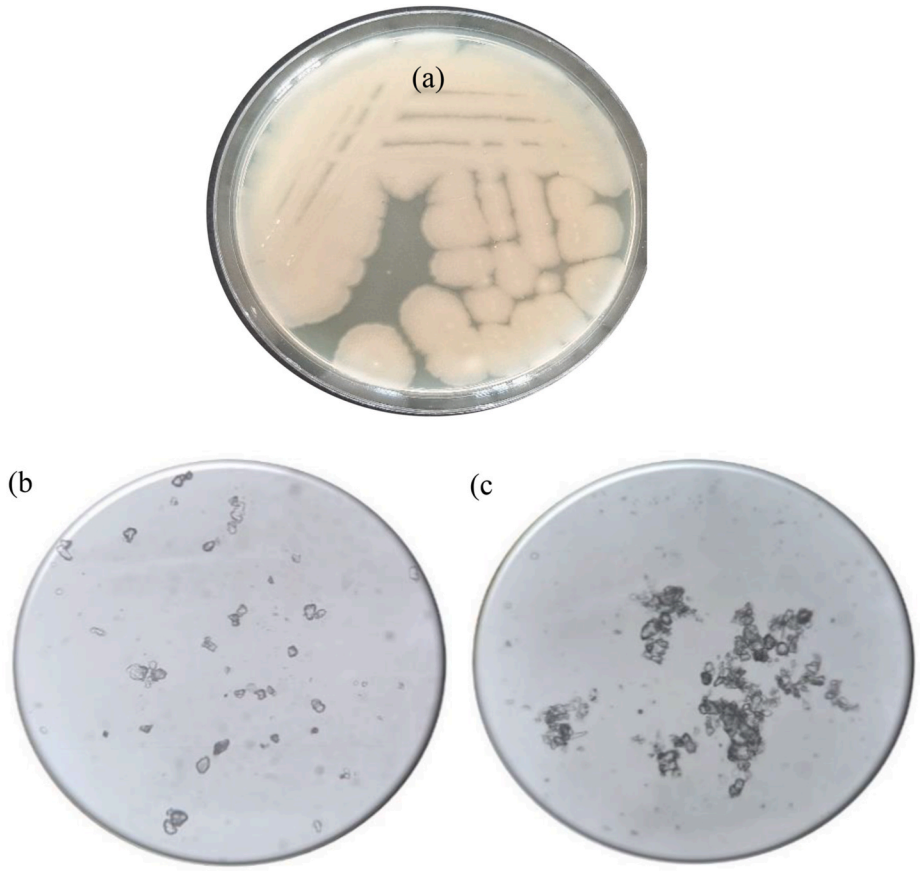

Fig. 1. The soil isolate *B. cereus* (S55) on agar plate (a), microscopic observation of Kaolin (b) and Kaolin with bioflocculant (c).

Table 2
Biochemical Characterization of strain S55 was conducted using various tests, and the results are interpreted as follows: + Positive – Negative ± Weak Positive.

Isolate	*B. cereus* (S55)
Motility	Motile
Catalase	+
Indole Production	–
Voges-Proskauer	+
Starch Hydrolysis	+
Casein Hydrolysis	+
Gelatin Hydrolysis	+
Citrate	–
Oxidase	+
Glucose	+
Sucrose	+
Fructose	+
Mannitol	–
Maltose	+
Mannose	–
Galactose	–
Raffinose	–
Rhamnose	–
Arabinose	±
Xylose	±
Inositol	–
Lactose	–

decline to 49 % at pH 11. Optimal bioflocculant production was observed at pH 7. Consequently, a neutral pH (pH 7 ± 0.2) was identified as optimal for subsequent experiments to maximize bioflocculant output. Bisht and Lal[48] detailed *Bacillus* sp. TERI VB2, isolated from soil, demonstrated optimal bioflocculant activity at a pH scale of 7–9, peaking at neutral pH of 7. The initial pH of a production medium significantly regulates factors, including oxidation-reduction reactions and electrostatic charge on microbes and affects enzyme reactions and nutrient uptake by microbes, resulting in reduced flocculation activity. Similarly, *Bacillus subtilis* OL818309, isolated from lake water, exhibited its highest bioflocculant activity at an optimized pH of 7.[7] *Bacillus* species BF9 was isolated from aquaculture ponds and demonstrated optimal flocculating activity at pH 8.[45]

The impact of cultivation temperature on the flocculation of *B. cereus* (S55) is elucidated in Fig. 5d. The strain showed enhanced flocculation activity at 30 °C, achieving a rate of 92.9 %. In contrast, their flocculation activity declined to 80.7 % at 40 °C. The activity was significantly reduced at 50 °C, suggesting possible thermal denaturation of essential enzymes or destabilization of cell structure at elevated temperatures. At the lower end of the spectrum, flocculation activities at 20 °C were moderate, indicating that sub-optimal temperatures also hampered bioflocculant efficacy. In a similar vein, *Citrobacter koseri*, isolated from tapioca wastewater, demonstrated its highest flocculation activity at 30 °C, as reported by Suryani et al.[14] The metabolic process of organisms is linked to growing temperature, with maximum enzymatic activity typically attained within an optimal temperature range.[29] If the cultivation temperature was too low, the microbial strain might enter a dormant state, resulting in incomplete activation of the enzyme systems crucial for bioflocculant production.[52] Conversely, excessively high culture temperatures can disrupt the bacterial strains' nucleic acids and enzyme systems, thereby hindering productivity.

