

Studies on Biofloculant Production by *Bacillus* sp. AEMREG7

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Abstract

The detrimental effects of inorganic and organic synthetic flocculants on humans and their recalcitrance in the environment have necessitated the search for safe, eco-friendly alternatives, including microbial flocculants. A biofloculant-producing bacteria was isolated from sediment samples of Algoa Bay in the Eastern Cape Province of South Africa. The bacteria was identified through partial 16S ribosomal ribonucleic acids (rRNA), nucleotide sequencing, and BLAST analysis of the gene sequence that showed the bacteria to have 98% similarity to *Bacillus algicola* strain QD43 and deposited in GenBank as *Bacillus* sp. AEMREG7 with accession number KF933697.1. The effect of culture conditions on MBF-UFH production revealed optimally at inoculum size of 3% (v/v), glucose, a mixed nitrogen source [$\text{NH}_4(\text{SO}_4)_2$ + urea + yeast extract], and pH 6 for inoculum size, carbon and nitrogen sources, and initial medium pH for the fermentation process, respectively. The spent culture broth-rich biofloculant attained the highest flocculating activity of 88.5% after 72 h of fermentation during the exponential phase of bacterial growth. It showed a high flocculating activity for kaolin clay suspension over a wide pH range of 4-10 with maximum flocculating activity observed at pH 6; and also retained 70.5% flocculating activity after heating at 100°C for 60 min. These findings suggest that MBF-UFH has a great potential to substitute hazardous chemical flocculants commonly used in water treatment, hence reducing deformities among individuals in society.

Keywords: *Bacillus* sp. AEMREG7, MBF-UFH, biodegradable, glycoprotein, flocculating activity

Introduction

Flocculants are used in waste/drinking water treatment processes as well as in downstream processing [1]. They are usually grouped as synthetic organic, inorganic, and naturally occurring flocculants [2], and owing to

the efficacy and cost-effectiveness of synthetic organic and inorganic flocculants, they find important uses in industries [3, 4]. Aluminium salts are among the most widely utilized inorganic flocculants due to the high efficiency of flocculation and cost effectiveness relative to other flocculants [5]; nevertheless, the problem of residual aluminium in water has raised serious public health concerns [6]. Besides, a large aluminium salt dosage is

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required for effective flocculation, which always results in large volumes of sludge [7]. The challenge of sludge generated by aluminium salts has been abolished by the use of polyacrylamide. Nevertheless, major consequences have been associated with polyacrylamide, including poor biodegradability and production of carcinogenic monomer [8].

As a result of these demerits allied with chemical flocculants, it is imperative to appraise water quality on a perpetual basis [9]. In order to adequately provide these services to meet people's demands, it is incumbent upon governments and society at large to develop, among other things, appropriate scientific strategies in wastewater treatment technology that are not only environmentally friendly but also cost-effective. Therefore, the development of safe and biodegradable flocculants cannot be overemphasized [9].

Bioflocculants, on the other hand, have enormous advantages such as biodegradability, lack of secondary pollution from intermediate degradation, and being innocuous to humans, hence their relevance in water-treatment technology has gained much popularity [10]. Bioflocculants are secondary metabolites secreted by microorganisms during growth [11]. Over the decades, many microorganisms, including bacteria, fungi, and algae have been reported to produce bioflocculants with different chemical compositions such as polysaccharide, protein, glycoprotein, and nucleic acids [12]. Gao et al. [13] through several studies have been reported on bioflocculant production from different microbes [14], with high production cost being the major limiting factor for large-scale production for industrial applications [15, 16]. Therefore, it would be economically favourable, as a cost-cutting measure, to utilize cost-effective substrates for bioflocculant production on an industrial scale [17].

In this paper, a bioflocculant-producing bacteria was isolated from the sediment samples of Algoa Bay in the Eastern Cape Province of South Africa. The bacteria was identified by 16S rRNA sequence analysis and the various factors influencing MBF-UFH production were investigated to determine the cost-optimal culture conditions favourable for the bacteria.

Materials and Methods

Sample Collection and Isolation of Bacterial Strain

The sediment samples of Algoa Bay (a marine environment) were collected and processed according to Jensen et al. [18] with some modifications. A half gram (0.5 g) of wet sample was diluted with 5 ml of sterile seawater. The suspension was vortexed and allowed to sediment for 60 s, out of which 100 µl of the suspension was inoculated onto the surface of R2A agar plates, spread with a sterile glass rod and incubated for 96 h. The distinct isolates were picked and streaked onto nutrient agar plates to obtain their purity and separate from mixed populations.

