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Isolation and screening of bioflocculant-producing bacteria from Richards Bay Harbour marine environment in South Africa

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The search for biodegradable, economical and sustainable extracellular biopolymers from microorganism has been on the rise globally since chemical flocculants have been found to be hazardous to the environment. Marine sediments have been reported to contain a wide array of unique, diversified and novel organisms than those found in terrestrial and extreme environments that were once thought to be unfavourable to support their growth and the production of secondary metabolites as useful tools in biotechnology. Bacterial strains were isolated from soil, water and sediment samples collected from Richards Bay Harbour, Kwazulu Natal, South Africa. Their physiological and colonial characteristics were observed and recorded. Most of the isolates showed different range of white, yellow, pink, creamy, green and brown pigmented small to medium swarming colonies that were either raised or flat at the centre. Out of the initial mixed cultures, 22 pure isolates were screened for flocculating abilities using their crude extract. Among the screened isolates, three isolates showed significant flocculating activities greater than 70% in kaolin clay suspension (4.0 g/L). The selected three isolates with their corresponding flocculating activities were preliminarily named as S1 (94%), S13 (97%) and S15 (74%), respectively. 16S rRNA identification revealed that S1, S13 and S15 were identified as *Bacillus safensis*, *Bacillus pumilus* and *Bacillus atrophaeus*, respectively. These three species of *Bacillus* have been observed to produce representative resistant endospore of the bacillus species and have been extensively studied in econiches of spacecraft and biodefence control interplanetary stations. The phylogenetic tree of the three organisms was constructed to show common ancestry as Bacillus species. However, based on the literature, unlike the *Bacillus safensis* and *Bacillus pumilus*; *Bacillus atrophaeus* was found for the first time to produce bioflocculants. The results showed that Richards Bay marine environment in South Africa as a reservoir for bioflocculants-producing bacteria with a potential to produce flocculants. The biotechnological importance of these isolated microorganism can be further achieved through optimisation of their culture conditions for bioflocculants production, extraction and characterisation of the produced bioflocculants.

Keywords: Marine; kaolin clay suspension; Bioflocculant; flocculating activity

INTRODUCTION

Water is an important natural resource that is continually being polluted due to various processes leading to contamination of water with agricultural, industrial and domestic wastes (Cosa et al. 2013). Flocculation is one of the methods of choice for removing contaminants and organic matter from water using flocculating agents (Lee et al. 2012; Lee et al. 2017). The search for biodegradable, economical and sustainable extracellular biopolymers has been on the rise globally (More et al. 2012; Subudhi et al. 2015). Naturally occurring flocculants are made up of natural polymers such as: cellulose, starch, chitosan, natural gums, mucilage and bioflocculants (Li et al. 2009). Bioflocculants, as an example of naturally occurring flocculants are secreted by microorganisms during their growth phase and exist in forms such as polysaccharides, proteins, uronic acids, and glycoproteins (Salehizadeh and Yan, 2014). Bioflocculants that have been produced by microbes are subjected to further screening for ecotoxicity and antimicrobial activity (Aljuboori et al. 2015). In terms of their action, bioflocculants can work with or without the presence of a cation.

Microorganisms that possess the potential of secreting bioflocculants (flocculation biopolymer) have been studied and are steadily receiving more scientific attention (Xiong et al. 2010). Microorganisms are sources of many useful primary and secondary metabolites ranging from filamentous bacteria of the genus Streptomyces (Actinomycetes) and fungi. These microorganisms such as fungi, actinomycetes, algae and bacteria have been isolated (Gong et al. 2008; Xia et al. 2008; Xiong et al. 2010) from extreme environments that were once thought to be unfavourable to support their growth and production of secondary metabolites as useful tools in biotechnology (Wang et al. 2011). Various samples were collected from sampling sites and grown on different selective and enriched media. Pure colonies were obtained from sub culturing are stored in liquid nitrogen or freeze-dried in the presence of a cryopreservative (Seidel, 2006).