Fig. 5e illustrates the impact of various cations on the flocculation activity of *B. cereus* (S55). It exhibited moderate flocculation activity with monovalent and divalent cations but significantly reduced strain activity with trivalent cations. The highest flocculation activity for *B. cereus* (S55) was observed with $CaCl_2$, achieving 93.7 %. The control exhibited moderate flocculation. The escalated activity observed with

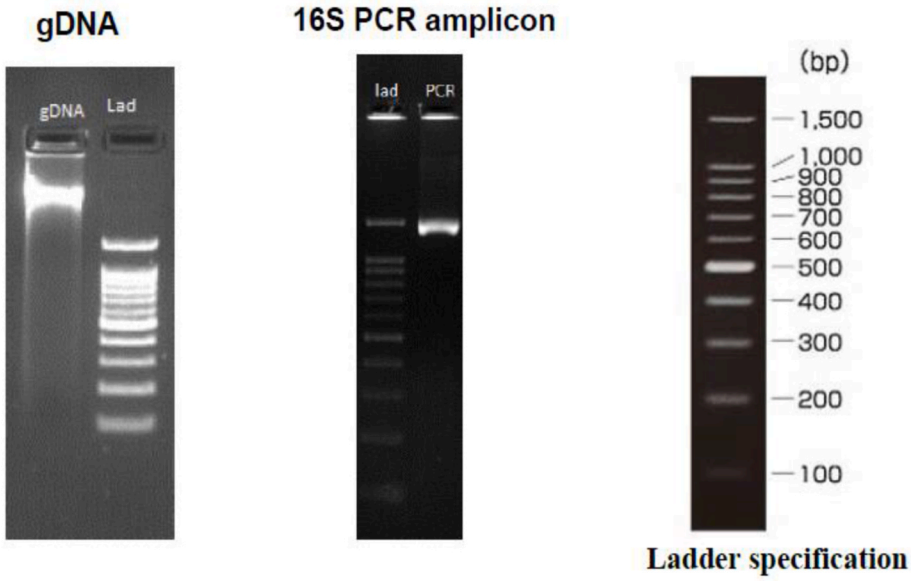

Fig. 2. QC Data of genomic DNA isolated from S55. The agarose gel image shows a single, high-molecular-weight DNA band of strain S55.

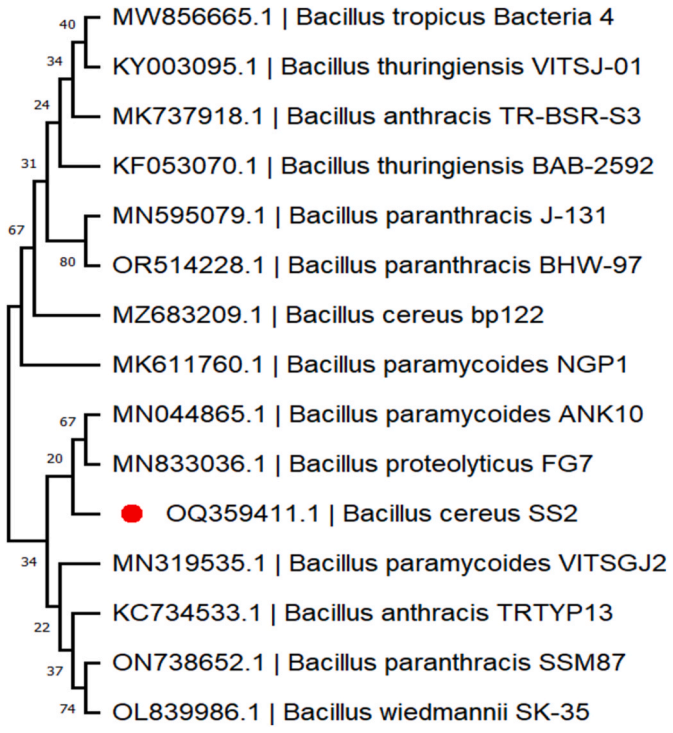

Fig. 3. Construction of 16s rRNA-based phylogeny of *Bacillus cereus* S55 (OQ359411) and similar isolates. **Note:** The red bulletin indicates our target isolates.

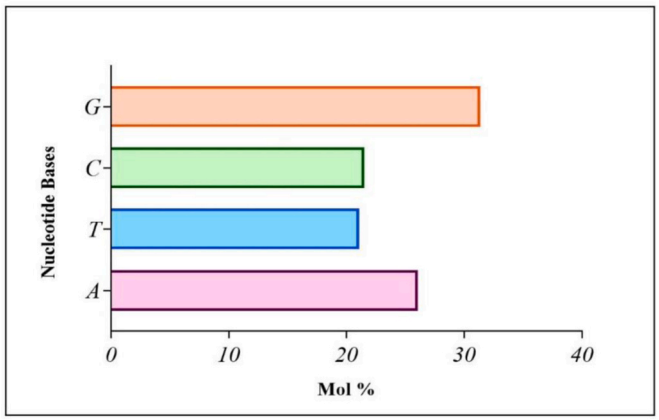

Fig. 4. Predicted nucleotide composition of *B. cereus* S55 based on 16s rRNA gene.

$CaCl_2$ can be ascribed to cations as coagulants; they neutralize the electrokinetic potential of kaolin solution, inevitably facilitating the attraction and aggregation of kaolin particles before flocculation.[53] Divalent cations demonstrated the highest flocculation activity against *Bacillus* spp. UPMB13, isolated from oil palm roots, as reported by Zulkeflee et al.[53] Similarly, Li et al.[54] documented effective flocculation in *Bacillus circulans* isolated from the soil when $CaCl_2$ was utilized during the kaolin assay.

Fig. 5f illustrates the resultant shaking rate on the flocculation of *B. cereus* (S55). *B. cereus* (S55) displayed a gradual increase in flocculation activity, peaking at 140 rpm with an efficiency of 96.02 %, before declining to 57.88 % at 200 rpm. Dissolved oxygen mass, which is crucial for nutrient subsuming and enzyme effects, is significantly influenced by shaking speed.[52] Similarly, Adebayo-Tayo and Adebami[55] reported that *Alcaligenes aquatilis* AP4, isolated from palm oil mill effluent, demonstrated high flocculation activity at a shaking speed of 140 rpm. Li et al.[56] identified an optimal shaking speed range of 140–160 rpm/min for the effective growth of *Bacillus licheniformis* X14.

