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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2020 17(3): 1610-1625. **OPEN ACCESS**

Wastewater treatment by a novel bioflocculant from a consortium of *Bacillus pumilus* JX860616 and *Bacillus subtilis* CSM5

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In recent years, researchers are focusing on improving bioflocculant yields through isolation of novel bioflocculant-producers and the use of microbial consortia. This study aimed at producing, characterising and applying a bioflocculant from a consortium of *Bacillus pumilus* JX860616 and *Bacillus subtilis* CSM5. After optimisation, glucose and ammonium sulphate were the preferred nutrient sources. The yield of 3.1 g/l was produced when optimum culture conditions were; 30 °C, initial pH 6, 165 rpm, after 72 hours. The obtained bioflocculant is cation dependent, thermostable and effective at the dosage size of 0.2 mg/ml. Fourier transform infrared (IR) spectrum was indicative of hydroxyl, amide and amino groups. It demonstrated a margin of safety as 100% of breast cancer cells were obtained after treated with 200 µl/mg of the bioflocculant. Its flocculation mechanism was mediated by Ba^{2+}, which stimulated rapid flocculation by neutralising the residual negative charges of the bioflocculant and kaolin particles, enabling bridging mechanism. Its removal efficiencies on wastewater on biochemical oxygen demand, chemical oxygen demand and phosphorus were; 99, 84 and 75%, respectively. The results suggest the potential use of consortium for improvement of yields and the industrial impotence of the bioflocculant.

Keywords: *B. pumilus* JX860616, *B. subtilis* CSM5, bioflocculant, flocculating activity

INTRODUCTION

Water pollution is a serious concern in developing countries due to the disposal of untreated effluents from municipal and industrial sources (Wu et al., 2012 & Connor et al., 2017). In developing countries, merely 8% of the generated wastewater undergoes treatment of any kind (Sato et al., 2013). The challenge of wastewater treatment is to reduce high levels of chemical oxygen demand, biological oxygen demand and nutrients (Teh et al., 2016). To combat this challenge, a number of highly efficient treatment methods such as flocculation are employed (Dao et al., 2016). Flocculation is a process whereby destabilized colloidal and suspended particles agglomerate to form large flocs using flocculating agents such as; (1) inorganic salts (alum, ferric sulfate and ferric chloride), (2) synthetic organic polymers (polyaluminium chloride and polyethylene imine) and (3) natural flocculants (bioflocculants) (Guoa et al., 2018). The flocs formed can be effectively and easily removed by sedimentation, flotation or filtration processes (Yang et al., 2016).

In practice, inorganic flocculants are used predominately due to their cost-effectiveness (Piyo et al., 2011). However, they have moderate solubility in water and require large dosage sizes. Moreover, they are highly sensitive to pH variations and low temperatures (Brostow et al.,

2009). Due to the shortcomings observed from inorganic flocculants, organic synthetic flocculants have been used in as alternatives (Mabinya et al., 2011). Organic synthetic flocculants are convenient to utilize, highly soluble in aqueous solutions and are unaffected by pH changes. In addition, they have a well-defined and understood flocculation mechanisms (Buczek et al., 2017). Nevertheless, their monomers have been reported to be mutagenic, neurotoxic and carcinogenic (Salehizadeha et al., 2018). Moreover, they are resistance to degradation, thereby constituting to environmental hazards (Mishra et al., 2018). Consequently, their use in many industries are discouraged.

Biopolymers produced from biomaterials and microorganisms have gained a considerable scientific attention recently (Sun et al., 2015). Microbial flocculants, generally known as bioflocculants are macromolecules composing of polysaccharides, proteins, glycoproteins or proteoglycans. They are secreted by bacteria or fungi as a result of substrate metabolism or microbial growth (Zulkeflee et al., 2016). They have been effectively used as reducing and stabilizing templates during metallic nanoparticle synthesis (Dlamini et al., 2019), to flocculate inorganic solid suspensions (Pua et al., 2014) and in the removal of the heavy metals (Dih et al., 2019). Their utilization is due to the fact that they are nontoxic in nature (Chopra and Ruhi, 2016) and lack secondary pollution due to their hydrolysable functional groups (Ayangbenro et al., 2019). However, low flocculating properties, production yields and the poorly understood kinetics of flocculation underlying their actions, do constraint their industrial applicability (Salehizadeh and Shojaosadati, 2001).

Cosa and Okoh. (2014) and Okaiyeto et al. (2013) have reported that combination of microbial strains in fermentation can improve bioflocculant yields. The improved yield is hypothesized to be due to the symbiosis or synergism relationship among the microorganisms (Zhang et al., 2007). Genus *Bacillus* consists of diversity of bacterial species known to secrete a variety of biofloccuants and other important metabolites in larger quantities (Mahmoud et al., 2015). In our previous study, we isolated and identified bioflocculant-producing *Bacillus pumilus* JX860616 and *Bacillus subtilis* CSM5 from marine water (Maliehe et al., 2016). At optimal culture conditions, each bacterium produced 2.4 g/l and 1.5 g/l of bioflocculants, respectively.

In this study, *Bacillus pumilus* JX860616 and *Bacillus subtilis* CSM5 were used to construct a consortium. The medium composition and culture conditions of the consortium were optimized and the bioflocculant was extracted. Lastly, the bioflocculant was characterised, its flocculating mechanism was evaluated and it was applied in wastewater treatment.

MATERIALS AND METHODS

Microorganisms and production medium

The bacteria were obtained at the Department of Biochemistry and Microbiology at the University of Zululand, South Africa. They were previously isolated from a sediment sample from Sodwana Bay in KwaZulu-Natal, South Africa (28 °45 ′S31 °54 ′E) and identified by 16S-rRNA nucleotide sequencing analysis as; *Bacillus pumilus* JX860616 and *Bacillus subtilis* CSM5. All chemicals and reagents used were procured from Sigma-Aldrich (St Louis, MO, USA) and water used was glass distilled. The standard production medium by Zhang et al. (2007) was used for bioflocculant production. The medium composed of glucose (20.0 g), KH_2PO_4 (2.0 g), K_2HPO_4 (5.0 g), $(NH_4)_2SO_4$ (0.2 g), NaCl (0.1 g), CH_4N_2O (0.5 g), $MgSO_4$ (0.2 g) and yeast extract (0.5 g) in a litter of filtered seawater and was autoclaved (121 ^oC for 15 minutes).