Marine sediments have been reported to contain a wide array of unique, diversified and novel organisms than those found in terrestrial environments (Cosa and Okoh, 2012). Similar work has been carried out at Mtunzini and Sodwana Bay in Northern Kwazulu Natal, South Africa (Maliehe et al. 2016; Ugbenyen et al. 2017; Ugbenyen et al. 2018). Over 100 species of microorganism that have the ability to produce bioflocculants have been isolated and reported over the past decades (Zhang et al. 2010). *Rhodoccocus erythropolis* was reported by Kurane et al. (1994) to produce a proteinous bioflocculant but loses its activity to flocculate when exposed to enzyme digestion (Subudhi et al. 2015). Some bioflocculants have been reported to be produced by a number of bacteria including *Alcaligenes cupidus* KT-201 (Kurane and Nohata, 1991), *Bacillus sp.* Gilbert (Piyo et al. 2011), *Cobetia* sp. OAUIFE (Ugbenyen et al. 2012), *Bacillus sp*. AS-101 and *Bacillus firmus* from the soil (Salehizadeh and Yan, 2014). B*acillus mucilaginosus* was reported by Deng et al. *(*2003) to have a flocculating rate of 99.65% for kaolin suspension at a dosage size of only 0.1 mL/L. Ugbenyen *et al*. (2012) reported a thermally stable crude bioflocculant produced by *Cobetia* sp. OAUIFE that shows a residual flocculating activity of about 78% after heating for 25 min at 100 ^{o}C. Ugbenyen et al. (2017) reported a novel bioflocculant producing *Pantoea* sp. isolated from the Mtunzini beach, Kwazulu Natal with a flocculating activity of 92.4% and inoculum size of 3% (v/v). Most of these reported microorganisms producing bioflocculants were obtained from marine water and soil sediments (Lam, 2006).

Halophiles (salt loving) organisms find their most adaptable habitat in the marine environment and are distinguished by their characteristics of adapting to different concentrations of salt for their growth and have evolved physiological and genetic features to cope with osmotic stress exerted by this halophilic environments (Edbeib et al. 2016). Halophiles maintain their cytoplasmic osmotic balance by either accumulating high

concentrations of inorganic ions or organic osmotic solutes (Ventosa et al. 1998). Halophilic microorganisms have been reported to produce various types of biomolecules such as extracellular polysaccharide, pigments and intracellular polyester polyhydroxyalkanoates due to their hypersaline deleterious nature. This has been the focus of various scientists and biotechnologists across the globe (Biswas and Paul, 2017). Some microorganisms including *Bacillus safensis* and *Bacillus pumilus* (member of the firmicutes) in this study, have been reported as halophilic microorganisms (Edbeib et al. 2016).

Bioflocculation as a dynamic process involving living cells, is an important and effective process of separation of microorganism in a treated wastewater effluent (Laspidou and Rittman, 2002; Lachhwani, 2005; Salehizadeh and Yan, 2014). Bioflocculant can be classified as glycoprotein, polysaccharides and nucleic acids in nature (Ugbenyen et al. 2012). The biochemical composition of EPS is influenced by various factors such as carbon source, microbial species, the downstream extraction methods and nutrient supplementation (Nouha et al. 2017).

Bioflocculant producing microbes have been reported to possess organised clustered genes that are highly conserved and are involved in bioflocculant synthesis (Bai et al. 2008; Zhang et al. 2014). Gene clusters responsible for this synthesis have been reported in both Gram negative and Gram-positive bacteria (Stingele et al. 1996). Some bacteria have been studied intensively for the production of secondary metabolites. These includes *Bacillus Licheniformis, R. radiobacter* and *Streptomyces* species, the Gram-positive bacteria, particularly for its antibiotics activity (Yan et al. 2013; Zhang et al. 2014). The optimisation of the gene cluster contained in the gene sequence of identified bioflocculant microbes can be optimised using the molecular biological technique to improve the production of the bioflocculant that have consistent or desirable properties in the fermentation systems or conditions (Stingele et al. 1996; Ates et al. 2011; Ates et al. 2013; Delbarre et al. 2014). However, the gains of these microbial produced bioflocculant has remained overshadowed by the high energy cost due to substrate required, growth enhancement and survival of the microbial producer (Wolfaardt et al. 1999).

The focus of recent biotechnology has been to seek the basis of knowledge of the genomic sequence and gene clusters that encodes the enzymatic production of these secondary metabolites. This can be helpful when manipulated, to externally regulate and improve the production of bioflocculant (Yang et al. 2007; Finore et al. 2014). The gains of these knowledge can be explored in advance genetic engineering (Bajaj et al. 2007; Rehm 2009). There is already a report of transfer of the complete Extracellular Polymeric Substances (EPS) gene from bioflocculant producing LAB strain into a non EPS producing heterologous host to facilitate bioflocculant production. This advanced technology has its basis in that the heterologous host must possess all necessary genetic information for precursor synthesis (Germond et al. 2001). Boels et al. (2003) has further described that a homologous overexpression of complete EPS gene cluster led to increased EPS production in *Lactococcus lactis* after a manipulated overexpression of the NIZO B40 eps gene cluster of a complete eps gene cluster in *Lactococcus lactis*. Many researches have been focused on the various applications of EPS in recent years (Gupta and Thakur, 2016). These extracellular polysaccharides play different roles and functions such as preventing desiccation and protection from environmental stress (Limoli et al. 2015), especially to halophilic microorganisms that are being studied in this current study. EPS also helps in the adherence to surfaces and taking in of nutrient from the environments (Caruso et al. 2018). This study mainly focused on the isolation of novel bacterial isolates from marine environments with high polymer yield of bioflocculants with flocculating activity.