Fig. 5g illustrates the impact of inoculum dosage on bioflocculant production of *B. cereus* (S55), demonstrated optimal flocculation activity at an inoculum dosage of 2 %, achieving 95.63 %, with a notable decline in activity at higher dosages. Excessive inoculum size can create a competitive niche among bioflocculant producers, suppressing overall bioflocculant production and activity.[57] Conversely, lower inoculum percentages enhanced production and flocculation activity more efficiently. *Virgibacillus* sp., isolated from a marine environment, achieved its highest flocculation activity at an inoculum size of 2 %, with subsequent higher sizes showing reduced activity.[47] Balancing the inoculum size is essential to avoid underperformance due to extended lag phases and suppression of flocculant activity due to excessive microbial competition. *Bacillus licheniformis* X14 exhibited enhanced flocculation

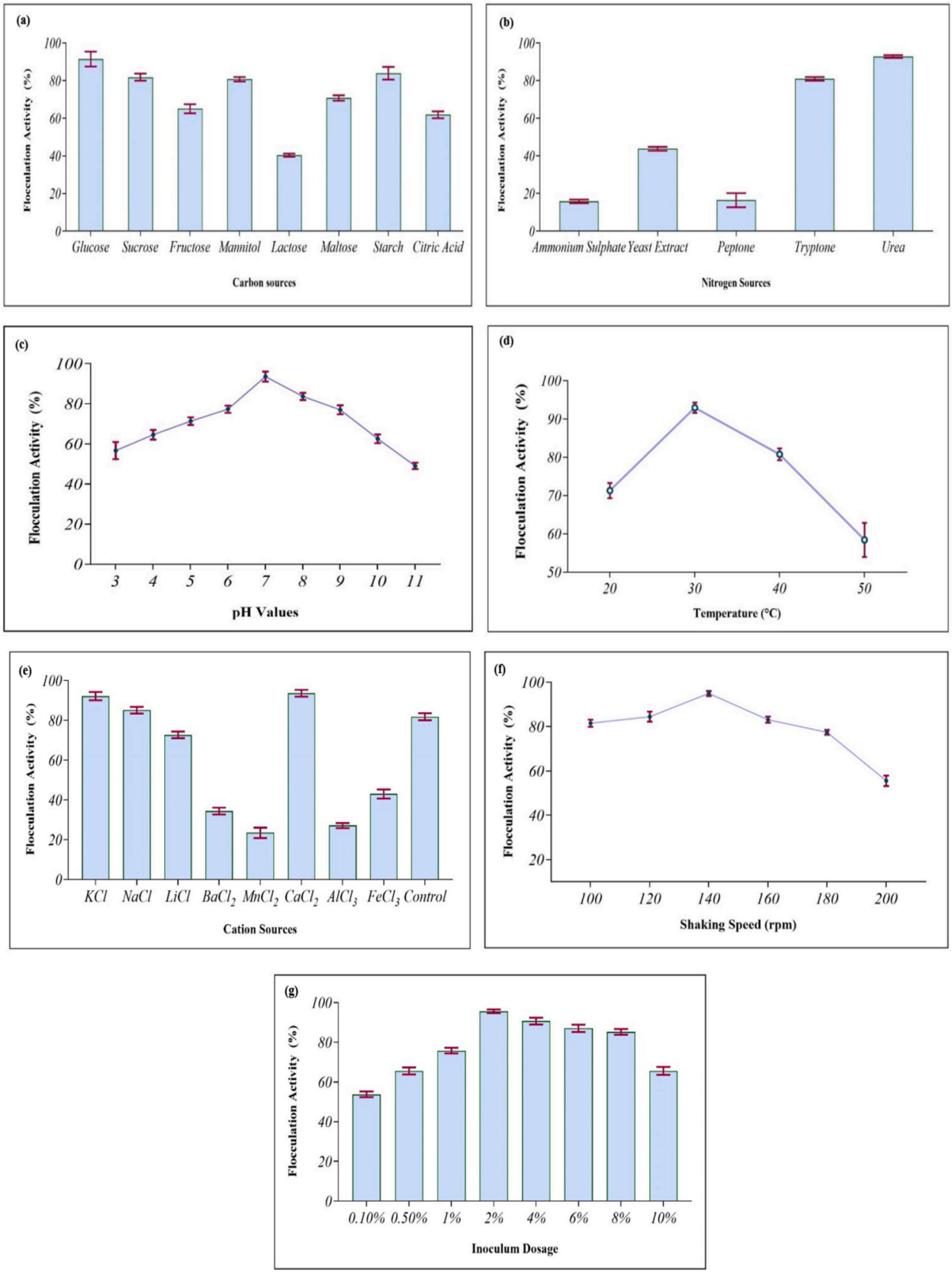

Fig. 5. Effect of various factors on flocculant efficiency in kaolin clay suspension including carbon (a) and nitrogen (b) pH values (c) and temperature (d) cation sources (e) and shaking speed (f) dosage (g).

activity and productivity with the inoculum dosage of 1 % used in a 100 ml production medium.[56]

3.7. Time course assay

The study observed that cell growth was not observed in the initial 12-h lag phase. Following the initial phase, there was a gradual increase in both cell growth and flocculation activity. At 72 h, the growth reached a steady state, coinciding with a flocculation activity peak, marking the stationary phase's onset depicted in Fig. 6. Subsequently, the highest activity at 72 h represented the stationary phase; after 84 h, cell density and flocculation activity gradually declined because of cell autolysis and decreased enzyme activity. This study demonstrates that the production of bioflocculant was the outcome of the bacterial growth phase rather than cell autolysis.[58] The pH of the production medium was initially adjusted to neutral and was observed to decrease over time. This decrease may be attributed to cell metabolism through glucose utilization in the medium or to the production of organic acids during bacterial metabolism.[10]

Similarly, Abu Tawila et al. showed that *Bacillus salmalaya* 139SI-7 reached its peak flocculation activity during the late stationary phase at 72 h.[10] Gong et al.[59] stated that flocculation activity was higher in the early stationary phase and declined after 84 h for organism *Serratia ficaria*.