Media and Cultivation Conditions

The composition of activation medium included (per litre): beef extract 3 g, Tryptone 10 g, and NaCl 5 g [19]. Production medium composed (per litre): glucose 20 g, K₂HPO₄ 5 g, KH₂PO₄ 2 g, NH₄(SO₄)₂ 0.3 g, urea 0.5 g, yeast extract 0.5 g, MgSO₄·7H₂O 0.3 g, and NaCl 0.1 g [20]. The medium for a slant included (per litre): glucose 20 g, K₂HPO₄ 5 g, KH₂PO₄ 2 g, NH₄(SO₄)₂ 0.3 g, urea 0.5 g, yeast extract 0.5 g, MgSO₄·7H₂O 0.3 g, NaCl 0.1 g, and agar 20 g [21]. All media were prepared using filtered seawater and sterilized by autoclaving at 121°C for 15 min. For all experiments, the cultivations were carried out at 28°C and 160 rpm.

Screening of Bioflocculant-Producing Bacteria

About 48 bacterial isolates were obtained from sediment samples of Algoa Bay and screened for bioflocculant production as follows. Two loopfulls of the isolate from a nutrient agar plate were inoculated into 50 ml of activation medium and incubated for 24 h. One millilitre of the activation medium was inoculated into a 250 ml flask containing 50 ml of production medium and incubated at 28°C in a rotary shaker at 160 rpm for 72 h. Two millilitres of the fermented broth were carefully withdrawn and centrifuged at 4,000 rpm for 30 min; the cell-free supernatant was used to determine the flocculating activity according to the description of Kurane et al. [22]. The isolate with the highest flocculating activity (MBF-UFH) was preserved in 20% glycerol stock and stored at -80°C for future studies.

Determination of Flocculating Activity

The flocculating activity of MBF-UFH was determined according to the description of Kurane et al. [22]. Kaolin clay was used as test material in preparing the water suspension at a concentration of 4 g/l. One-hundred ml of the kaolin suspension were measured into a 250 ml conical flask. Three ml of 1% CaCl were added, followed by 2 ml of MBF-UFH. The solution was agitated for 60 s, transferred into a graduated measuring cylinder, and allowed to sediment for 5 min. A control was prepared in a similar way, but MBF-UFH was replaced with un-inoculated production medium. The flocculating activity was calculated using the formula:

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

...where A = optical density of the control at 550 nm and B = optical density of a sample at 550 nm.

16S rRNA Sequence Identification of Bacteria

DNA extraction was conducted using the boiling method described by Cosa et al. [23], whereby two to three colonies were suspended in 70 µl of sterile double-distilled

water. The samples were heated in a water bath at 100°C for 10 min, cooled for 5 min, and centrifuged at 3,000 rpm for 5 min. The supernatant was transferred to a clean tube and stored at 4°C. This serves as the template in the PCR assay. PCR was carried out in 50 µl reaction volume containing 2 mM MgCl₂, 2U Supertherm Taq polymerase, 150 mM of each dNTP, 0.5 mM of each primer (F1: 59-AGAGTTTGTATCITGGCTCAG-39; I = inosine and primer R5: 59-ACGGITACCTTGTTACGACTT-39), and 2 µl template DNA. The primers in this study were used to amplify nearly full-length 16S rRNA sequences. The PCR programme used was an initial denaturation (96°C for 2 min), 30 cycles of denaturation (96°C for 45 s), annealing (56°C for 30 s), extension (72°C for 2 min), and a final extension (72°C for 5 min). Gel electrophoresis of PCR products was conducted on 1% agarose gels to confirm that a fragment of the correct size had been applied.

Optimization of Culture Conditions for MBF-UFH Production

Different inoculum sizes ranging 1-5% of the seed culture were used to inoculate the production medium and the effect of each on MBF-UFH production was assessed according to the description of Zhang et al. [20]. The effects of the medium compositions on produced MBF-UFH were also investigated. We assessed the effect of different carbon sources on MBF-UFH production by replacing the glucose in the production medium by one of the following carbon sources (20 g/l): fructose, starch, sucrose, maltose, lactose, and Na₂CO₃. Both inorganic and organic nitrogen sources were also evaluated for their effects on biofloculant production. The nitrogen sources (1.3 g/l) included: yeast extract, urea, NH₄SO₄, NH₄NO₃, peptone, tryptone, and mixed nitrogen source ((NH₄)₂SO₄ + urea + yeast extract). The impact of initial pH of the production medium on MBF-UFH production and flocculating activity were also examined by adjusting the pH from 4-10, with 0.1 M HCl and NaOH [14]. The effect of cations on the flocculating activity of MBF-UFH was appraised by replacing the CaCl₂ solution [24] in the flocculation assay with various metal solutions, which included: Li⁺, Na⁺, K⁺, Ca²⁺, Mg²⁺, Al³⁺, and Fe³⁺, and the thermal stability of the crude MBF-UFH was investigated as described by Gong et al. [21] with the following modifications: A 72 h old culture was centrifuged at 4,000 rpm for 30 min to obtain cell-free supernatant. Two ml of MBF-UFH were heated in a water bath for 1 h at 50-100°C.