MATERIALS AND METHODS

3.1 Description of study site

Richards Bay lies at sea edge almost halfway

between Durban and Kosi Bay. It is situated on a 30 square kilometre lagoon of the Umhlathuze River within the coordinates (S 28˚ 47' 34,23 E 32 ˚ 4' 51,564). It is one of the largest and deepest harbour in the African continent and it is the gateway to Zululand. It is a fast-growing industrial centre with various mining activities being carried out along the surrounding of the harbour by companies. These activities play a major role in the impact of water pollution and there is a challenge to be able to maintain its ecological diversity.

3.2 Sample collection, bacterial isolation and identification

3.2.1 Sample collection

Five different marine sediment samples randomly collected including soil, water and sediment samples from Richards Bay harbour, Kwazulu Natal, Republic of South Africa. Samples were collected aseptically using sterile plastic bottles of 250 mL capacity and McCartney bottles (25 mL), transported for analysis in a cooler box with ice to the Microbiology Laboratory of the University of Zululand, KwaDlangezwa, South Africa, and analysed within six hours after collection.

3.2.2 Cultivation Media

Nutrient agar, yeast extract agar (YEA) and M1 medium (YEA + starch) were used for the cultivation of microorganisms. In the cultivation of microorganism, non-selective media such as Nutrient agar medium is used for general cultivation and maintenance. However, there are also fastidious organism that grows on enriched

media such as yeast extract agar and M1 medium (YEA + starch). M1 agar and YEA agar were used for the isolation of actinomycetes with modifications (Ogunmwonyi et al. 2010). The basal ingredient in nutrient agar per litre consists of meat extract (1.0 g), peptone (5.0 g), yeast extract (2.0 g), sodium chloride (8.0 g), and bacteriological agar (15 g) as solidifying agent. All ingredients were dissolved in 1 Litre of filtered seawater. M1 consist of 10 g starch, 4 g yeast extract, 2 g peptone and 18 g bacteriological agar were dissolved in 1 Litre of filtered seawater as described by Ogunmwonyi et al. (2010) and Mincer et al. (2002). Yeast extract agar composed of yeast extract (3 g), peptone (5 g) and bacteriological agar (15 g) supplemented with 10% starch (2.3 g) in 1 Litre of filtered marine water.

3.2.3 Isolation of Microorganisms

Ten-fold serial dilutions of the water samples were prepared with sterile (0.85%) saline solution. One hundred microliter (100 µL) of the diluted water samples was inoculated to nutrient agar, yeast extract agar and M1 agar plates using spread plate techniques. To isolate microorganisms from the sediment samples, the dry/dilute and dry/stamp method slightly modified were used (Jensen et al. 2005). All plates were incubated at 37 ^{o}C for 24 - 72 hours after which the plates were observed for growth. Single colonies were selected randomly based on their colour, size, structure, morphology, subcultured onto nutrient agar plates and incubated at 37 ^{o}C for 24 - 72 hours to obtain pure cultures. Pure colonies obtained after the incubation (or sub culturing) for 24 - 72 hours at 37 ^{o}C were used for screening of bioflocculant producing bacteria and identification. Identification was done based on their morphology, cultural characteristics using Bergey's manual of systematic bacteriology (Christen, 2008).

3.3 Screening for bioflocculant production

3.3.1. Production medium and bacterial growth

The production medium composed of ingredients listed in Table 3 was prepared as described by Zhang et al. (2007) in marine water. The production medium was prepared by dissolving the ingredients (Table 1) in marine water and autoclaved for 15 minutes at 121 ^{o}C. One loopful of bacterial colony was inoculated into 50 mL of sterile production medium in a 100 mL conical flask.

Table 1: Production medium for screening microorganism for production of bioflocculant.