Construction of the consortium and determination of flocculating activity

A consortium *of B. subtilis* CSM5 and of *B. pumilus* JX860616 was constructed. A loopful of each strain was inoculated separately into 150 ml Erlenmeyer flasks containing 50 ml of the production medium. They were incubated at 30 ºC, at a shaking speed of 165 rpm for 72 hours. Thereafter, 1 ml of each bacterial strain was inoculated into 150 ml Erlenmeyer flasks containing 50 ml production medium for construction of bacterial consortium. They were then incubated at 30 ºC at a shaking speed of 165 rpm. After 72 hours, the culture broth was centrifuged at 8000 rpm for 30 minutes at 4°C and the supernatant was used to assess the flocculating activity. Flocculating activity was evaluated using kaolin suspensions as described by Kurane et al. (1994). Briefly, 100 ml of kaolin suspension (4 g/l) was poured into 250 ml Erlenmeyer flask. Three milliliter of 1% w/v $CaCl_2$ and 2 ml of a cell free supernatant were poured into the suspension. The mixture was shaken for 60 seconds and poured into a 100 ml measuring cylinder. It was allowed to stand for 5

minutes at room temperature. The sample was withdrawn and its optical density ($OD_{550\ nm}$) was measured. Flocculating activity was evaluated as follows: Flocculating Activity (%) = [(A − B/A)] × 100, where A denotes the optical density of the control (kaolin solution) and B the optical density of a sample.

Optimisation of the medium composition and culture conditions

The establishment of optimum medium composition and growth conditions for bioflocculant production were obtained by varying parameters such as: inoculum size (%, v/v), nutrient sources, cations, shaking speed, initial pH of the production medium, temperature and time.

Evaluation of the inoculum size and nutrient sources

The effect of inoculum size on flocculating activity was evaluated. Different inoculum sizes ranging from 1 to 5% (v/v) were inoculated into the production medium. The effect of each inoculum size on flocculating activity was determined spectrophotometrically as detailed previously. The effect of different carbon sources on the flocculating activity was investigated. Bacterial consortium was cultured on various carbon sources (glucose, fructose, sucrose, lactose, maltose, starch and molasses). Thereafter, the flocculating activity was determined. The effect of organic nitrogen sources (casein, yeast extract, urea, tryptone and peptone) and inorganic nitrogen source ($(NH_4)_2SO_4$) was also evaluated. The nitrogen sources (1.2 g/l) were individually incorporated into the production medium replacing the initial multiple nitrogen sources (yeast extract (0.5 g), urea (0.5 g) and $(NH_4)_2SO_4$ (0.2 g) in an equivalent amount (Nwodo et al., 2016).

Effect of cations, shaking speed, initial pH and temperature on flocculating activity

Different cations (NaCl, KCl, LiCl, $MnCl_2$, $BaCl_2$ and $FeCl_3$) were used to assess their effect on flocculating activity. Three milliliter of calcium chloride (1%) used as a standard cation was replaced with these cations. The control was kaolin solution without addition of cation. The flocculating activity was determined spectrophotometrically at a wavelength of 550 nm. The shaking speed the culture was varied in a range of 0-220 rpm to evaluate its effect on the flocculating activity. The effect of initial pH on the flocculating activity was also investigated by adjusting the initial pH of the growth medium in a range of pH 3-12 by 1N HCl and 1N NaOH using pH meter (Eutech Instruments pH 700, Singapore). The optimum temperature for high flocculating activity was evaluated by incubating the consortium at varied temperatures (i.e. 20, 25, 30, 35, and 50 ^oC). At the end of incubation period, flocculating activity was measured as stated previously (Okaiyeto et al., 2016).

Time course assay

The effect of growth time on flocculating activity, pH and cell number was assessed in accordance to a previous study by Cosa et al. (2013). The consortium was cultured under the obtained investigated optimal growth conditions. Samples were withdrawn aseptically every 12 hours interval for 120 hours. The flocculating activity, optical densities (OD_{550}) of the growth broths, representing bacterial growth and the pH were also determined by the spectrophotometer (Pharo 300, Merck KGaA, Germany) and a pH meter (Eutech Instruments-pH 700), respectively.

Extraction and purification of the bioflocculant

The bioflocculant was extracted and purified in accordance to the method by Chang et al. (1998). The consortium was cultured at the obtained optimum medium composition and culture conditions. After 72 hours of incubation, the growth broth was centrifuged (8000 rpm, 4°C, 30 minutes). The distilled water (1 volume) was added to the supernatant phase and centrifuged (8000 rpm, 30 minutes, 4°C) to remove the insoluble substances. Ethanol (2 volumes) was added to the supernatant, agitated and then allowed to precipitate for 12 hours at 4 °C. The yielded precipitate was vacuum-dried and the crude product was dissolved in distilled water (100 ml). One volume of the mixture of chloroform and butanol (5:2 v/v*)* was then added, agitated and left to settle for 12 hours at room temperature. Thereafter, the cell-free supernatant was centrifuged (4000 rpm, 4 °C, 30 minutes) and vacuum-dried.

Characterisation of the bioflocculant

Different bioflocculant concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) were prepared in distilled water. Their flocculating activities were accessed subsequently to determine the optimum concentration (Luo et al., 2014). The elemental analysis was carried out with scanning electron microscope-energy dispersive X-ray detector (SEM-EDX) (Oxford Instruments-X-Max^N) (Dlamini

et al., 2019). The functional groups of the bioflocculant were analyzed by Fourier transform infrared (IR) spectroscopy (Perkin Elmer UATR TWO, 2000, Germany). The bioflocculant (10 mg) was heated within a range of 22 to 900 °C at a heating rate of 10 °C per minute under a constant flow of nitrogen gas in order to assess its pyrolysis profile (Ravindrana et al., 2018).

