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Assessment of the flocculating potentials of *Alcaligenes faecalis* Isolated from the Estuary of Sodwana Bay

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Abstract

Alcaligenes faecalis was previously isolated from Sodwana Bay, South Africa and was shown to be a bioflocculant producing microorganism. The bioflocculant production potential was further assessed through the optimization of the standardized culture media. The production of biofloculant as well as the flocculation was evaluated using different variables such as the size of inoculum, sources of carbon and nitrogen, time course and pH. Through optimization *A. faecalis* showed an improvement in the production of its bioflocculant and also flocculating activity for the following factors: flocculating activity of 71% for an inoculum size of 1%. The bioflocculant produced when maltose was used as source of carbon, showed flocculating activity of 91%, urea, as the most efficient nitrogen source, showed a flocculating activity of 97%, the optimum pH was 9. The time courses analysis between 60 and 72 hours showed the peak for flocculation and by implication highest level of bioflocculant production.

Keywords: Bioflocculant; *Alcaligene;* Flocculating Activity; Inoculum size.

1.0 Introduction

Flocculation as defined by Droste, (1997); is a means by which disrupted particles in suspension are assembled into bigger flocs so that they can be separated from suspension. The use of microorganisms to produce flocculants that are capable of bringing colloidal particles of suspension together in form of flocs (bioflocculation) is an increasing biotechnological process for water treatment, fermentation and processes in the downstream industries (Nakata and Kurane, 1999; Ugbenyen and Okoh, 2014). Bioflocculants are secondary metabolite produced by microorganisms during their growth. It is composed of high polymers such as extracellular polysaccharide, glycoprotein, cellulose, protein, and nucleic acid (Piyo *et al*. 2011).

A number of flocculants, such as inorganic, organic synthetic and natural flocculants, have been used for water treatment, fermentation and processes in the downstream industries (Kurane *et al*. 1986, Fujita *et al*. 2000). These flocculants are employed for different reasons according to their chemical characteristics and toxicity. Among them, is poly-aluminum chloride (PAC), which is commonly used for water and wastewater treatment. However, a major health associated problem with the use of PAC is Alzheimer's disease (Kowall *et al*. 1989). Another widely used organic flocculant is Poly-acrylamide (PAA) because of it high efficiency and low cost. However, PAA has the challenge of not been biodegraded easily and the monomeric unit is associated with both neurotoxicity and carcinogenicity (Vanhorick and Moens 1983, Dearfield and Abermathy 1988). Therefore, the development of biodegradable and safe flocculants is essential as an alternative. In contrast, microbial polymers that have flocculating activity are generally biodegraded readily and are relatively inert in nature, indicating their potential to replace the existing inorganic and organic synthetic flocculants.

Water is one of the most important sources of life and it is important to preserve it and purify it to suite not only human but also environmental needs. Therefore it is important to clean waste and industrial water before releasing it to nearby streams, rivers and oceans. According to the European Commission the maximum level of contaminant of aluminum in drinking water has been set at 200 g/l, with a target concentration of 50 g/l. (Zheng *et al.,* 2008; Salehizadeh and Shojaosadati, 2001).

This study was done to improve and optimize, the bioflocculant production potential of *Alcaligenes faecalis*, which is a gram negative, rod shaped, and motile bacteria. The effects of various parameters namely temperature, pH, cationic inducer, inoculum size and flocculation time was studied.

2.0 Experimental

2.1 Description of study site

Samples were taken at depths from 1 to 5 meters from the shore break into the sea at both the estuarine mouths and from beach of Sodwana Bay located on the east coast of South Africa, between St. Lucia and Lake Sibhayi with coordinates 27º32'S and 32º40'E . These sample locations were chosen near or at the river mouths as these areas are believed to have excess suspended particles. In areas like this microbes (with bioflocculant properties) thrive in such environments to flocculant suspended materials.

2.2 Sample collection and processing

Six different marine water and sediment samples were randomly collected from approximately 0–20 m directly offshore of the beach. The samples collected were transported in ice to the hydrology and microbiology laboratories of the University of Zululand, KwaDlangezwa. Sediment samples were dried overnight at 30ºC.