Ingredients	Amount
Glucose	20.0 g
Yeast Extract powder	0.5 g
Urea	0.5 g
$(NH_4)_2SO4$	0.2 g
K_2HPO_4	5.0 g
KH_2PO4	2.0 g
NaCl	0.1 g
$MgSO_4.7H_20$	0.2 g
Marine water (Filtered)	1 Litre

A standardised inoculum was hereafter used after the inoculum size for the selected bacterium was obtained. The inoculated medium was incubated at 30 ^oC for 72 hours in a shaking incubator with a rotating speed of 160 rpm. After the incubation period, 2 mL of the fermentation broth was then centrifuged (8000 rcf for 30 minutes at 4 ^oC) to remove the cells, while the cell-free supernatant was used as crude bioflocculant to determine the flocculating activity (Xia et al. 2008).

3.3.2 Determination of flocculating activity.

The flocculating activity tests were carried out according to the method previously described by Kurane et al. (1994) with slight modifications as described by Ugbenyen and Okoh (2013). Four gram per litre of kaolin clay suspension was used as a test material. Three millilitres (3 mL) of 1% $CaCl_2$ and 2 mL of cell-free supernatant were added to 100 mL of kaolin clay suspension (4.0 g/L) contained in a 250 mL conical flask. The mixture was agitated vigorously, poured into a 100 mL measuring cylinder and allowed to stand for 5 minutes at room temperature for sedimentation. A control experiment was prepared using the same method but the bioflocculant solution was replaced by sterile culture medium. The top clear solution (1 cm) was used to determine the optical density at 550 nm with a spectrophotometer (Pharo 100, Merck KGaA, Germany) (Xia et al. 2008).

Flocculating activity was calculated using the following equation:

Flocculating Activity = {(A - B)/A} x 100%

Where *A* = optical density at 550 nm (OD_{550}) of control and *B* = optical density at 550 nm (OD_{550}) of a sample.

3.4 Molecular identification using the organism 16S rRNA gene

The bacterial isolates were cultured in 50 mL fresh Luria Broth (LB) and incubated at 37 ^oC on rotatory shaker speed of 200 rpm for 16 hours. The bacteria were further identified through DNA sequencing using 16S rRNA molecular techniques. For amplification of 16S target region of the bacteria, the universal primers 27F and 1492R were used to amplify the 16S target region of the bacteria (Lane et al. 1991; Turner et al. 1999). The bacterial strains were analysed using the 16S rRNA. The amplified and purified PCR products were used to determine the sequence of the bacteria. The findings were compared with the National Centre for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov). The phylogenetic tree was thereafter constructed as described by Okaiyeto et al. (2013) using similar sequences found in Gen Bank. Nucleotide sequence analysis was carried using the MAFFT (Multiple Alignment using Fast Fourier Transform) with fasta sequence of the 16S rRNA. These isolates were stored in the 20% glycerol broth at -80 ^oC freezer in the Department of Biochemistry and Microbiology, University of Zululand, KwaDlangezwa, Kwazulu-Natal, South Africa.

3.5 Optimisation of culture conditions and extraction

To enhance and increase the yield and flocculating efficiency of a bioflocculant, the conditions in the culture broth for the selected bacteria were optimised by varying factors such as carbon and nitrogen sources, metal ions, aeration ratio, initial pH effect, inoculum size, culture incubation time and temperature effect. The extraction and purification of the bioflocculant was carried out as described by Chang et al. (1998) and Cosa et al. (2011) after optimisation. After optimal hours of fermentation, the bioflocculant from the production medium was harvested by centrifugation for 15 minutes at 5000

g at 4 ^{o}C. For isolation of EPS, one volume of distilled water was added to the supernatant phase and further centrifuged for 15 minutes at 5000 g at 4 ^{o}C to remove insoluble substances. Two (2) volumes of ice-cold ethanol was added to the culture supernatant, agitated properly and stored for 12 hours at 4 ^{o}C. The precipitate was vacuum-dried to obtain the crude bioflocculant. The crude bioflocculant obtained was re-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. The mixture was shaken vigorously and left to stand at room temperature for 12 hours. The supernatant was centrifuged at 5000 g for 15 minutes at 4 ^{o}C and vacuum dried to obtain a pure bioflocculant. The weight of the dried bioflocculant was expressed in g l^{-1} culture (Poli et al. 2009; Okaiyeto et al. 2013; Gupta & Thakur (2016).

3.6 Statistical analysis

All data were conducted and collected in triplicates with results expressed as mean and standard deviation values. These data were subjected to one-way analysis of variance (ANOVA) using Graph Pad Prism ™ 6.1. Differences were considered at a significant level of p<0,05..