In vitro cytotoxicity assay of the bioflocculant

The cell cytotoxicity was measured according to Mosman (1983). Breast cancer cells (MCF7) were grown to confluency in 25 cm^3 flasks. They were trypsinised and plated into 48 well plates and incubated overnight at 37 ^oC. The medium was substituted with the fresh medium (MEM + Glutmax + antibiotics). The bioflocculant was added and incubated at 37 ^oC. After 4 hours, the medium was replaced by complete medium (MEM + Glutmax + antibiotics +10 % Fetal bovine serum). After 48 hours cells were exposed to the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Percentage viability of the cells treated with the bioflocculant was calculated, thereafter.

Mechanism of flocculation

The flocculation mechanism of the bioflocculant was proposed after the zeta potentials of the kaolin particles, mixture of kaolin particles and NaCl, kaolin particles flocculated by the bioflocculant in the presence of NaCl and the bioflocculants were measured by Zetasizer Nano (Malvern, UK) at 25 °C (Aljuboori et al., 2015).

Removal efficiencies of the bioflocculant on wastewater

The biological oxygen demand (BOD), chemical oxygen demand (COD), and phosphorus (P) in wastewater from the Nhlabane Estuary, South Africa (28 °45 ′S31 °54 ′E), were measured with spectro-quant (Pharo 300, Merck KGaA, Germany), before and after application of the bioflocculant. The Jar test was used in accordance to the method by Okaiyeto et al. (2016). Three millilitres of 1% (w/v) NaCl and 2 ml of the bioflocculant solution were both poured into 100 ml wastewater sample. The mixture was shaken at 200 rpm for 3 min; thereafter, the speed was reduced to 40 rpm and allowed to shake for 5 minutes. The removal efficiencies of the bioflocculant on BOD, COD and P were measured as follows:

Removal efficiency (%) = $(E_o – D/ E_o) \times 100$

where E_o and D, are the initial and final values obtained before and after treatment with the bioflocculant, respectively. Alum and ferric chloride were used as positive controls.

Software and statistical analysis

All the experimentations were done in triplicates and the error bars in the figures shows the standard deviations of the data. Data were subjected to one-way analysis of variance using Graph Pad Prism ™ 6.1. Values with different alphabets show significant differences ($p \leq 0.05$).

RESULTS AND DISCUSSION

Rarely do microbial strains isolated from nature produce bioflocculants at sufficiently high concentrations for commercialisation (Salehizadeh et al., 2018*).* Of recent, microbial species in consortia have been reported to improve bioflocculant yield when compared to pure strains (Zhang et al. 2007). Thus, the bacterial consortium of *Bacillus pumilus* JX860616 and *Bacillus subtilis* CSM5 was constructed in order to improve bioflocculant yield.

Effect of inoculum size

Inoculum size effects microbial growth and bioflocculant production. Optimum inoculum size is favourable for bioflocculant-producers to adapt to the medium. It shortens the lag phase and promotes bioflocculant production (Nwodo et al., 2013)**.** The inoculum size of 2% gave the highest flocculating activity of 92.8% (Table 1). However, since there was no significant difference between 92.8% flocculating activity and 86.3% that was achieved at 1% (v/v) inoculum size, the inoculum size of 1% was preferred and used for subsequent inoculations. The preferable inoculum sizes range from 1 to 5% (v/v) (Okoh et al., 2012). Small inoculum sizes turn to prolong the lag growth phase while large inoculum sizes usually results in a niche overlapping of the microorganisms, thereby suppressing bioflocculant production (Nurul‑Adela et al., 2016). The bacterial consortium fell within the accepted range and thus is economical. The results were better compared to those of *Citrobacter sp*, which showed the optimum bioflocculant production at an inoculum size of 5% (Jang et al., 2001).

Effect of carbon and nitrogen sources

Bacterial strains prefer different carbon and nitrogen sources for multiplication and production of bioflocculants. The effect carbon and nitrogen sources on bioflocculant production was

evaluated and the results are displayed in Table 1. Glucose was the suitable carbon source with a flocculating activity of 92.8%. Starch was the least preferred carbon source with a flocculating activity of 48.1%. The results contradicted those of Zhang et al. (2002) whereby glucose inhibited bioflocculant production by *Sorangium cellulosum*. Nevertheless, the findings are similar to those observed by Ogunsade et al. (2015) whereby glucose enhanced bioflocculant production by *Bacillus amyloliquefaciens* ABL 19. Generally, all nitrogen sources were good sources for bacteria and showed flocculating activities above 80%. Ammonia sulphate was the best nitrogen source with a flocculating activity of 98.1%. The observations were in agreement to those of Okaiyeto et al. (2016) whereby ammonium sulphate promoted the growth of marine *Bacillus* sp and yielded high bioflocculant.

Effect of shaking speed on flocculating activity

The function of agitation is to distribute the microbial cells and nutrients evenly throughout the medium and to ensure oxygen absorption into the medium. The effect of shaking speed on the flocculating activity is shown in Figure 1. A shaking speed of 165 rpm was the most conducive speed, giving the highest flocculating activity of 95%. The shaking speeds above or below 165 rpm led to a decrease in the flocculating activity. Thus, the shaking speed of 165 rpm enabled sufficient absorbance of oxygen into the medium and distribution of nutrients. The results were in close conformity with those reported by Piyo et al. (2011) whereby the best flocculation was obtained at 160 rpm. The similarity may be due to the same oxygen demand by bacteria at different growth stages (Li et al., 2009).

Effect of temperature on flocculating activity

Metabolic reactions take place effectively at optimum temperatures for microbial growth and bioflocculant production (Shahadata et al., 2017). A rise in temperature increases growth, metabolic function and bioflocculant production until the point where denaturation reactions set. Low temperatures may decrease the microbial growth rate and cell wall polymer synthesis (More et al., 2014). In this study, the effect of temperature on flocculating activity was assessed and the results are shown in Figure 2. The maximum flocculating activity of 98% was observed at a culture temperature of 30 ^{o}C. The temperature above or below 30 ^{o}C led to a decrease in flocculating activity. The results corroborate the observation by Luo et al. (2016) whereby *Bacillus megaterium* sp1 preferred 30 ^{o}C to efficiently produce sufficient bioflocculant. However, they were contrary to those of Giri et al. (2015) whereby 40 °C was the optimum temperature for bioflocculant production by *Bacillus subtilis* F9.