Time Course of MBF-UFH Production

Optimum culture conditions were used for time course of MBF-UFH production in accordance with the method described by Liu et al. [14] and Nwodo et al. [25], with some modifications. The seed culture was prepared by inoculating two loopfulls of bacterial colonies into 50 ml of activation medium and incubated overnight in a rotary shaker at 28°C. The fermented broth was

diluted with sterile saline water to an optical density of 0.1 at OD₆₆₀ [26]. The standardized bacterial suspension was inoculated into 200 ml of the production medium in 500 ml flasks and incubated in a rotary shaker at 160 rpm and 28°C for 192 h of cultivation. 10 ml of the samples were withdrawn periodically at time intervals of 24 h; 2 ml of the fermented broth were centrifuged and the supernatant was used to determine MBF-UFH in accordance with the method of Kurane et al. [22]. The growth of the bacteria was monitored by bacterial counts using a standard spread plate technique and optical density OD₆₆₀, pH, and flocculating activity of MBF-UFH with time.

Extraction and Purification of MBF-UFH

The extraction and purification of the biofloculant were carried out in accordance with Cosa et al. [27] and Li et al. [28]. Optimal culture conditions were used to produce MBF-UFH over a growth period of 72 h and the fermented broth was centrifuged at 4,000 rpm for 30 min. One volume of sterile distilled water was added to the supernatant and centrifuged at 4,000 rpm for 30 min. Two volumes of ethanol were added to the supernatant and allowed to stand overnight at 4°C. The precipitate was collected by centrifuge and vacuum dried. The crude MBF-UFH was weighed and further purified by being dissolved in 100 ml of distilled water and one volume of a mixture of chloroform and n-butyl alcohol (5:2, v/v) was added and agitated for 60 s. The solution was left standing at room temperature for 12 h prior to dialysing against distilled water overnight. Two volumes of ethanol were added to the dialysed solution and the recovered precipitate was dissolved in distilled water and vacuum dried.

Statistical Analysis

Triplicate values were obtained, averaged, and statistically analysed using SPSS version 8. The error bars represent the standard deviation (SD) of the data.

Results

A total of 48 bacterial isolates were obtained from the sediment samples of Algoa Bay. This isolate showed good biofloculant production potential with flocculating activity of over 60% for kaolin clay suspension. It appeared yellowish, filamentous with branching, and spore-forming on the nutrient agar. Amplification of its 16S rRNA gene yielded the expected amplicon size of appropriately 1.5 kb. The partial sequence (1073 nucleotides) 16S rRNA gene was aligned with all related sequences in NCBI database by the BLASTN program and showed about 98% similar to *Bacillus algicola* strain QD43 and deposited as *Bacillus* sp. AEMREG7 in the GenBank with accession number KF933697.1. A phylogenetic tree was constructed according to the neighbour-joining algorithm as shown in Fig. 1.

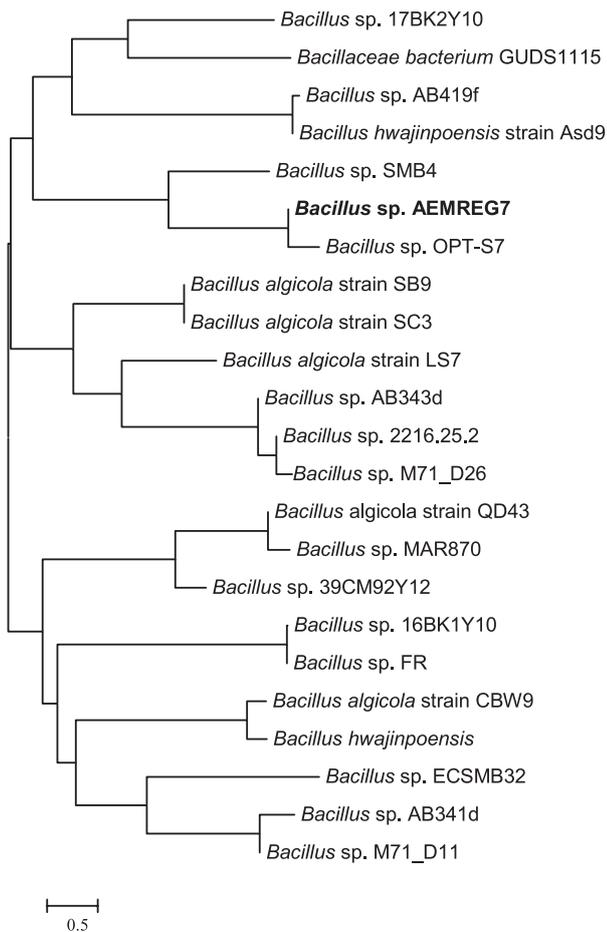


Fig. 1. Phylogenetic tree showing the relationship among *Bacillus* sp. AEMREG7 and published 16S rRNA sequence.