RESULTS AND DISCUSSION

2.1 Isolation, screening and identification of bioflocculant producing bacteria

In this study, twenty-two bacterial isolates classified according to their colony, colour, size and structural morphologies were obtained from marine sediments, water and soil samples of Richard Bay Harbour in the Kwazulu Natal, South Africa. These isolates were screened for bioflocculant production using kaolin clay suspension as shown in Table 1. The description of the isolates morphologically and their observed flocculating activity were shown in Table 2. Table 2 shows the key to the labelling of the specimen depending on the types or location of samples

The pure isolates were screened to observe the presence of bioflocculant and tested against 4.0 g/L of kaolin clay suspension. Figure 1 shows the corresponding flocculating activities of the twenty-two screened isolates labelled S1, S2 to S22 respectively. Among the twenty-two screened isolates, three isolates showed significant flocculating activities greater than 70% in kaolin clay suspension (Figure 1).

The selected three isolates with their corresponding flocculating activities were named as S1 (94%), S13 (97%) and S15 (74%), respectively. The selected bioflocculant-producing bacteria (S1 & S15) were isolated from the soil sample while S13 was isolated from water sample with epiphytic plant near by Tuzi Gazi harbour beach line, Richards Bay. The Tuzi Gazi harbour beach is an example of a marine environment that has been reported to be a habitat to moderately halophilic microorganisms (Biswas & Paul, 2017).

The bacteria were further identified through DNA sequencing using 16S rRNA molecular techniques. For amplification of 16S target region of the bacterium, the universal primers 27F and 1492R were used to amplify the 16S target region of these bacteria (Lane et al. 1991; Turner et al. 1999). The three selected bacterial strains were analysed further using the 16S rRNA and Nucleotide sequence analysis based on gyrase A gene using the Basic Local Alignment Search Tool (BLAST) of the 16S rRNA, this revealed S1, S13 and S15 as *Bacillus safensis*, *Bacillus pumilus* and *Bacillus atrophaeus*, respectively. S15 bacterium has 98.64% similarity to *Bacillus atrophaeus* (*B. atrophaeus*) strain SRCM191359 with accession number CP021500.1. S1 bacterium has 100% similarity with *Bacillus safensis* FO-36b genome with accession number CP010405.1 while S13 was identified as *Bacillus pumilus* with accession number MH547393.1(Akapo , 2019).

Bacillus safensis

Bacillus safensis (*B. safensis*) is a Gram-positive, mesophilic, spore-forming, aerobic and chemo-heterotrophic bacterium (Kothari et al. 2013). It is a rod-shaped and motile bacterium with high tolerance for salt, heavy metals, and ultraviolet and gamma radiations (Satomi et al. 2006; Raja and Omine 2012). *B. safensis* was

originally isolated from a spacecraft in Florida and was supposed to have been transported to planet Mars on a spacecraft. The prefix SAF in the name "safensis" was arbitrarily derived from "Spacecraft-Assembly Facility" in the USA from where the organism was first isolated (Satomi et al. 2006; Lateef et al. 2015). *B. safensis* has been reported to colonize various habitats including terrestrial and marine environments (Liu et al. 2013).

Table 2: Morphological description of isolates and labels showing their isolation sources.

S/N	Flocculating activity	Location label	Colony characteristics
S1	94%	MSS5	Small whitish colonies with serrated edges
S2	<0%	3SC4	Creamy white colour
S3	<0%	MSS3	Small yellow colony with rough edges
S4	12%	ICS2	Shining translucent colony
S5	11%	MSS4	Raised and swarming yellow colony with irregular edges
S6	<1%	MSS1	Yellowish round colony
S7	<0%	5WS5	Pink colony with round colony with depressed centre
S8	<1%	2SP4	Translucent colony with raised colony
S9	<0%	MSS 7	Whitish creamy small colonies with round edges and slow growth on nutrient agar
S10	5%	4SW1	Milky translucent colony
S11	5%	3SC2	Green pigmented swarming tiny colonies on NA
S12	<3%	ICSc	Milky coloured colonies
S13	97%	1CS3	Dried serrated edges with mixed organism/ white colony
S14	48%	CSS2	Translucent colony with raised colonies
S15	74%	MSS	Mucoid translucent shining colonies, draws on touch
S16	<1%	2SP	Light brown coloured and translucent
S17	36%	CSS1	Small tiny whitish colony with round shaped colonies
S18	17%	5WS	Medium round colonies with flat centre
S19	<1%	CSS	Creamy serrated edge colonies
S20	<1%	MSS3	Mucoid brown shining colonies, white edges and brown centre
S21	46%	ICS	Light brown colonies with medium regular shape with raised centre
S22	2%	5WSb	Swarming whitish creamy colony with serrated edges

Table 3: Key detailing Samples Description and sites from where collected.