Effect of the initial pH on flocculating activity

Initial pH of the growth medium determines the electrification of the microbial cells and oxidation–reduction potential (Xia et al., 2008). Thus, the alteration in pH can directly or indirectly affect absorption of nutrients in the production medium, metabolic reactions and bioflocculant production. The effect of the initial pH of the growth medium on flocculating activity is shown in Figure 3. The highest flocculating activity of 95% was obtained when the initial pH of the culture medium was 6. However, the flocculating activity was more significant in acidic conditions and gave flocculating activities above 80%. With the initial pH of 6, bacteria were able to multiply and to produce sufficient bioflocculant. The findings were in agreement with those reported by Zhang et al. (2007) whereby the initial pH of 6 was the best pH for bioflocculant production by strains of BAFRT4, HXCS2, HXTD2, CYGS1 and CYGS4 in consortium.

Time course assay

Bioflocculant production takes place at different growth stages among microbial strains (Liu et al., 2015). The effect of time on flocculating activity (FA), optical density (OD) of culture broth (which indicated cell number) and initial pH are shown in Figure 4. The flocculating activity increased relatively to the optical density until 72 hours. The flocculating activity of 90% was obtained within 72 hours. After 72 hours, the OD decreased while there was an insignificant increase in flocculating activity. Therefore, 72 hours of growth was preferred. Arafa et al. (2014) found similar results whereby the flocculating activity of the bioflocculant from *B. cereus* was maximum within 72 hours of the growth period. Furthermore, the observations implied that the bioflocculant was produced by biosynthesis during growth and not by cell autolysis (Ntsangani et al., 2017). The initial pH of the growth broth decreased from the initial pH value of 6.00 to pH 3.2.

Table 1: Effect of inoculum size, carbon sources, nitrogen sources and cations on flocculation

Inoculum size (%)	FA (%) ± SD	Carbon source	FA (%) ± SD	Nitrogen source	FA (%) ± SD
1	86.3±3.60[a]	Fructose	48.3±2.74[a]	Yeast extract	86.8±2.71[a]
2	92.8±2.73[a]	Starch	48.1±8.65[a]	Urea	93.5±2.22[b]
3	92.4±2.49[a]	Xylose	48.9±4.93[a]	Peptone	94.1±1.03[b]
4	80.7±12.13[a]	Molasses	76.4±10.12[b]	Casein	95.8±2.28[b]
5	76.8±7.09[a]	Lactose	76.8±8.54[b]	Ammonium sulphate	98.1±0.45[b]
-	-	Sucrose	82.6±6.01[b]	-	-
-	-	Maltose	83.1±10.21[b]	-	-
-	-	Glucose	92.8±2.73[b]	-	-

FA denotes flocculating activity while SD denotes standard deviation.

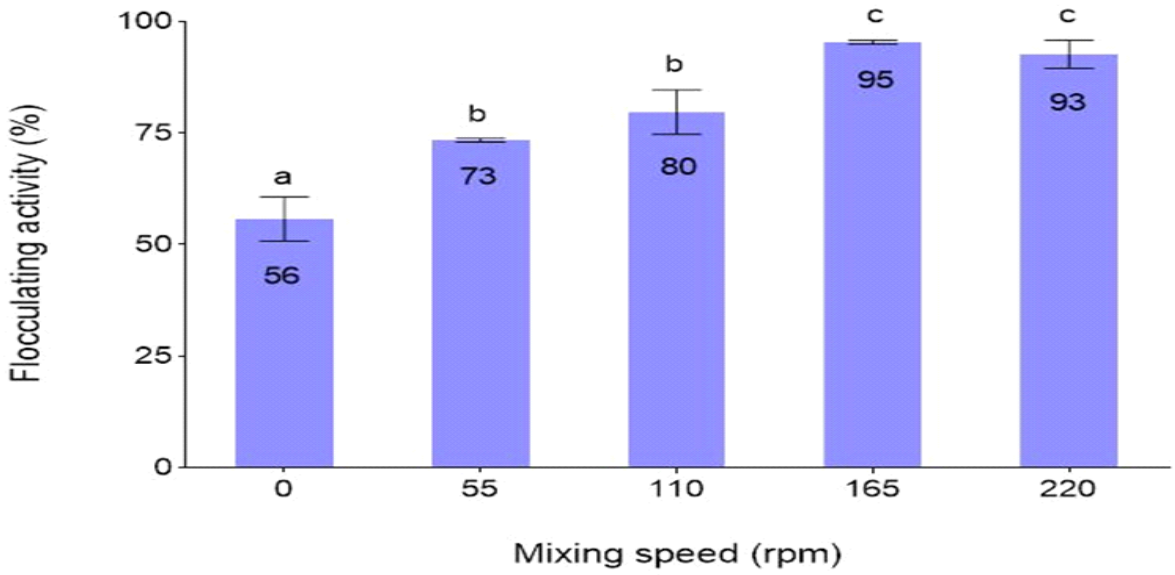

Figure 1: Effect of shaking speed on flocculating activity.

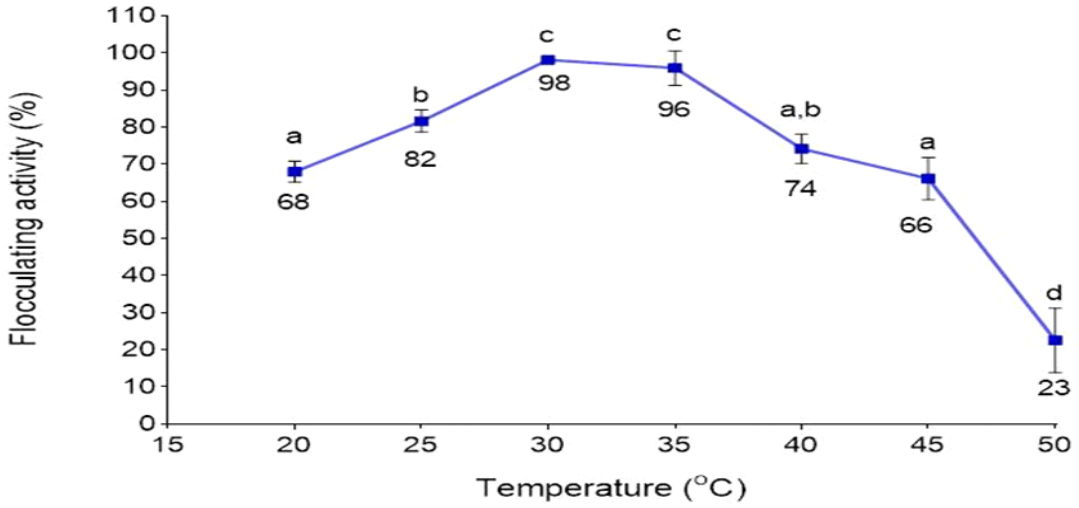

Figure 2: Effect of temperature on flocculating activity.