Effect of Inoculum Size on MBF-UFH Production

The relationship between inoculum size and MBF-UFH production was investigated and the results are presented in Fig. 2. It was observed that the increase in inoculum size of the seed culture from 1 to 3 (%v/v) resulted in an

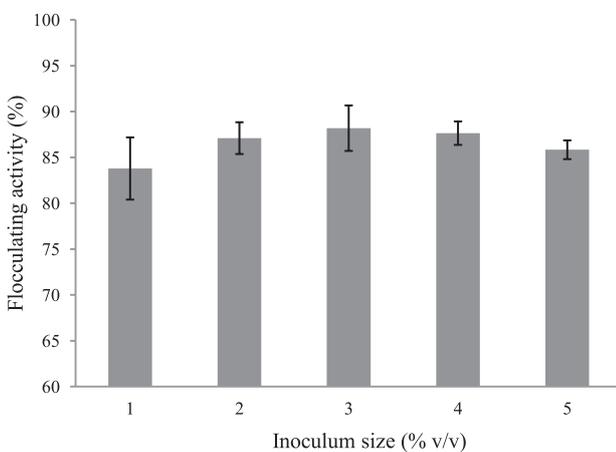


Fig. 2. Effect of inoculum size on MBF-UFH production by *Bacillus* sp. AEMREG7.

Table 1. Effect of carbon and nitrogen source on MBF-UFH production by *Bacillus* sp. AEMREG7.

Carbon source	FA±SD	Nitrogen source	FA±SD
Glucose	88.03±2.37	Peptone	71.50±2.98
Fructose	46.64±8.34	Tryptone	37.85±5.74
Maltose	34.16±3.37	Urea	72.12±8.50
Sucrose	70.34±5.27	Yeast extract	79.02±3.92
Lactose	67.03±2.27	NH ₄ SO ₄	53.58±2.862
Starch	6.10±0.92	NH ₄ NO ₃	54.36±5.85
Na ₂ CO ₃	55.62±2.65	Mixed nitrogen	86.35±1.72

* FA = Flocculating activity, SD = Standard deviation, mixed nitrogen = [NH₄(SO₄)₂ + urea + yeast extract]

increase in flocculating activity (83.7-88.2%), which is an indication of MBF-UFH production. However, a further increase in inoculum size led to a steady decrease in flocculating activity culminating at 85.8% observed at 5% v/v. Inoculum size of 3% (v/v), which resulted in optimum MBF-UFH production and was used in all subsequent experiments.

Effect of Carbon and Nitrogen Source on MBF-UFH Production

The effect of different carbon sources on MBF-UFH production was evaluated and represented in Table 1. Culture media were separately supplemented with different carbon sources cultivated at agitation speed of 160 rpm and 28°C for 72 h. The microorganism showed preference for glucose as an effective carbon source for MBF-UFH production with the highest flocculating activity of 88.03%, followed by sucrose (70.34%) and lactose (67.03%). The medium containing inorganic carbon source Na₂CO₃ supported MBF-UFH production

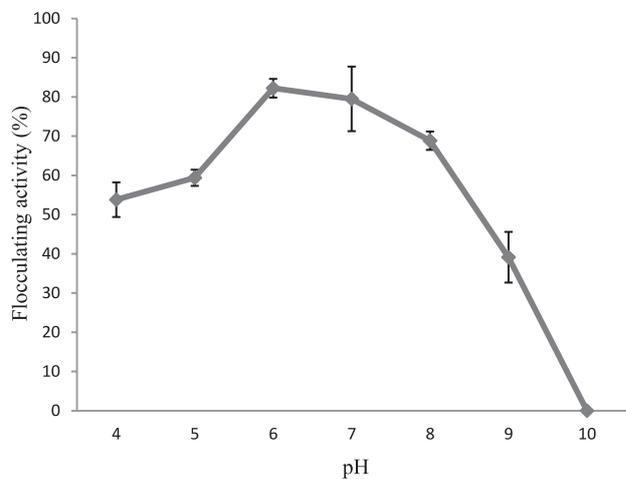


Fig. 3. Effect of initial pH of growth medium on MBF-UFH production by *Bacillus* sp. AEMREG7

Table 2. Effect of cations and pH on the flocculating activity of MBF-UFH produced by *Bacillus* sp. AEMREG7.