S/N	Description of Sample Source	Key labels
1	Water sediment sample with epiphytic plants	1CS
2	Water sample with coral shell on rocks at Tuzi Gazi	2SP
3	Sediment sample/water near Cubana	3SC
4	Estuary 2 sediment/water sample near Alkaanstrand	4SW
5	Marine/Estuary sediment/water sample	5WS
A	Marine/Estuary water	CSS
C	Soil sample from the Tuzi Gazi beach	MSS

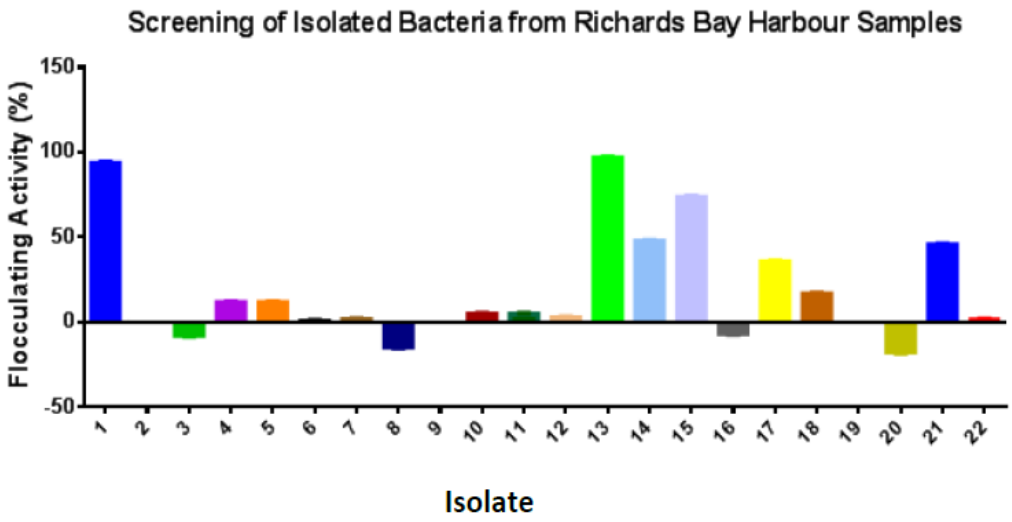

Figure 1: Flocculating activity of the 22 isolated bacterial strains from Richards Bay Harbour.