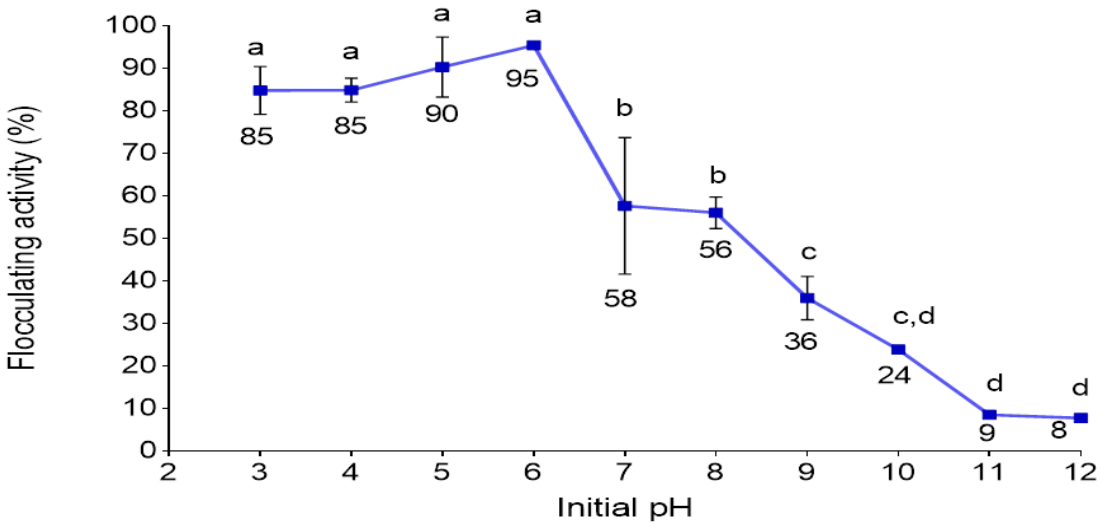

Figure 3: Effect of initial pH on flocculating activity.

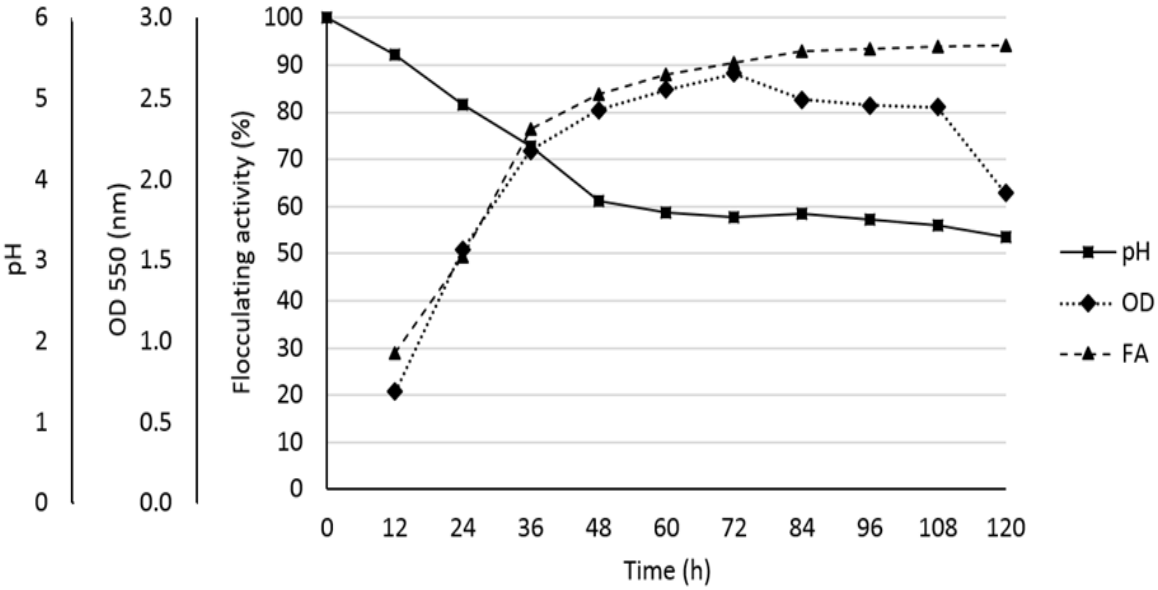

Figure 4: Effect of time course on FA, OD and initial pH.

The decrease might have been owed to the fact that organic acidic components were excreted by the bacteria, consequently lowering the pH (Okaiyeto et al., 2013).

Bioflocculant yield

Low yield of the bioflocculants is a major challenge. After fermenting 1 L of a of growth broth of the consortium containing a consortium for 72 hours, about 3.1 g of the purified bioflocculant was obtained. The yield of the consortium was higher than of the individual bacterial strains (*B. pumilus* JX860616: 2.4 g/l; *B. subtilis* CSM5: 1.5 g/l). The increase might be due to the synergistic relationship between the strains. Moreover, it was higher than of other pure bacterial strains from other studies (Xiong et al., 2010). The results confirm the study by Zhang et al. (2007) and Pau et al. (2014) who reported improved yields as a result of combination of microbial strains in fermentation.