Cations	FA(%)±SD	pH	FA(%)±SD
Na ⁺	85.03±3.96	4	53.78±4.41
K ⁺	83.76±1.99	5	59.57±2.06
Li ⁺	86.57±1.74	6	82.20±2.39
Mg ²⁺	80.59±3.51	7	79.49±8.23
Mn ²⁺	86.27±1.01	8	68.84±2.36
Ca ²⁺	87.20±2.11	9	39.12±6.46
Fe ³⁺	-	10	-
Al ³⁺	92.19±3.03		

* FA = Flocculating activity, SD = Standard deviation

with flocculating activity of 55.62% compared to fructose and maltose. The lowest flocculating activity of 6.1% was observed with starch as a carbon source.

The effect of different nitrogen sources was investigated to find the suitable nitrogen supplement for MBF-UFH production and the results are presented in Table 1. Apart from tryptone, which had the lowest flocculating activity (37.85%), other organic nitrogen sources such as peptone (71.5%), urea (72.12%), and yeast extract (79.02%) favoured the production of MBF-UFH. Both inorganic nitrogen sources tested moderately buttressed

MBF-UFH production with flocculating activity of 53.58% and 54.36% for NH₄(SO₄)₂ and NH₄NO₃, respectively. The highest flocculating activity of 86.3% was observed when the mixed nitrogen source [NH₄(SO₄)₂ 0.3 g, + urea 0.5 g, + yeast extract 0.5 g] was used as a supplement.

Effect of Initial pH of Growth Medium on MBF-UFH Production

The pH of growth medium has a great influence on biofloculant production [22]. The effect of initial pH of growth medium was assessed (Fig. 3). It was noticed that the production of MBF-UFH was optimal at pH 6 with flocculating activity of about 82.2%, followed by pH 7 with flocculating activity of 79.5%. The production of MBF-UFH was completely inhibited at pH 10 and, as a result, pH 6 was selected for the subsequent experiments.

Effect of Cations and pH on the Flocculating Activity of MBF-UFH

The synergistic effect of various cations on MBF-UFH produced by *Bacillus* sp. AEMREG7 is depicted in Table 2. All the cations evaluated enhanced flocculating activity above 80% except for Fe³⁺, which inhibited the flocculating activity of MBF-UFH. The highest flocculating activity of 92.2% was observed with Al³⁺, followed by Ca²⁺ at 87.2%.

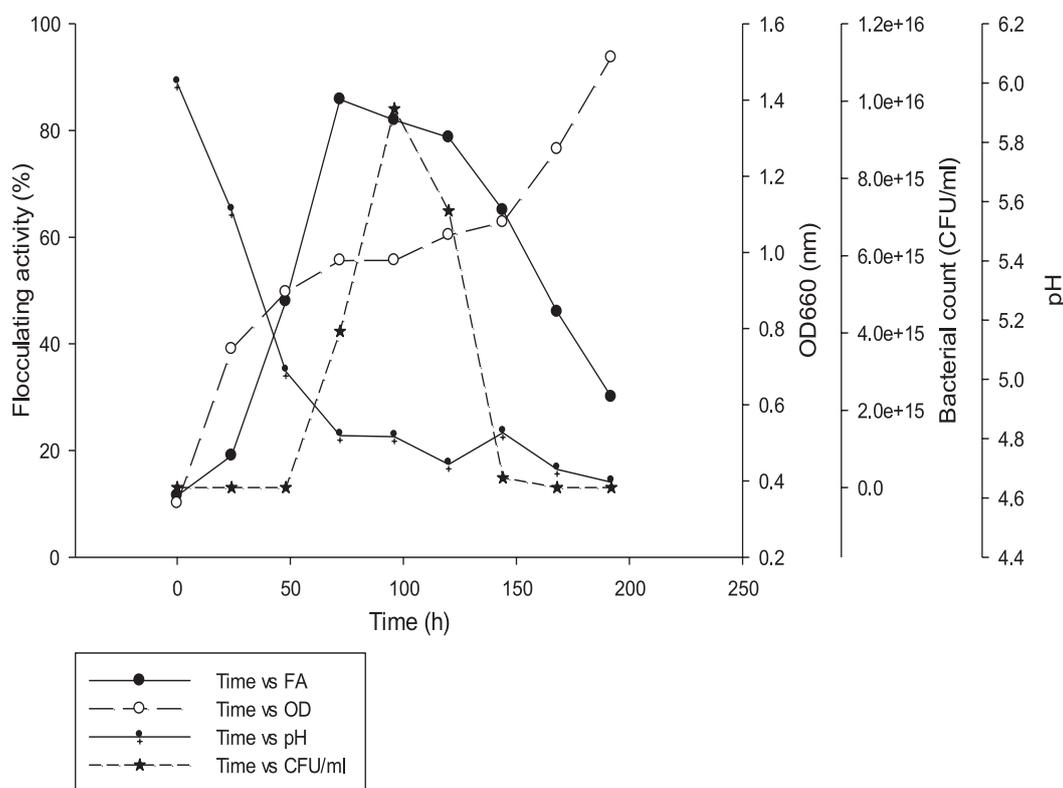


Fig. 4. Time course of MBF-UFH production by *Bacillus* sp. AEMREG7, the bacteria growth was monitored over 192 h (bacterial count expressed in CFU/ml and OD660), flocculating activity (FA) and pH measured over cultivation time.