2.3 Isolation and screening of microorganisms

Bioflocculant-producing microorganisms were isolated in cultured agar plate with medium composition as follows: meat extract,1.0 g; peptone,5.0 g; yeast extract, 2.0 g; NaCl, 8.0 g; and agar, 15.0 g per litre of filtered sea water in accordance to Jensen et al. (1991). These microorganisms were originally screened on the basis of colony morphology. Two loopfuls of bacterial colonies were then grown in 50 mL of screening medium described by Zhang et al. (2007) and Ugbenyen et al. (2012), with slight modifications, on a rotary shaker (160 rpm) at 30°C for 72 h. The medium contained (g/L of filtered natural sea water): glucose, 20; casein, 0.7; yeast extract, 0.5; KH_2PO_4, 2; K_2HPO_4, 5; NaCl, 0.1 and $MgSO_4 \cdot 7H_2O$, 0.2. After the period of incubation was completed, 2 mL of the fermentation broth was centrifuged (8 000 g, 30 min) to separate the cells, after which the cell-free culture supernatant was analyzed for flocculating activity. At the end of flocculation, the cell-free culture supernatant of one isolate showed good flocculating activity for kaolin clay. The isolate was then selected for further study. The pre-culture was stored at 4°C and used for subsequent inoculations.

2.4 Flocculating activity experiment

Flocculating activity was measured according to the method described elsewhere (Kurane et al., 1986; Zhang et al., 2007; Cosa et al., 2011; Ugbenyen et al., 2012) with modifications. Briefly, 3 mL of 1% $CaCl_2$ and 2 mL of cell-free supernatant were added to 100 mL kaolin suspended solution (4 g/L) in a 250 mL flask. The mixture was stirred vigorously, poured into a 100 mL cylinder and allowed to stand for 5 min. The absorbance at 550 nm of the clarifying solution was measured spectrophotometrically. A control experiment was performed using the same method, but with fresh culture medium replacing the cell-free supernatant. The flocculating activity was calculated according to the equation:

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

where A is the absorbance of the sample at 550 nm; B is the absorbance of the control experiment at 550 nm.

2.5 Identification of Bioflocculant producing microorganisms

2.5.1 DNA Extraction

DNA extraction was done for selected isolates showing good potential for the production of bioflocculant using ZR fungal/bacterial DNA MiniPrep™ Kit from Zymo Research, Inc. The extraction protocol was performed according to the instruction of the manufacturer.

2.5.2 Amplification of 16S rRNA gene (Polymerase chain reaction (PCR))

Gene amplification was carried out in 50 µL reaction volume of Dream Taq™ Green PCR Master Mix containing 0.4 mM of each dNTP, 4 mM $MgCl_2$, 0.2 µL Dream Taq™ polymerase, 1 µM of each forward and reverse universal primer (F:5'ATGCCATAGCATTTTTATCC3'), (R:5'GATTTAATCTGTATCAGG-3') and 1 µg template DNA. The condition for polymerase chain reaction includes an initial denaturation (95ºC for 3 min), 25 cycles of denaturation (95ºC for 30 sec), annealing (56ºC for 30 sec) and extension (72ºC for 1 min), and a final extension (72ºC for 5 min).

2.5.3 PCR Products Sequence

The PCR product sequence was done at the facilities of Inqaba Biotech Laboratories, Pretoria, South Africa using a sequencer. The 16S rRNA

gene sequences obtained was compared with others in the NCBI genebank database with Basic Alignment Search Tool (BLAST) according to Stephen *et al*. (1997).

2.6 Inoculum size experiment

An amount of 50 mL of production medium in 100 mL flasks was separately inoculated with 0.5, 1.0, 1.5, 2.0, and 2.5 mL of the pre-culture, representing 1, 2, 3, 4,and 5 % inoculum size of the test bacteria, respectively, cultivated at 30°C and 160 rpm for 72 h. After the period of incubation was completed, the fermentation broths were centrifuged (8 000 g, 30 min) to separate the cells. The cell-free culture supernatants were analyzed for flocculating activity.

2.7 Effects of carbon and nitrogen sources

The effects of carbon sources on bioflocculant production by the *Alcaligenes faecalis* were assessed. The carbon sources included glucose, sucrose, maltose, starch, fructose, lactose and molasses. Nitrogen sources (namely, urea, ammonium sulphate, peptone, casein and yeast extract) were assessed also for their effect on bioflocculant production. In the production medium, casein and yeast extract were replaced with one of the nitrogen sources in equivalent amounts.