3.8. Production and purification of bioflocculant

The bioflocculant production under optimized conditions yielded approximately 5.311 g/L for *B. cereus*, beyond the yields reported by Okaiyeto et al.[60] and Jayaprakash et al.[7] The study by Okaiyeto et al. yielded a 1.6 g MBF-UFH purified bioflocculant extracted from *Bacillus* sp. AEMREG7 under optimal conditions.[60] Jayaprakash et al. disclosed a yield of 4.619 g/L purified bioflocculant from *B. subtilis* OL818309.[7] This comparison underscores the effectiveness of the optimized conditions employed in this study, leading to a significantly higher bioflocculant yield.

3.9. Chemical assay and characterization studies of purified bioflocculant

The chemical assay of *B. cereus* was found to have 56.1 % polysaccharide and 10.92 % protein. The bioflocculant was found to be composed of polysaccharides.[61]

The FTIR analysis of *B. cereus* bioflocculant revealed distinct absorption peaks between 400 and 4000 cm^{-1}, as illustrated in Fig. 7. *B. cereus* displayed prominent peaks at 3348.42, 2098.55, 1635.64, 1550.77, 1188.15, 678.94, 601.79, 470.63 and 408.91 cm^{-1}. The broad O–H stretching vibrations observed at 3348.42 cm^{-1} indicated hydroxyl groups commonly associated with polysaccharides.[62] Weak peaks 2600.19 cm^{-1} conform to C–H stretching bands.[63] It exhibited a peak in the region of the amide I band, specifically at 1635.64 cm^{-1}, attributable to C]O stretching vibrations in protein groups, confirming the presence of proteins in the bioflocculant and the presence of carboxylate ions in the polysaccharide.[64] A weak stretching band at 1550.77 cm^{-1} of *B. cereus* suggests aromatic rings in the bioflocculant product.[63] Bands of absorption in the region of 1188.15 cm^{-1} correspond to C–O and C–*O*–C linkages, being polysaccharides as well as other sugar derivatives.[12,63,65] The 678.94 cm^{-1} and 601.79 cm^{-1} peaks were identified as alkyl halides.[48] Furthermore, low-intensity peaks at 470.63 cm^{-1} and 408.91 cm^{-1} were remarking to metal-oxygen stretching, indicating the potential metal-binding capacity of the bacterial bioflocculant. Functional moieties like hydroxyl, amine and carboxylate have been extensively documented for their role in enhancing the flocculation properties of bioflocculants by improving their ability to bind with suspended particles and promote aggregation.[34]

The surface morphology of the purified bioflocculants produced by *B. cereus* was analyzed using SEM, as shown in Fig. 8a–c. The SEM images of the bioflocculant-produced *B. cereus* (Fig. 8b) displayed an irregular, compact, clumpy crystalline pattern. The surface exhibited pores and textures that contributed to the kaolin coupling, directing the conformation of flocs (Fig. 8c). These observations were like previous

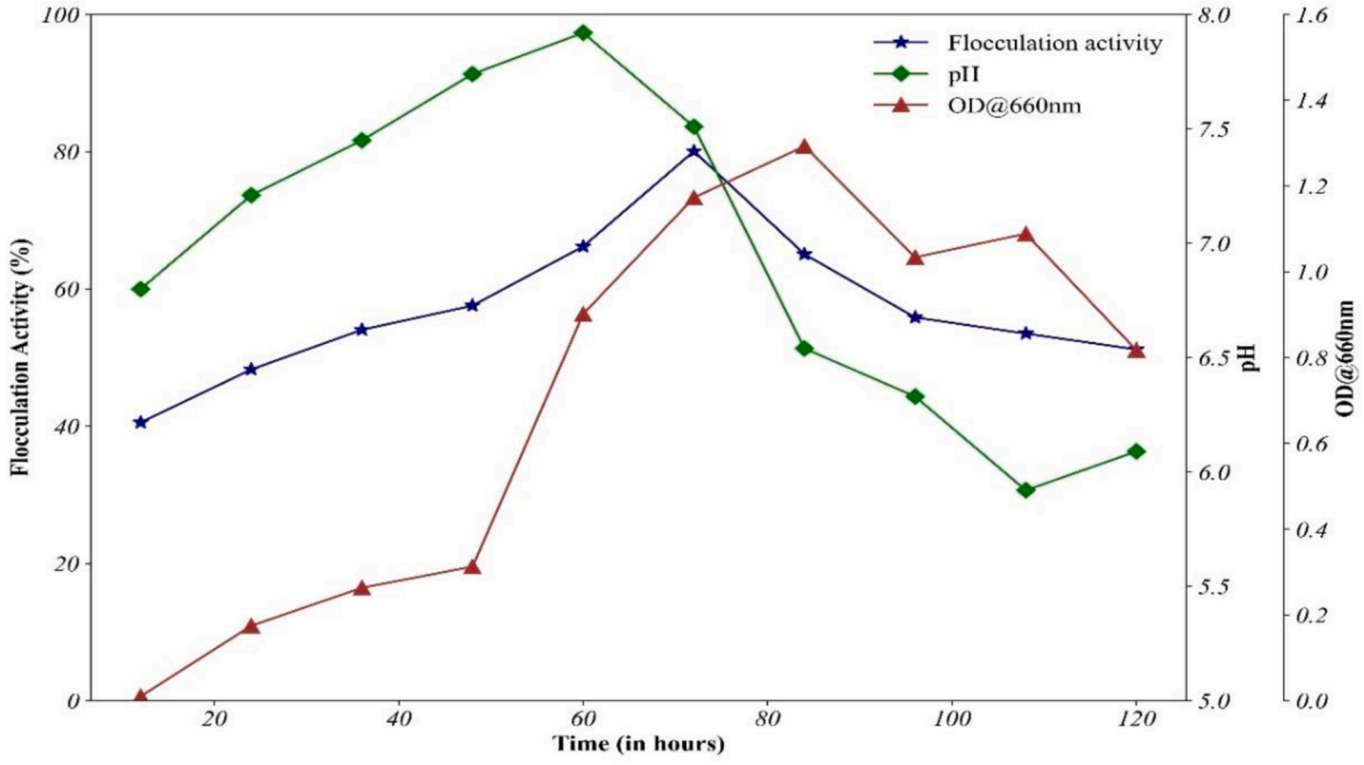

Fig. 6. Time Course assay of bioflocculant production by *B. cereus*.