Table 3. Thermal stability of crude MBF-UFH produced by *Bacillus* sp. AEMREG7

Temperature (°C)	FA (%)±SD
50	79.7+4.23
60	85.1+4.54
70	85.4+3.47
80	83.1+2.72
90	82.9+0.50
100	70.5+5.32

* FA = Flocculating activity, SD = Standard deviation

Evaluation of the effect of pH on the flocculating activity of MBF-UFH is represented in Table 2. MBF-UFH exhibited a wide pH range from acidic (pH 4) to alkaline (pH 9) with the flocculation rate marginally better at weak acidic condition. The highest and lowest flocculating activities of 87.8% and 48.8% were achieved at pH 6 and 10, respectively. There was no significant difference in flocculating activity from pH 4-6 as against a noticeable sharp decline between pH 9 and 10.

Time Course of MBF-UFH Production

Fig. 4 shows the time course of MBF-UFH by *Bacillus* sp. AEMREG7. The bacterial growth was monitored over a period of 192 h. Initially, a corresponding increase in flocculating activity with cultivation time was observed with a maximum activity of 85.8% attained at 72 h during the active phase of cell growth. It was observed that a further increase in cultivation time led to a decrease in flocculating activity as well as the number of viable cells expressed as CFU/ml; and at the same time, the optical density of the culture increased progressively with the increase in flocculating activity of the produced MBF-UFH. The pH of the culture broth decreased gradually throughout the cultivation time from 6-4.65. A yield of about 1.6 g of the crude bioflocculant was obtained under optimum culture conditions.

Thermal Stability of Crude MBF-UFH

Results of thermal stability of crude MBF-UFH are represented in Table 3. It was found that the increase in temperature (from 50 to 70°C) resulted in an increase in flocculating activity of MBF-UFH from 79.7-85.5%. Further increase in temperature resulted in a decline in activity with a residual flocculating activity of about 70.5% recorded at 100°C.

Discussion

Water is one of the most precious resources without which life on earth is not possible [7]. It is a basic need

connected with the very survival of human beings. Access to safe drinking water is a basic human right and essential for achieving gender equality, sustainable development, and poverty alleviation. Lack of safe water and basic sanitation is an acute problem for women and girls who live in poor overcrowded urban slums – as women are usually the ones who face the burden of water collection in homes – and consequently lessening the burden on women who are usually the caregivers to people crippled by drinking chemically treated water.

Every industrial process is a potential source of pollution and requires a specific treatment of the wastes produced [29]. The introduction of waste treatment(s) processes increases the plant-running costs, thus making any effort to improve their efficiency difficult to achieve. Cost-effective treatment such as flocculation by microbial flocculants have gained huge biotechnological attention in recent years [1]. In view of this, isolation of bioflocculant-producing microorganisms from diverse environments has become imperative.

Water milieu remains a good source of microbes with novel metabolites [30]. Depending on specific environmental conditions, microbes can be manipulated to produce exopolymeric substances of a particular composition, and physiochemical properties that promote the survival of the microbial population [31]. Nonetheless, marine environments have been documented supplying interesting bacteria-producing unique bioactive compounds such as bioflocculants that have various industrial applications [32].

Identification of MBF-UFH-Producing Microorganisms

In this study, a marine bacteria was isolated from sediment samples of Algoa Bay and – based on 16S rRNA sequence analysis – the BLAST results showed about 98% similarity to *Bacillus algicola* strain QD43 and were deposited as *Bacillus* sp. AEMREG7 in the GenBank with accession number KF933697.1. The results obtained from the blasted the nucleotide sequence of the bacterial gene was compared with those in the Genebank as shown in Fig. 1.

Our findings were in accordance with many previous studies that reported several bacterial strains closely related to the genus *Bacillus* that have been explored in bioflocculant production [1, 12, 27, 29, 33-35]. However, no report in literature has explored the location of *Bacillus* sp. AEMREG7 in bioflocculant production.