2.8 Effect of pH and cations

The effects of these parameters on production of bioflocculant by the test bacteria *Alcaligenes faecalis* were assessed by the method of Liu et al. (2010). Variation of the initial pH of the production medium over a wide range of 3–12 was achieved using HCl and NaOH, whereas the cations that were varied included Li^+, Na^+, K^+, Mn^{2+}, Fe^{2+} and Al^{3+}. For cation assays, flocculant tests was done by replacing $CaCl_2$ solution with a solution of LiCl, NaCl, KCl, $MnCl_2$, $FeCl_2$ and $AlCl_3$ and their various flocculating activity was measured.

2.9 Time profile assay

Medium composition for the time profile assay was as described earlier, with slight modification (Zhang et al., 2007; Ugbenyen et al., 2012). With the optimum growth conditions previously determined, the medium for bioflocculant production was inoculated with pre-culture of the test bacteria *Alcaligenes faecalis*. 2 mL of samples were withdrawn at 12-h intervals over a period of 108 h, centrifuged (8 000 g, 30 min) and the cell-free supernatant was used to determine the flocculating activity. The optical density (OD) of the broth at 660 nm was also recorded.

2.10 Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) using the GraphPad PRISM 5 statistical package. A significance level of $p < 0.05$ was used. The experiments were carried out in triplicate.

3.0 Result

3.1. Effect of inoculum size

Alcaligenes faecalis has the capability to produce bioflocculant using the inoculum sizes1%-5% (v/v) as shown in Figure 1. Optimal flocculating activity was above 70% at inoculum sizes1, 4 and 5% (v/v) respectively.

3.2. Effect of carbon sources

The effect of various carbon sources on flocculating activity is shown in Figure 2. Maltose was the preferred carbon source for the production of the bioflocculant by the bacterium due to its highest flocculating activity of 91%. However *A. faecalis* can utilize various carbon sources as shown in Figure 2 as they all have a flocculating activity of above 60% accept for molasses which showed a flocculating activity of 39%.

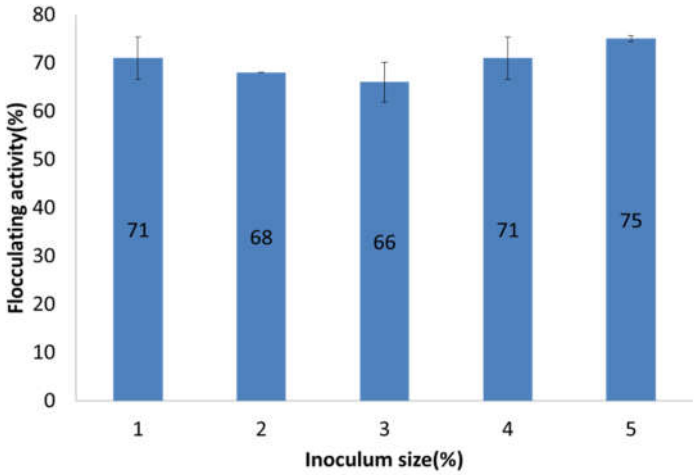

Figure 1: *Effect of inoculum size on flocculating activity of Alcaligenes faecalis. The results are represented as mean value of triplicate ± Standard deviation. Error bars represent the standard deviation of mean value of triplicate.*

3.3. Effect of nitrogen sources

In Figure 3, urea was the prefered nitrogen source for *A. faecalis* for the production of bioflocculant with a flocculating activity of 97% followed by ammonium sulphate. Urea and peptone yielded the highest flocculating activity in terms of organic nitrogen sources whereas ammonium sulphate was the only tested inorganic nitrogen source with an optimal of 89%. Yeast extract and casein showed that they were not a preferred source for bioflocculant production by the bacterium with low flocculating activity.

3.4. Effect of pH

Figure 4, showed the effect of pH on the bioflocculant production potential of *A. faecalis*. The bacterium could produce bioflocculant over a wide range of pH as revealed by the flocculating activity. The maximum flocculating activity was observed with the highest flocculating activity between 80-85% at pH 8 and 9. Thus *A .faecalis* showed optimal growth at alkaline pH.