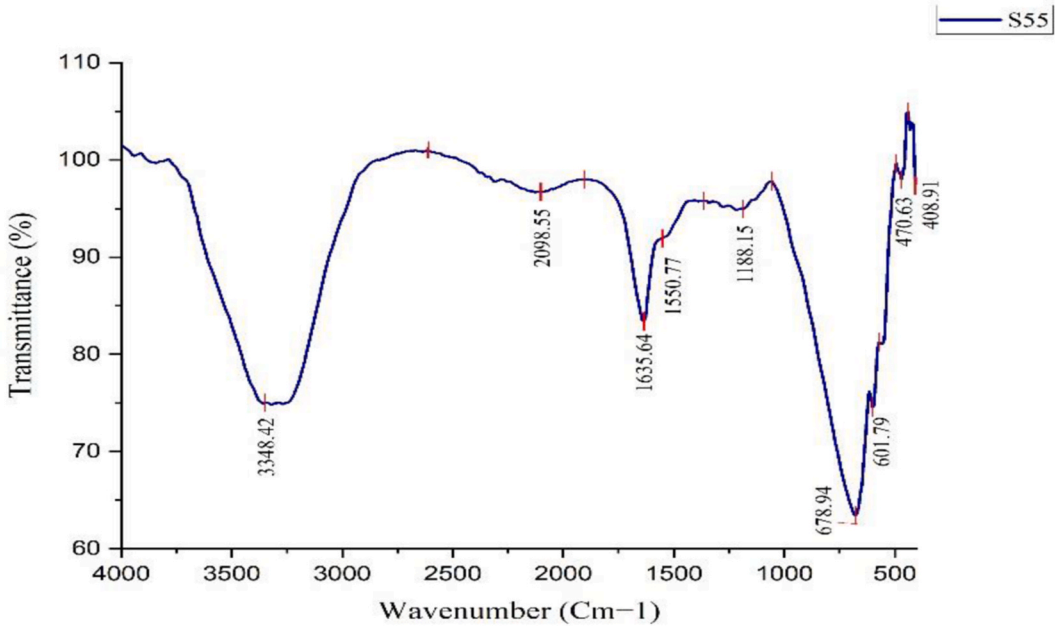

Fig. 7. Fourier Transform infrared Spectrum of Purified Bioflocculant *B. cereus* (S55).

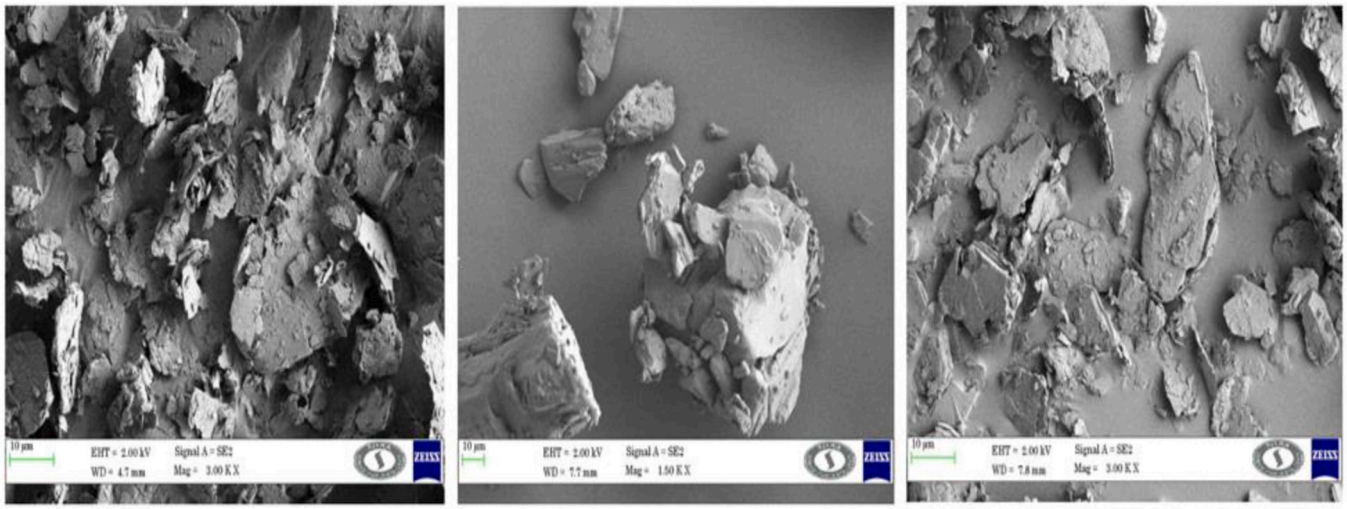

Fig. 8. SEM Analysis of Kaolin particles (a), and purified bioflocculant of *B. cereus* (S55) (b) Flocculated kaolin particles with *B. cereus* bioflocculant (c).

reports on *Bacillus velezensis*[66] and *Alcaligenes faecalis* HCB2,[57] where the bioflocculants exhibited similar morphological characteristics conducive to efficient flocculation.

The elemental composition of the bioflocculants produced by *B. cereus* was depicted in Fig. 9. The analysis revealed the presence of elements, quantified as weight percentages (% *wt*). *B. cereus* contains C (47.62 %), O (52.30 %), and Al (0.08 %). Following the flocculation process, the elemental composition of *B. cereus* was found to primarily consist of O (54.04 %) and Si (26.49 %), along with minor elements such as Mg, Al, K, Ca, Ti, Fe, and Cu making up 19.47 %.[6] These compounds were believed to come significantly with bioflocculant firmness and pliability.[8]

The XRD patterns of *B. cereus* are illustrated in Fig. 10. The XRD pattern of *B. cereus* displayed several 2θ angle peaks at 10° to 50° 2θ with prominent peaks in the spectrum confirming its crystalline structure.[67] These crystallinity characteristics underscore the structural integrity of the bioflocculants, which enhances their effectiveness in various applications, such as improved binding capacity and stability in the treatment process.