Optimization of Culture Conditions for MBF-UFH Production

Bacteria can utilize the nutrients in the culture medium to synthesize high molecular-weight polymers intracellularly under the action of specific enzymes, which can be excreted into the medium or on the surface of the bacteria as a capsule [31]. The bacteria produce a wide range of extracellular polymeric substances (EPS)

composed of polysaccharides, proteins, lipids, and other biological macromolecules [36]. Hence, optimization of media compositions and fermentation conditions, discovering new bioflocculant-producing bacterial strains, and utilizing cost-effective substrates have become logical approaches for improving the yields and production cost of these exopolymeric substances from different microbes [37].

Effect of Inoculum Size on MBF-UFH Production

It has been articulated in previous studies that small inoculum sizes prolong the stagnant phase of bacteria growth and a large inoculum size forms niche and inhibits bioflocculant production [20, 34]. In Fig. 2, the production of MBF-UFH was significantly enhanced when 3% inoculum size was used. A slight decrease in MBF-UFH production was noticed with further increase in inoculum sizes. Gong et al. [21] found that 1% inoculum size was favourable for bioflocculant production by *Serratia ficaria*. On the other hand, Wang et al. [17] observed that 5% inoculum size was found to be preferable for bioflocculant production by *Klebsiella mobilis*.

Effect of Carbon Source on MBF-UFH Production

The structure and composition of bioflocculants depend on a number of factors such as the type of carbon source used in the production medium and also fermentation conditions [38]. Optimization of fermentation conditions influences not only the yield of bioflocculants and physicochemical properties of bioflocculants, but also their chemical composition [14, 31]. In Table 1, glucose was the preferred carbon for MBF-UFH production by *Bacillus* sp. AEMREG7, followed by sucrose and lactose. Our findings were in accordance with some of the investigations reported by other researchers. For example, the production of bioflocculants by *Chryseobacterium daeguense* W6, a facultative oligotroph *Klebsiella* sp. PB12, *Azotobacter* sp. SSB81, and *Cordyceps ophioglossoides* L2 reached optimal in the medium containing glucose as a sole carbon source [14, 39-41]. In contrast, Aljuboori et al. [42] found that sucrose was more preferable to other carbon sources assessed for bioflocculant production by *Aspergillus flavus*. The production of bioflocculant was optimal in the medium where ethanol was used as a carbon source by *Pseudomonas aeruginosa* [43]. Furthermore, among other carbon sources evaluated, lactose was most favourable for the production of exopolysaccharide by lactic acid bacteria *Streptococcus phocae* PI80 [44].

Effect of Nitrogen Source on MBF-UFH Production

It has been well documented that the presence of nitrogen sources in the cultivation medium greatly influences bioflocculant production and cell growth

[45]. In Table 1, MBF-UFH production by *Bacillus* sp. AEMREG7 was optimal in the presence of mixed nitrogen sources followed by yeast extract. These results were in agreement with the observations made by Liu et al. [14], that organic nitrogen sources were better sources for bioflocculant production compared to inorganic nitrogen sources. The highest flocculating activity was observed with yeast extract followed by casein hydrolysate, tryptone, and beef extract. However, Ugbenyen et al. [34] found that all the nitrogen sources tested supported bioflocculant production by *Bacillus* sp. Gilbert. The highest flocculating activity was observed in the medium with potassium nitrogen (76.6%) as the nitrogen source.

Ismail and Nampoothiri [46] documented that yeast extract was a more favourable nitrogen source that enhanced EPS production by *L. plantarum* MTCC 9510. Cosa et al. [27] reported that tryptone was the nitrogen source of choice for bioflocculant production by *Oceanobacillus* sp. Pinky, whereas in the findings of Aljuboori et al. [42], peptone was the best nitrogen source that supported bioflocculant production by *Apergillus flavus* (among other nitrogen sources investigated). The production of extracellular polysaccharide by *Aureobasidium pullulans* was greatly supported in the medium containing NaNO₃ [47].

Effect of Initial Medium pH on MBF-UFH Production

Fig. 3 shows that pH 6 and 7 were more favourable for MBF-UFH production with the highest flocculating activity observed at pH 6. It has been well established in several studies that the initial pH of the production medium greatly influences bioflocculant production as it determines the electric charge of the cells and redox potential, which affects nutrient absorption as well as influencing the rate of enzymatic reaction [35, 48]. Similarly, Noghabi et al. [36] found that the production of extracellular biopolymer by *Pseudomonas fluorescens* BM07 was favourable between pH 6-8. Also, He et al. [49] found that pH 6 was more favourable for both cell growth and bioflocculant production by *Funalia trogii*. The optimum pH for cell growth and bioflocculant production by *Citrobacter* sp. TKF04 and *Aspergillus sojae* were pH 7.2-10 and 8, respectively [50-51].