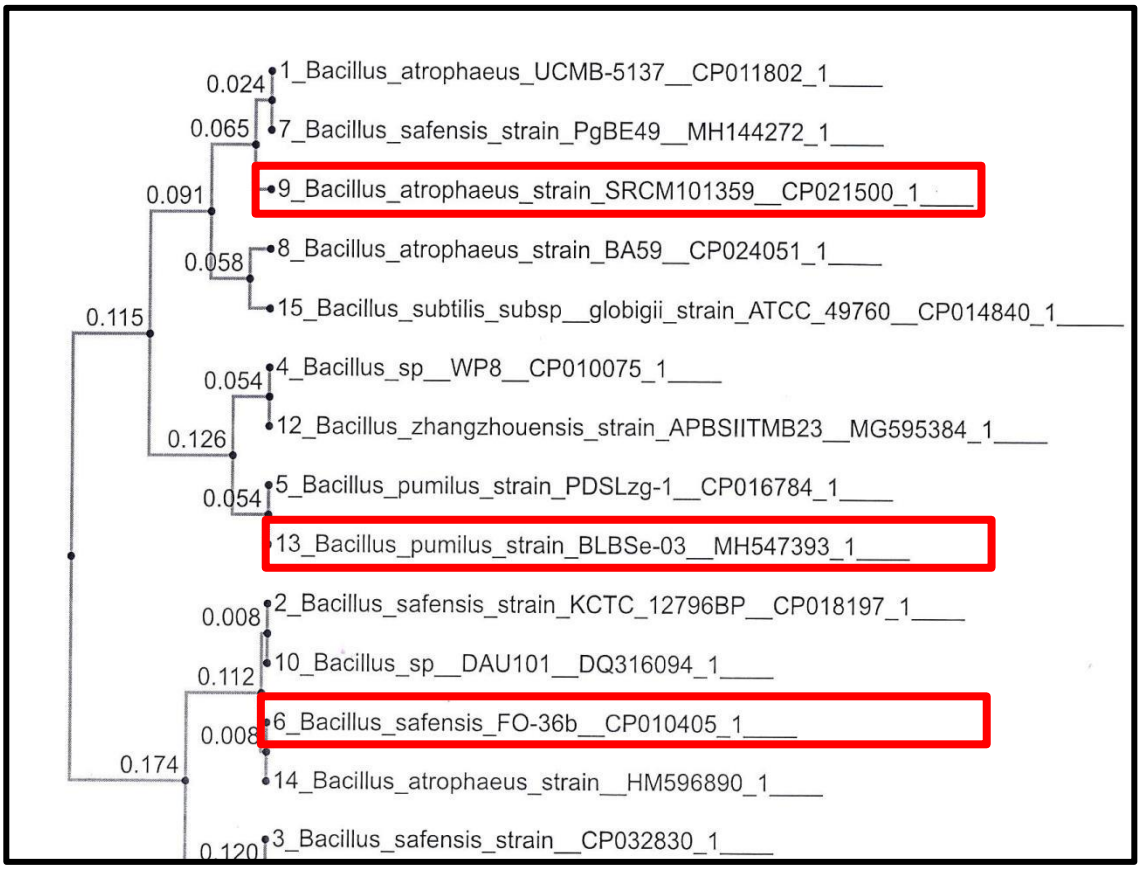

Figure 2: A phylogenetic tree showing the relationships among the selected isolates, *Bacillus atrophaeus* strain SRCM101359, *Bacillus pumilus* strain BLBSe-03 and *Bacillus safensis* FO-36b (highlighted in red lined rectangles) and other closely related sequences collected from the Gene bank analysed using MAFFT alignment tool and phylo.io.

B. safensis has been reported to produce flocculants in consortium with other Bacillus species (Harun et al. 2017). In this study, the optimisation of the bacterium condition for *B. safensis* produced a yield of 7.90 g impure and 2.18 g pure bioflocculant (labelled BSCP01), respectively. The bacterium metabolised glucose, complex nitrogen as carbon and nitrogen sources, respectively at an incubation temperature of 35 ^{o}C (165 rpm). The optimal incubation time was minimum of 24 hours and maximum 84 hours. The optimum inoculum size for the bioflocculant production for this bacterium was 3% v/v (Akapo et al. 2019).

2.1.2 Bacillus pumilus

Bacillus pumilus is a Gram-positive bacterium. It has been found to be the second most dominant species among the aerobic spore-forming bacteria (La Duc et al. 2004). *B. pumilus* has been placed using multiple genome comparison in microbial taxonomy as strains that are likely to belong to the *B. safensis* group (Tirumalai et al. 2018). Coli *et al* (2016) has reported that endospores of a strain of *B. pumilus* have been found to grow better on International Space Station (ISS) than here on Earth. This is a concern for forward and backward contamination in aerospace and this level of resistance in such closed environments could affect living conditions (Moissl-Eichinger et al. 2016). *Bacillus pumilus* is an example of halophilic organism. There have been few reports on *Bacillus pumilus* in the production of bioflocculants (Makapela et al. 2016) and in consortium with other microorganisms (Maliehe et al. 2016). Makapela et al. *(*2016) reported a thermostable bioflocculant with low dosage concentration of 0.1 mg/mL and a working pH range of 3-11 while Maliehe et al. (2016) observed a 3.0 g/L yield of bioflocculant TPT from a consortium of *Bacillus pumilus* JX860616 and *Alcaligenes faecalis*. After the optimisation of the culture condition of the bacterium identified as *Bacillus pumilus* strain BLBSe-03 ***(***MH547393.1) in this study, it produced a yield of 7.050 g impure and 6.800 g pure bioflocculant (labelled BPMH54), respectively. To produce the bioflocculant BPMH54, the bacterium metabolised lactose, yeast extract powder as carbon and nitrogen sources, respectively at an incubation temperature of 30 ^{o}C (110 rpm). The optimum inoculum size for the bioflocculant production for this bacterium was 3% v/v (Akapo, 2019)