Effect of dosage and cations on flocculating activity

Dosage size determines the flocculating activity of bioflocculants. Insufficient dosage sizes have the tendency not to neutralize negative charges on the colloidal particles in suspension, leading to a poor flocculation process (Gong et al., 2008). On the other hand, excessive amounts of bioflocculant concentrations in suspension can lead to re-stabilisation of colloidal pollutants and thereby reducing flocculating activity. The bioflocculant dosage size assay was done and the results are illustrated in Table 2. The highest flocculating activity of 77.2% was observed at a concentration of 0.8 mg/ml. However, there was no statistical difference ($p < 0.05$) observed between 0.2 mg/ml and 0.8 mg/ml, regarding the flocculating activities. Therefore, the concentration of 0.2 mg/ml was preferred in all experiments. The effectiveness of these bioflocculant at low concentration translates its economic friendliness. The findings were in conformity with those of Cosa and Okoh. (2014) whereby the concentration 0.2 mg/ml of the bioflocculant from a consortium of *Oceanobacillus* sp and *Halobacillus* sp was optimum for effective flocculation. Nevertheless, they were contradictory to those of the bioflocculant from *Azotobacter indicus* which preferred higher dosage size (8% v/w) (Patil et al., 2011),

Cations enhance the adsorption of bioflocculants onto the surface of colloidal particles by neutralizing and stabilising the negative charges of the bioflocculant and colloidal particles in suspension (Wong et al., 2012). The purified bioflocculant was highly effective when Ba^{2+} was used as a cation (Table 2). It gave the highest flocculating activity of 90.1%.This implied that Ba^{2+} effectively neutralised the negative surface charge on the bioflocculant and kaolin particles, and thereby shortened the distance between particles and the bioflocculant, consequently resulting in high flocculating activity. The results agreed with the generally accepted hypothesis that multivalent metal cations effectively bridge with negative functional groups on bioflocculant chains. Moreover, they were in accordance with the results observed by Okoh and Ugbenyen. (2014) whereby the flocculating activity was significantly stimulated by divalent cations (Mg^{2+}, Mn^{2+} and Ca^{2+}).

Elemental analysis of the bioflocculant

The elemental composition of the bioflocculant plays an important role in structure and flocculating activity of the bioflocculants (Bisht and Lal, 2019). Various elements bring about flexibility and stability. The composition of the elements in mass proportion (% w/t) is illustrated in Figure 5: The presence of carbon, oxygen and nitrogen confirmed the bioflocculant as a glycoprotein molecule (Devi et al., 2015). Moreover, the abundance of carbon and oxygen affirms the bioflocculant to be predominately a carbohydrate in nature. Okaiyeto et al. (2015) obtained almost similar results with a glycoprotein bioflocculant MBF-UFH which possessed different elements such as: C, O, Na, Mg, P, S, Cl, K and Ca.

Functional groups of the bioflocculant

The functional groups of the bioflocculants provide adsorption sites for colloids in suspension (Liang et al. 2018). Thus, the flocculating activity strongly depends on the number of functional groups available in bioflocculant chains. IR spectrum of the bioflocculant displays the obtained functional groups (Figure 6). It revealed the presence of hydroxyl (3309 cm^{-1}), amide (1648 cm^{-1}) and amino (1090 cm^{-1}) groups. These functional groups resulted in the observed high flocculating activity (Bisht and Lal, 2019). Moreover, the detected functional groups affirm the bioflocculant as a glycoprotein molecule. The results were similar to those obtained in other studies (Abd-El-Haleem et al., 2008 & Tang et al., 2014).

Pyrolysis property of the bioflocculant

According to Wang et al. (2011) thermal degradation of bioflocculants usually occurs in two steps. The first step involves an increase in temperature to about 150 ^{o}C resulting in a loss of moisture by bioflocculants. The second step entails depolymerisation of the bioflocculant structures at higher temperatures. Figure 7 illustrates the pyrolysis profile of the test bioflocculant. There was a decrease in weight between 35 and 150 ^{o}C. The weight loss was attributed to a loss of moisture content from the hydroxyl group. Further decline in weight was attributed to the degradation of the bioflocculant. The degradation temperature (*T*d) of this bioflocculant was first observed at 155 ^{o}C and then at 170, 294 and 384 °C. More than 75% was obtained after exposure to 900 ^{o}C. This affirmed the bioflocculant as a thermal stable molecule.

Table 2: Effect of dosage size and cations on flocculating activity

Dosage size (mg/ml)	FA (%) ± SD	Cations	FA (%) ± SD
0.2	72.9±6.20[a]	Li^{+}	86.5±1.37[a,c]
0.4	71.7±4,70[a]	Na^{+}	80.4±2.36[a,d]
0.6	70.9±2.25[a]	K^{+}	78.1±4.51[b,d]
0.8	77.2±2.21[a]	Ba^{2+}	90.1±2.35[c]
1	72.8±2.34[a]	Mn^{2+}	85.8±3.11[a,b,c]
		Ca^{2+}	77.2±2.21[d]
		Fe^{3+}	47.4±3.59[e]
		Control (Without cation)	71.0 ±1.37[d]

FA denotes flocculating activity while SD denotes standard deviation.