Effect of Cations on the Flocculating Activity of MBF-UFH

As shown in Table 2, the flocculating activity of MBF-UFH was stimulated in the presence of the cations evaluated – except Fe³⁺, which inhibited the flocculation of MBF-UFH. Cations play a vital role in flocculation in neutralizing and stabilising the residual negative charge of both functional groups of MBF-UFH and the surface charge of the suspended particles, and consequently weaken electrostatic repulsion between particles, thus enhancing flocculation [27, 52]. From our findings, the

highest flocculating activity was observed with Al^{3+} and followed by Ca^{2+} . However, many previous studies have reported on human health being implicated in aluminum residual water [53]. As a result, calcium chloride was chosen as a cation of choice in this study.

Effect of pH on the Flocculating Activity of MBF-UFH

The flocculating activity of bioflocculant is highly influenced by the pH of the medium [33]. The relationship between pH of the kaolin suspension and the flocculating activity of MBF-UFH is represented in Table 2. MBF-UFH flocculated better at acidic conditions compared to neutral and strong alkaline conditions with the maximum flocculating activity at pH 6. At high pH, the flocculating activity of MBF-UFH was inhibited, thereby resulting in lower flocculation. The hydroxyl ions (OH^-) adsorbed at high alkaline medium may interfere with the complex formed between MBF-UFH and the kaolin particles mediated by Ca^{2+} , thereby resulting in destabilization of the suspended particles [54]. A similar trend was equally observed by Okaiyeto et al. [24], where the bioflocculant produced by *Micrococcus* sp. Leo flocculated well in acidic condition from pH 2-6. In contrast, the flocculating activity of the exopolysaccharide produced by the deep-sea psychrophilic bacterium *Pseudoalteromonas* sp. SM9913 was optimal at pH 7 [54]. Gong et al. [21] reported that the crude bioflocculant produced by *Serratia ficaria* flocculated well at pH 5-7, whereas Gao et al. [10] and Zhu et al. [55] found that the flocculating activity of bioflocculants produced by *Rothia* sp. and *Chlamydomonas reinhardtii* were maximal at pH 9 and 10, respectively.

Time Course of MBF-UFH Production

The time course of MBF-UFH production by *Bacillus* sp. AEMREG7 is depicted in Fig. 4. The flocculating activity curve was parallel to a typical bacteria growth pattern – an indication that the production of MBF-UFH was associated with cell growth and not cell autolysis [56]. Some reports showed that most bioflocculants are produced by microorganisms during growth [57]. Fig. 4 also shows that further increase in cultivation time to 192 h resulted in a decrease in flocculating activity with a concomitant decrease in the viable cells and optical density (OD), which was observed as the nutrient got depleted. As the metabolic activity changes, cell death as well as the accumulation of toxic metabolic wastes negatively affect the production rate of MBF-UFH. Furthermore, the deflocculation enzymes released as a result of cell autolysis may have degraded MBF-UFH in the medium, thereby reducing their flocculating activity [51, 58]. Similar trends were observed by Gong et al. [21], Elkady et al. [29], and Ugbenyen et al. [59], with bioflocculants produced by *Serratia ficaria*, *Bacillus mojavensis* strain 32A, and *Cobetia* sp., which attained maximum flocculating activity at 72 h, after which flocculating activity declined with cultivation time. Contrary to the above, Zheng et al. [35]

found that the production of bioflocculant by *Bacillus* sp. F19 increases with an increase in cultivation time with the maximum flocculating activity reached after 36 h. In the findings of Gao et al. [13], the flocculating activity of the bioflocculant produced by *Vagococcus* sp. W31 increased with cultivation time and reached maximum at 60 h.

Effect of Temperature on MBF-UFH Flocculating Activity

Flocculation rate has been reported to increase with an increase in reaction temperature [60]. Table 3 shows the stability of Elkady et al. [29] with increasing temperature. Chemical analyses of purified MBF-UFH showed that it is a glycoprotein composed of polysaccharide (76%) and protein (14%). The observed thermal stability is an indication of MBF-UFH predominantly composed of a polysaccharide [42, 44]. The decrease in flocculating activity noticed at 100°C might be due to the denaturation of the protein component of MBF-UFH [11]. Similar findings were reported for a thermostable bioflocculant produced by *Klebsiella pneumonia* YZ-6, which retained more than 70% of its residual flocculating activity at 70°C [61].

Conclusions

The high flocculating activity possessed by the studied bioflocculant (MBF-UFH) under different physicochemical conditions indicates that this microorganism has the potential to be utilized on an industrial scale for bioflocculant production, which could serve as a possible substitute for hazardous chemical flocculants commonly utilized in water treatment processes. Furthermore, frequent use of microbial flocculants will reduce waterborne diseases caused by chemically treated water and, consequently, reduce deformities among infants, the elderly, and immunocompromised persons, hence ensuring proper growth and development of all individuals.

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