3.10. Safety assessment of isolated strain

Table 3 comprehensively details the antibiotic susceptibility profiles. *B. cereus* exhibited resistance to antibiotics such as Ampicillin and Penicillin. However, when exposed to other clinically significant antibiotics, they demonstrated varying degrees of sensitivity, ranging from intermediate to sensitive. This thorough assessment provided critical insights into these bacterial isolates' sensitivity and resistance patterns, facilitating a deeper understanding of their potential applications and limitations in clinical and environmental contexts. Soil is inhabited by a diverse range of microbial populations that possess the capability to evolve and adapt to their specific environmental conditions. Antibiotic

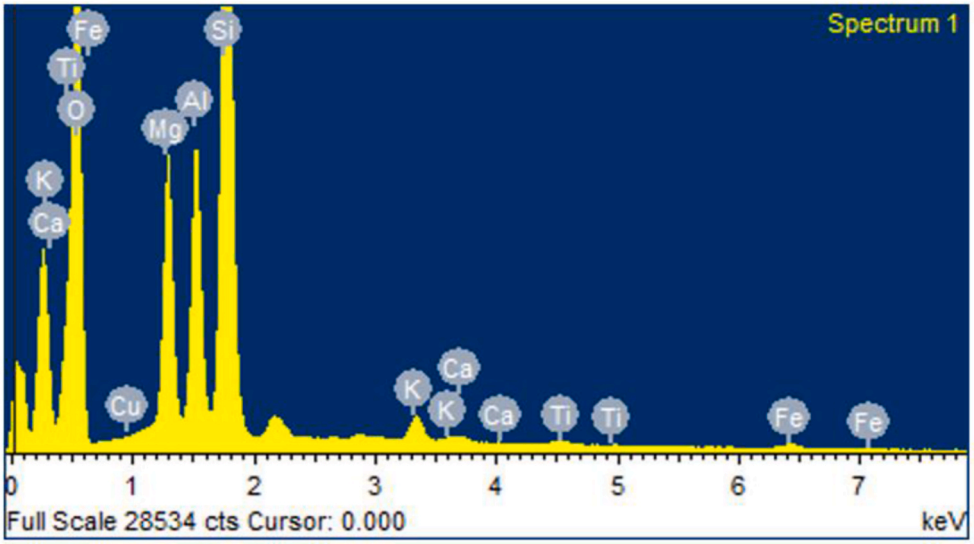

Fig. 9. Elemental composition of *B. cereus* (S55) purified bioflocculant.

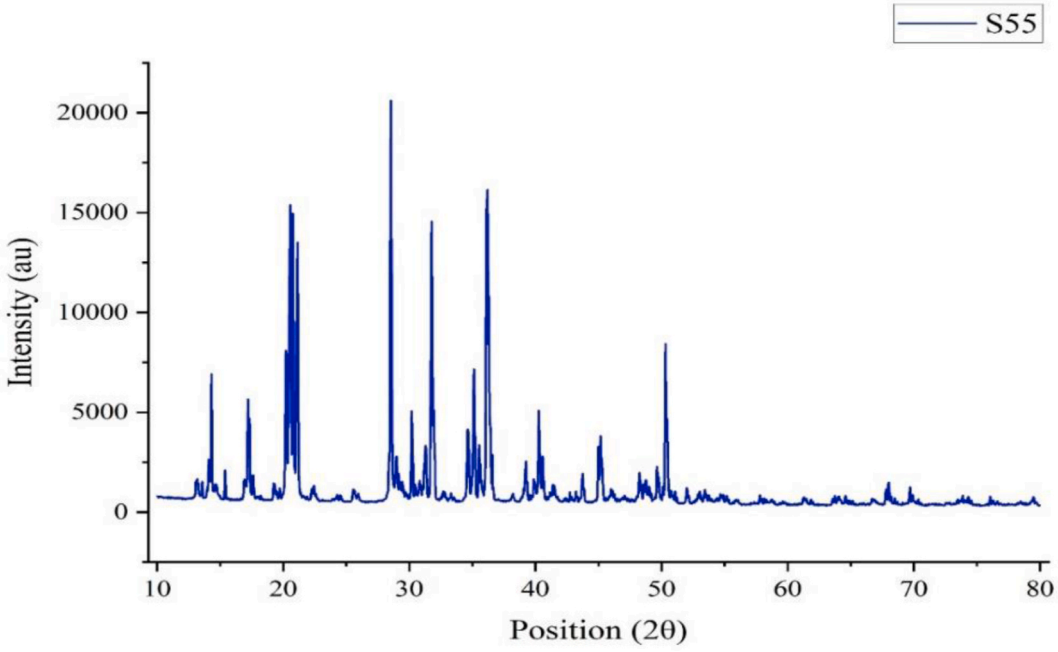

Fig. 10. X-ray diffraction patterns of the purified bioflocculant *B. cereus* (S55).

sensitivity testing is important for understanding the development of resistance among these organisms in natural environments.[68]

3.11. Flocculation properties of a purified bioflocculant

3.11.1. Effect of dosage concentration of the purified bioflocculant

The purified bioflocculant showed an optimal flocculation activity of 95.41 % at a concentration of 0.4 mg/ml, as illustrated in Table 4. Upon increasing the dosage to 1 mg/ml, flocculation activity peaked at optimal dosage and slightly declined to 83.83 %. At higher concentrations, the reduction in flocculation activity can be attributed to potential adverse interactions among the surface charge of the bioflocculant, a phenomenon likely caused by saturation effects. In such cases, the available surface area of the bioflocculant for particle binding diminishes, and excessive amounts of bioflocculant may disrupt the charge distribution within the solution.[69] Agunbidae et al. reported that an optimal dosage of 0.2 mg/ml resulted in the highest activity of purified bioflocculant, achieving 94.88 % effectiveness.[46] This bioflocculant was produced by *Streptomyces platensis*. Similarly, Luvuyo et al. found that the highest flocculation activity was observed at a concentration of 1 mg/ml, produced by a consortium of *Actinobacterium* sp. and *Methylobacterium* sp.[69] Furthermore, Okaiyeto et al. indicated the optimum activity occurred at a concentration of 0.1 mg/ml produced by the consortium *Halomonas* sp. Okoh and *Micrococcus* sp. Leo.[70]