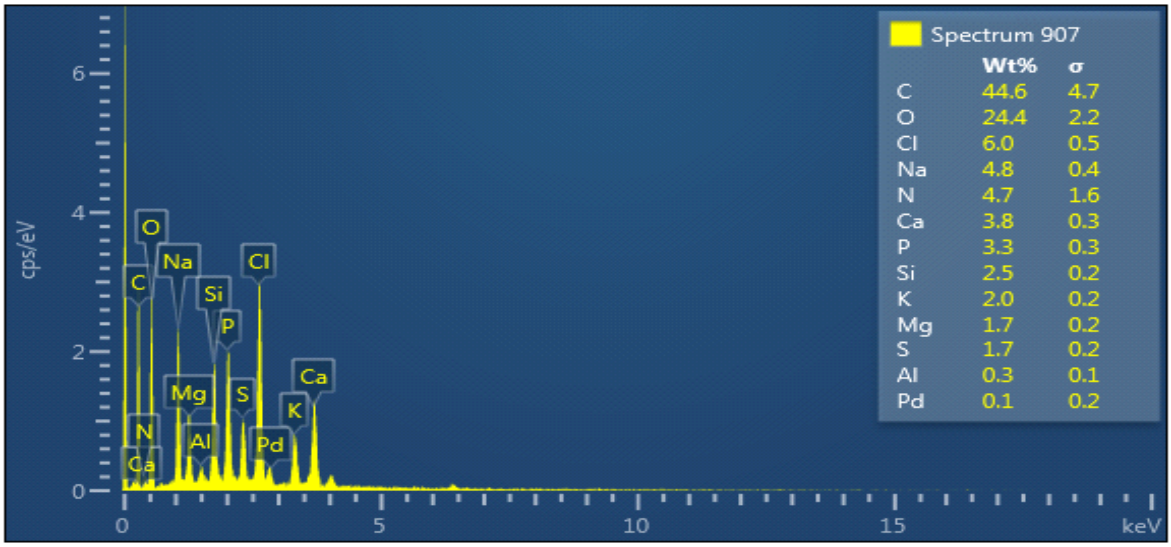

Figure 5: Elemental analyses of the bioflocculant.

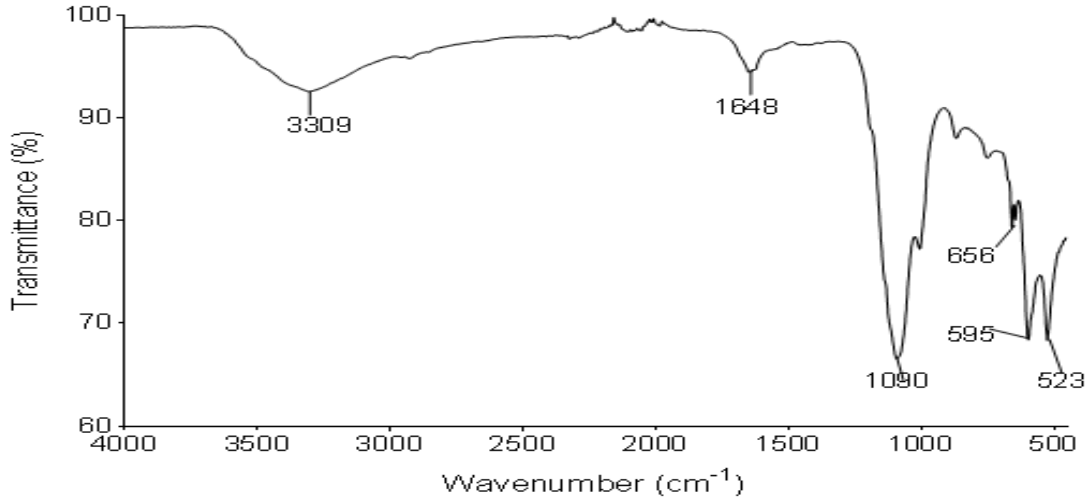

Figure 6: IR spectrum of the bioflocculant.

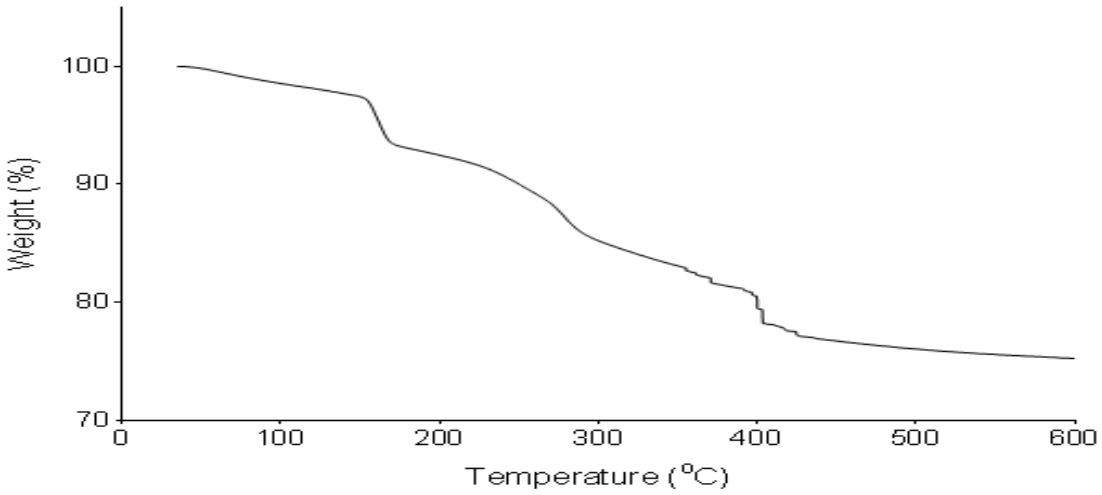

Figure 7: Pyrolysis property of the bioflocculant.

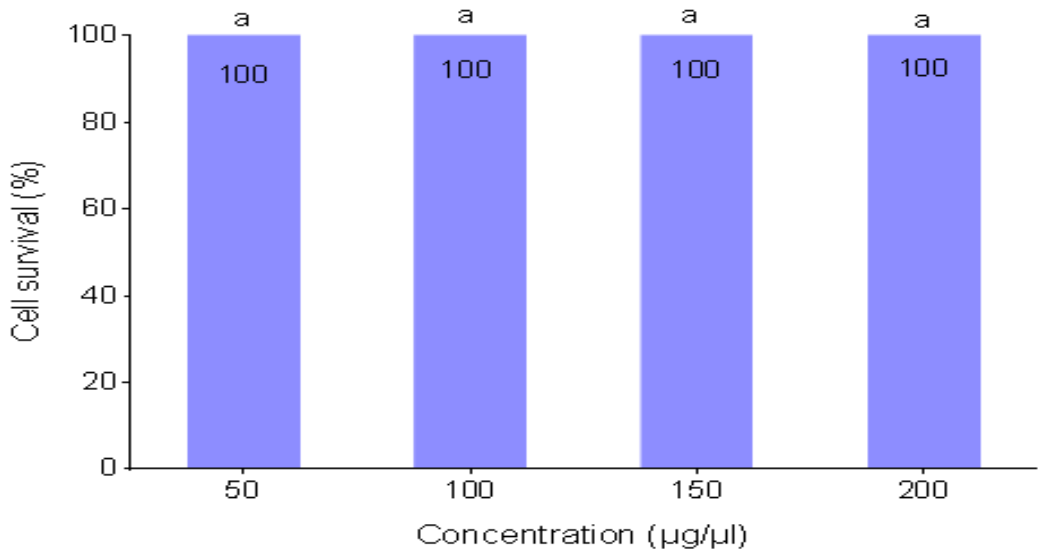

Figure 8: *In vitro* cytotoxicity of the bioflocculant on MCF7 cells.