3.5. Effect of cations

The effect of various metal ions on flocculating activity of the bioflocculant produced by *Alcaligenes faecalis* is shown in Figure 5, both monovalent (K^+, Li^+, and Na^+) and divalent (Ca^{2+}, Fe^{2+},and Mn^{2+}) cations yielded a flocculating activity of above 60% except Fe^{2+} with a flocculating activity of 46% whereas trivalent cation (Al^{3+}) showed the lowest flocculating activity 43%. K^+ showed an optimal flocculating activity of 78% thus was the preferred cation followed by Li^+ and Ca^{2+}.

3.6. Time profile

The flocculating activity of *A.faecalis* peaked at 60 hours with a flocculating activity of 68%. In the earliest hours from 0-36, the flocculating activity was less than 50%, and started to rise from 48 hours with a flocculating activity of 55% up to 84 hours with an activity of 62% then it dropped drastically to 26% at 108 hours. The highest flocculating activity was observed between 60-84 hours with a flocculating activity of more than 60%.

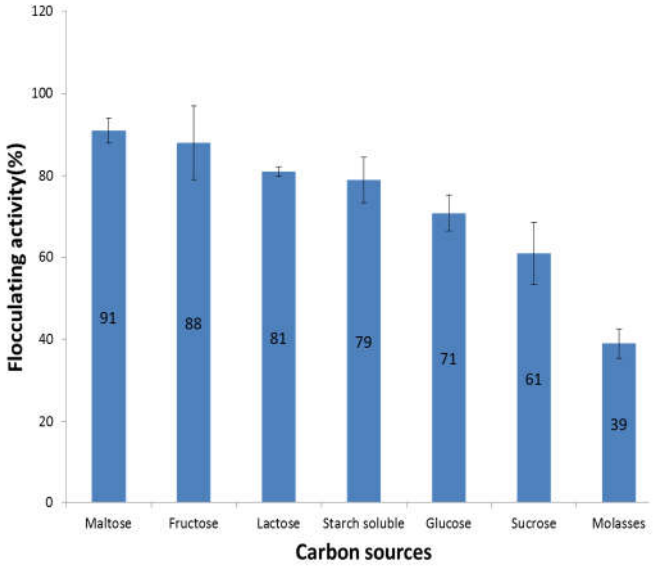

Figure 2: *Effect of different carbon sources on flocculating activity of Alcaligenes faecalis. The results are represented as mean value of triplicate ± Standard deviation. Error bars represent the standard deviation of mean value of triplicate.*

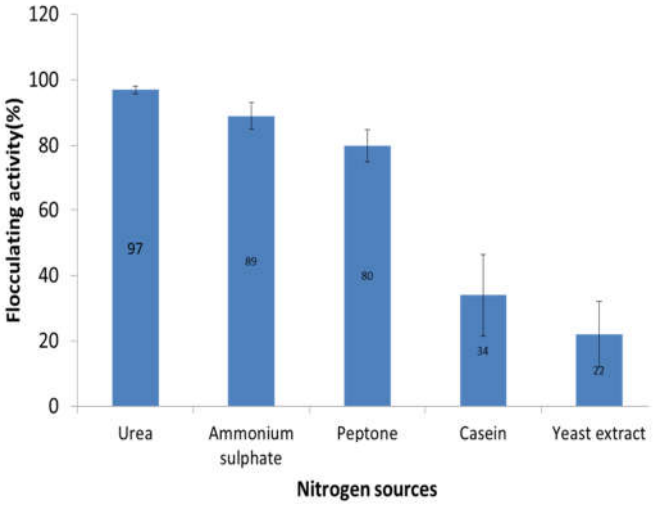

Figure 3: *Effect of nitrogen sources on flocculating activity of Alcaligenes Faecalis. The results are represented as mean value of triplicate ± Standard deviation. Error bars represent the standard deviation of mean value of triplicate.*

4.0 Discussion

Bioflocculant production potential differs with each microorganism having the capability to produce bioflocculant, hence growth parameters becomes highly significant in determining the amount of bioflocculant produced and in turn the flocculating activity. Various factors such as the types of carbon and nitrogen sources, pH and cations available in a growth media influence greatly the production and activity of a bioflocculant. According to some other study, it proved that the composition of the culture media and culture conditions do have an impact in the functionality of microorganisms to produce bioflocculant (Gboyega and Adebayo-Tayo, 2013).