2.1.3 Bacillus atrophaeus

Bacillus atrophaeus (formerly known and misclassified as *Bacillus subtilis var niger* or *Bacillus subtilis* subsp. Globigii) (simply called BG) belongs to the great diversity of industrially important *Bacillus* genus strains (Sella et al. 2015). Nakamura (1989) first proposed the species *B*. *atrophaeus* after examining a number of pigmented and non-pigmented strains of *B*. *subtilis.* Burke *et al.* (2004) reported that a cluster of strains ATCC 9372 was designated as a new sub-species, *Bacillus globigii*. *Bacillus atrophaeus* has been identified to belong to a group of useful bacteria with known production of biomolecules (Ma et al. 2018). *Bacillus atrophaeus (atrophaeus – black, phalis – brown, atrophaelis, dark brown)* is a Gram-positive rod shaped bacterium, motile, facultative anaerobic and produces ellipsoidal endospores (Nakamura, 1989; Burke et al. 2004). Reports have shown that multiple environmental signals trigger the onset of the production of spores such as nutrient starvation and high cell densities, where a set of cells diverts into a genetic programme (Veening, 2007). *Bacillus atrophaeus* on nutrient agar plate, appeared to be circular and possess smooth and milky white colonies, with irregular edges, mostly flat. It also possesses mucoid and ropy colony morphology which has been reported to be the basic identification properties for bioflocculant producing potential such as *Bacillus* sp. UPMB13 (Amir et al. 2003; Zulkeflee et al. 2012). In a related study, an isolated bacterium, identified as *Bacillus atrophaeus SRCM 101359 (98% identity) was* found as a *Bacillus atrophaeus* strain for the first time to produce bioflocculants based on available journal searches (Akapo et al, 2019). On optimisation of the bacterium condition, it produced a yield of 3.16 g of bioflocculant optimally whilst metabolising glucose, ammonium

chloride as carbon and nitrogen sources, respectively. Further culture conditions include incubation temperature of 35 ^{o}C, 110 rpm. The optimal incubation time was 96 hours and inoculum size of 4% (Akapo, 2019).

The phylogeny tree was constructed to show the ancestral origin of the bacillus species as seen in Figure 2. *Bacillus atrophaeus* SRCM 101359 is one of the members of the species with complete genome sequences. Few others include 1942, NRS 1221A, BA59 and GQJK17 which is the closest relative of strain SRCM 101359 (Ma et al. 2018). In a study, Ma *et al.* (2018) compared the genome sequence of *B. atrophaeus* strain GQJK17 with the other three complete genome sequences including *B. atrophaeus* SRCM 101359 and found out that the main gene clusters for producing some secondary metabolites are similar in all the four compared *Bacillus atrophaeus* strain. Tirumalai et al. (2018) reported in a recent phylogenetic study that *B. pumilus* and *B. safensis* strains (FO-36b), clustered together in a distinct group of *B. safensis* strains..

CONCLUSION

Twenty-two pure isolates were isolated from marine environment of Richards Bay Harbour, KwaZulu-Natal province of RSA and screened. Out of the screened isolates, three of the isolates of Bacillus species were identified through 16S rRNA nucleotide sequence and BLAST analyses as *Bacillus safensis (S1)*, *Bacillus pumilus(S13)* and *Bacillus atrophaeus (*S15) respectively. These all showed strong potential of bioflocculant production with flocculating activities of *Bacillus safensis* (94%), *Bacillus pumilus* (97%) and *Bacillus atrophaeus* (74%) respectively in kaolin clay suspension initially before optimisation. However, unlike the *Bacillus safensis* and *Bacillus pumilus*, *Bacillus atrophaeus SRCM 101359 (98% identity)* was found for the first time to produce bioflocculants based on available journal searches. It was significantly noted in this study that after optimisation, the flocculating activity for *Bacillus atrophaeus* showed a significant difference between the initial flocculating activity of 74% and 92% for the crude bioflocculant at the pH of 9. These results showed Richards Bay marine environment in South Africa as a reservoir of bioflocculants-producing bacteria with potential to produce flocculants. The biotechnological importance of these isolated microorganism was further achieved through optimisation of the culture conditions for their bioflocculants production, extraction and characterisation. The selected three isolates *Bacillus safensis*, *Bacillus pumilus* and *Bacillus atrophaeus* on further optimisation showed better flocculating activity and bioflocculant production yield..

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Conception, CSOA, AKB, ZGN.; Methodology & Design, C.S.O.A., ZGN, OOA, VSRP, AKB; Resources, A.K.B.; Data writing—original draft preparation, CSOA., ZGN., OOA., VSRP, AKB; supervision, ZGN, AKB.; funding acquisition, AKB. CSOA collected data and prepared the first draft of the manuscript. Proofreading: CSOA., ZGN., OOA, VSRP and AKB;

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