The detected carboxyl group in an amide group and hydroxyl groups, as shown by IR spectrum, might have led to formation of hydrogen bonds, which might be responsible for its thermal stability.

In-vitro cytotoxicity of the bioflocculant

MTT cell proliferation assay was used to assess cell viability of MCF7 cells after being treated with different concentrations of the purified bioflocculant. The bioflocculant showed no cytotoxic effects on MCF7 cell line. The cell line exhibited 100% growth in all tested concentrations (Figure 8). The toxicity threshold level at mean lethal concentration (LC_{50}) was not determined as the cells showed high percentage viability after MTT assay. The results affirmed the probable safe utilisation of the bioflocculant in different biotechnological applications. The results were in agreement with those of Sharma et al. (2017) whereby the exopolymer produced by *Acinetobacter haemolyticus* showed no toxicity on sheep blood cells. Moreover, there were no clinical symptoms observed on rats when the same biopolymer was used.

Flocculation mechanisms of the bioflocculant

Depending on the size of the bioflocculant and the surface properties of the colloidal particles in solution, two major flocculation mechanisms may happen: (1) bridging and (2) charge neutralization (Li et al., 2009). Charge neutralization takes place when the bioflocculant is oppositely charged, as compared to the colloids under treatment. Here, the surface charge density is compact by adsorption on the bioflocculant and the colloids. Bridging mechanism dominates when the bioflocculant extends into the mixture in a distance greater than the distance over which the colloids` repulsion can act. The zeta potentials were measured to assist in the determination of the flocculation mechanism of the bioflocculant and the results are shown in Table 3.

Table 3: Zeta potential of the samples

Samples	Zeta potential (mV)
Bioflocculant	-11.2±1.912
Kaolin particles	-6.59±3.000
Kaolin particles with Ba^{2+}	-7.01±0.992
Kaolin particles flocculated by the bioflocculant in the presence of Ba^{2+}	-5.02±2.754

The zeta potential values of the bioflocculant, kaolin suspension, kaolin plus Ba^{2+} and the flocculated kaolin suspension were all negative. However, the addition of Ba^{2+} to kaolin suspension and kaolin suspension plus bioflocculant resulted in the increase value of zeta-potentials. It was, therefore, concluded that Ba^{2+} stimulated rapid flocculation through neutralisation and stabilisation of residual negative charges of bioflocculant, forming the bridges that binds kaolin particles to each other.

The bridging mechanism was owed to the surface structures of the bioflocculant, chemical components and functional groups. The results were comparable to other studies (Guo et al., 2015).

Removal efficiency of the bioflocculant

High levels of COD and BOD often lead to anaerobic conditions, bad odours and stagnant waters that do not support aquatic life (Kamaruddin et al., 2013) while high concentrations of P induce eutrophication (Waajen et al., 2016). The removal efficiency of the bioflocculant on BOD, COD and P in wastewater from the Nhalabane Estuary was evaluated in comparison to conventional chemical flocculants (alum and ferric chloride). The bioflocculant showed good removal efficiencies of 99, 84 and 75% on BOD, COD and P, respectively (Table 4). It had better removal efficiencies on COD and P when compared to alum and ferric chloride. The results were comparable to other studies (Pua et al. 2014 & Cosa and Okoh 2014) whereby the extracted bioflocculants from bacterial consortia were more capable of efficiently reducing pollutants in wastewater when compared to chemical flocculants. The removal efficiencies showed by the bioflocculant implied that it has potential in industrial applicability.

Table 4: Removal efficiency of the bioflocculant

Type of flocculants	Water quality before treatment			Water quality after treatment			Removal efficiency (%)		
	BOD (mg/l)	COD (mg/l)	P (mg/l)	BOD (mg/l)	COD (mg/l)	P (mg/l)	BOD	COD	P
Bioflocculant	123.2±0.0	154±0.0	2.83±0.0	0.1±1.7	25±2	0.7±0.2	99[a]	84[a]	75[a]
Alum	123.2±0.0	154±0.0	2.83±0.0	0.2±3.4	55±7.2	1.3±0.0	99[a]	64[b]	54[b]
$FeCl_3$	123.2±0.0	154±0.0	2.83±0.0	0.1±1.7	51±1	1±0	99[a]	67[b]	65[b]

CONCLUSION

The bioflocculant from a consortium of *Bacillus pumilus* JX860616 and *Bacillus subtilis* CSM5 had high flocculating activity of 90% after 72 hours of fermentation at optimal conditions; glucose, ammonium sulphate, initial pH 7, 30 ^{o}C at a shaking speed of 165 rpm. The yield of 3.1 g/l was obtained. The yield was higher than those of the individual bacterial strains. The bioflocculant was cation dependent, thermostable and non-cytotoxic. The high flocculating activity was attributed to the detected functional groups such as carboxyl, amide and hydroxyl groups. The flocculation process was as a result of a double layer compression by cation Ba^{2+}, chemical reactions and bridging mechanisms. The bioflocculant effectively reduced COD, BOD and P levels in the treated wastewater. For further studies, the genes encoding for the production of the bioflocculant will be investigated. Moreover, metabolic pathways that are involved during bioflocculant production will also be investigated.

CONFLICT OF INTEREST

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

ACKNOWLEGEMENT

The authors acknowledge the Research and Innovation Committee of the University of Zululand, for funding of this research. The authors acknowledge the staff at University of Zululand and University of KwaZulu-Natal for their support.

AUTHOR CONTRIBUTIONS

Basson A.K. designed the project. Maliehe T.S and Singh M performed the experiments. Maliehe T.S wrote the manuscript. All authors read and approved the final version.

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