3.11.2. Effect of pH and heating stability of the purified bioflocculant

The pH of the kaolin solution was systematically adjusted, revealing flocculation activity exceeding 70 % across acidic, neutral and basic pH environments. Notably, the peak flocculation activity, measured at 90.74 %, occurred at neutral pH. In contrast, an elevated flocculation activity of 82.49 % was recorded at pH 4. Moderate flocculation was observed at pH levels 5,6 and 8, with these conditions maintaining

Table 3
The isolate was assessed against various antibiotic discs, and the results were categorized as Resistance (R), Intermediate (I) and Sensitive (S) based on the Zones inhibition (mm) according to the standards by the National Committee for Clinical Laboratory Standards. The data presented are the mean ± standard deviations.

Antibiotics	*B. cereus*
Amikacin	S
Amoxicillin	R
Ampicillin	R
Chloramphenicol	S
Ciprofloxacin	S
Erythromycin	S
Gentamycin	S
Imipenem	S
Kanamycin	S
Nalidixic Acid	I
Pencillin	R
Rifampicin	R
Streptomycin	S
Vancomycin	I
Tetracycline	I

stable flocculation rates. The bioflocculant's robust pH tolerance indicates its potential application in wastewater treatment processes without necessitating alterations to the pH of the wastewater.[18] Similarly, Okaiyeto et al. reported that the highest and most stable flocculation activity was achieved at pH 8, reaching 86 %.[70] Furthermore, Wang et al. identified the optimal pH range of 7–9, within which the biopolymer CBF–F26, derived from a mixed culture of Rhizobium *radiobacter* F2 and *Bacillus sphaeicis* F6, exhibited the highest flocculation activity.[71]

The optimal concentration of the purified bioflocculant was 0.4 mg/ml, which was dissolved in sterile distilled water and heated at various temperatures ranging from 20 °C to 120 °C for 30 min. Flocculation peaked from 20 °C to 60 °C, and a slight decline was observed up to 120 °C (Table 4). The bioflocculant maintained an activity level exceeding 85 % even at elevated temperatures, demonstrating significant thermal stability. In accordance with the findings of Luvuyo et al. maximum activity was observed at 80 °C with only a minor reduction in efficiency at 100 °C.[69] The decline in activity at high temperatures may be attributed to the denaturation of proteins within the bioflocculant. The stability of bioflocculant at elevated temperatures can be attributed to the formation of hydroxyl groups and hydrogen bonds.[18] Furthermore, it was noted that the bioflocculant produced by *Serratia ficaria* exhibited stability even after being subjected to heating at 100 °C for 15 min.[59]

3.11.3. Effect of divalent cation on the purified bioflocculant

The impact of calcium chloride at varying concentrations is presented in Table 4. Efficient flocculation activity was observed and dosages ranging from 0.5 % to 3 %. The bioflocculant activity gradually increased with rising dosages, peaking at 2 % before declining at higher concentrations. Cations are believed to reduce the negative charges on both the bioflocculant and the suspended particles, enhancing their interactions and promoting clumping. Furthermore, the consortium of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo exhibited the most significant activity when aluminium cations were present.[70] Additionally, flocculation was markedly improved with the introduction of calcium cations, resulting in a 91.7 % bioflocculant activity from *Virgibacillus* species, as reported by Sekelwa et al.[47]

3.12. Application of the bioflocculant in dye removal

The removal effectiveness of purified bioflocculant at various doses is illustrated in Fig. 11. Methylene blue and safranin demonstrated a reduction in dye intensity, whereas nigrosin and congo red displayed a progressive decline in removal efficiency. The removal increased gradually with dosage and reduced the intensity of the dye due to the absorbent area and absorbing sites.[50] As the concentration of the bioflocculant increased, the removal efficiency also increased. The decolourization ability of a dye molecule was influenced by the number of sulfonic groups it contains and its size. When larger molecules were absorbed onto the bioflocculant, they may prohibit others from being adsorbed. Additionally, a single molecule with more sulfonic groups would adsorb more bioflocculant. The attraction of colours to the bioflocculant was significantly affected by electrostatic interaction. When the functional groups of the bioflocculant molecules became completely deprotonated due to decreased pH, they exhibited negative characteristics. Consequently, the flocculation activity decreased because the electrostatic repulsion prevented the dyes from coming into close contact with the bioflocculant.[43] It is apparent from the chemical compositions of the dyes that they contain one or more sulfonic groups within a single molecule, leading to their anionic behaviour and solubility. The presence of carboxylate groups facilitated the positive charge carried by the flocculant molecules. Consequently, the electrostatic interaction between the flocculant molecules and the negatively charged dye molecules causes flocculation and decolourization.[72]

3.13. Application of *B. cereus* bioflocculant in domestic wastewater

In this study, the optimal purified bioflocculant significantly reduced BOD and COD in domestic wastewater by 48 % and 36.64 %, respectively. Other parameters, such as total dissolved solids, total suspended solids, and pH, showed notable reductions, as outlined in Table 5. These findings support microbial-based flocculants as a promising alternative for wastewater treatment, in contrast to chemical flocculants.[8] Furthermore, Selepe et al. reported that *Providencia huaxiensis* OR794369.1 effectively removed and reduced the contents of different sources of wastewater from coal mines and domestic sources.[8]

Table 4
Effect of various parameters on flocculation activity by purified bioflocculant.

Dosage		pH		Temperature		Cation dosage	
Concentration (mg/ml)	Flocculation Activity (%)	Values	Flocculation Activity (%)	Temperature (°C)	Flocculation Activity (%)	Ca^{2+} (%)	Flocculation Activity (%)
0.2	81.67 ± 1.89	3	75.16 ± 1.30	20	91.82 ± 0.65	0.5	87.55 ± 1.21
0.4	95.41 ± 1.66	4	82.49 ± 2.65	40	91.07 ± 1.12	1	83.48 ± 1.06
0.6	87.81 ± 1.30	5	78.46 ± 1.08	60	89.62 ± 1.12	1.5	89.19 ± 0.93
0.8	85.94 ± 1.42	6	78.56 ± 1.72	80	87.04 ± 1.31	2	96.17 ± 1.34
1	83.83 ± 1.42	7	90.74 ± 0.74	100	86.52 ± 0.98	2.5	84.64 ± 1.45
		8	78.08 ± 1.02	120	87. 46 ± 1.57	3	75.26 ± 1.76
		9	73.37 ± 0.56				
		10	72.14 ± 0.75				
		11	70.28 ± 1.60				

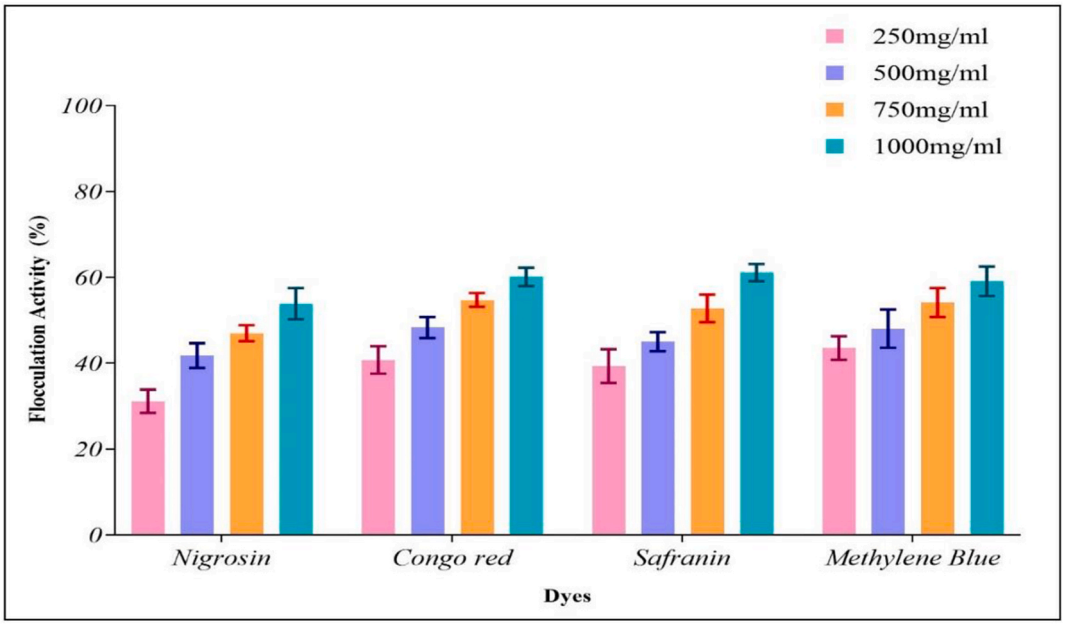

Fig. 11. Dye removal efficiency of bioflocculant produced by *B. cereus*.

Table 5
Removal rate of bioflocculants with domestic wastewater.

Parameters	Before Treatment	After Treatment
pH	8.12	6.67
TDS (ppm)	1064	927
BOD (mg/ml)	581.36	300.98
COD (mg/ml)	742	470.12
TSS (mg/ml)	310	140.38
Colour	Pale green	Pale green
Odor	Bad	Slightly Neutral
Flocculation Activity (%)	Very low	41.20 %

4. Conclusion

The research successfully isolated and characterized bacteria from the soil with the capacity to produce bioflocculant, identifying them as *B. cereus* (S55) through morphological analysis and 16S rRNA sequencing. The details of the strain were deposited in GenBank under accession number OQ359411. Culture conditions were optimized using the one-factor-at-a-time (OFAT) method, resulting in an increased bioflocculant yield. *B. cereus* exhibited optimal flocculating activity at a neutral pH, utilizing glucose and urea as nutrient sources, with an optimal stirring speed of 140 rpm. Chemical analysis revealed that *B. cereus* contains 56.1 % polysaccharides and 10.92 % protein. Infrared spectroscopy confirmed the presence of functional groups such as hydroxyl, amine, and carboxylate groups in the molecular structure of the bioflocculant. Structural insights acquired through SEM and XRD analyses revealed the crystalline properties of the bioflocculants. The efficacy of dye removal was also assessed, demonstrating the bioflocculant's effectiveness in removing contaminants from domestic wastewater. The encouraging outcomes of this study highlight the necessity for ongoing exploration and innovation in microbial technology, emphasizing sustainable solutions for global environmental challenges. These advancements can potentially transform industrial wastewater management, thereby contributing to cleaner water bodies and a healthier environment.

CRediT authorship contribution statement

Karthikeyan Harinisri: Writing – original draft, Visualization, Software, Resources, Methodology. **Ragothaman Prathiviraj:** Writing – review & editing, Software, Investigation, Formal analysis. **Balasubramanian Thamarai Selvi:** Validation, Supervision, Conceptualization.

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.

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Abbreviations used

BLAST	Basic Local Alignment Search Tool
CRA	Congo Red Agar
EPS	Extracellular polymeric substances
FA	Flocculation activity
FTIR	Fourier Transform Infrared Spectroscopy
MEGA	Molecular Evolutionary Genetics Analysis
MH	Muller-Hinton Broth
NCBI	National Center for Biotechnology Information
NCCLS	National Committee for Clinical Laboratory Standards
OFAT	One-factor-at-a-time
PCR	Polymerase chain reaction
R	Resistance
S	Sensitive
SEM	Scanning Electron Microscopy
VP	Voges-Proskauer
XRD	X-ray diffraction

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

org/10.1016/j.biotno.2024.11.003.

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