The inoculum size contributes to cell reproduction (cell proliferation) which impact on the amount of flocculant produce by the organism. In Figure 1, *A. faecalis* achieved the highest flocculating activity of 71% when the size of inoculum 1 %(v/v) was used. The flocculation decreased when 2 %, 3% (v/v) inoculation was used but increased with an inoculation of 4% and 5% (v/v).

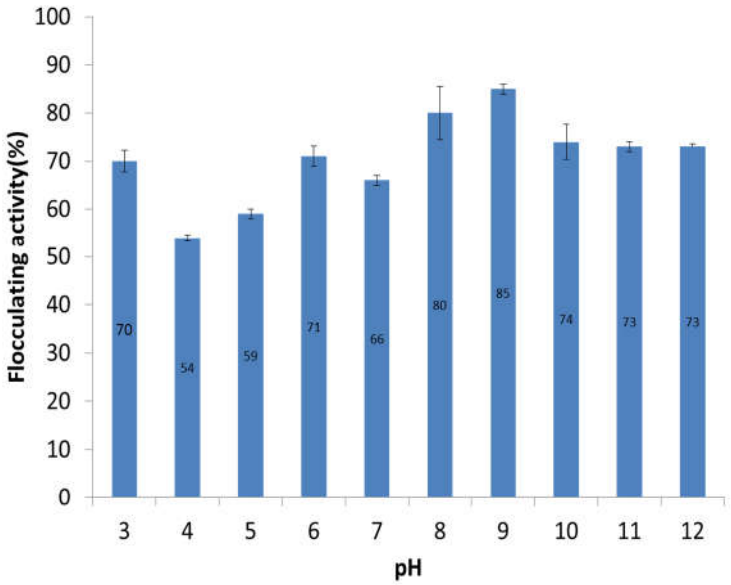

Figure 4: *Effect of pH on flocculating activity of Alcaligenes faecalis. The results are represented as mean value of triplicate ± Standard deviation. Error bars represent the standard deviation of mean value of triplicate.*

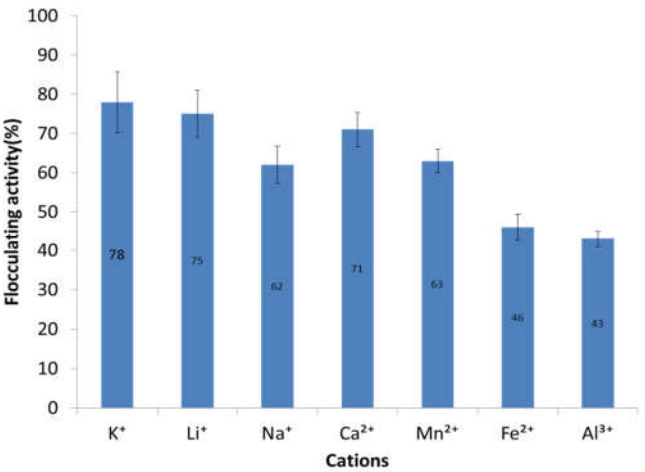

Figure 5: *Effect of cations on flocculating activity of Alcaligenes faecalis. The results are represented as mean value of triplicate ± Standard deviation. Error bars represent the standard deviation of mean value of triplicate.*

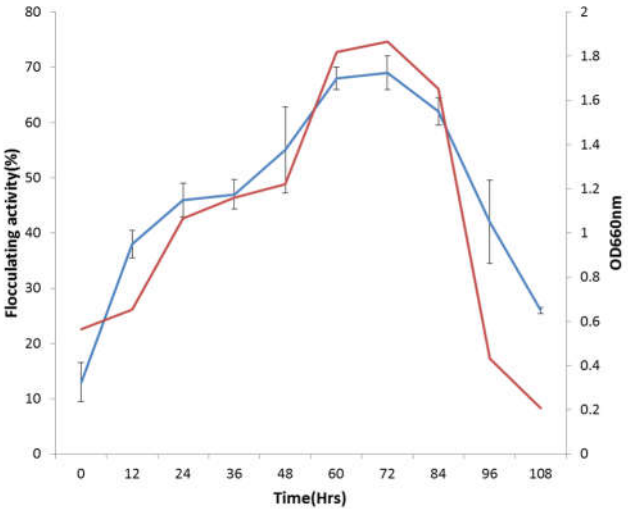

Figure 6: *Time profile of A. faecalis. The flocculating activities and the optical density were measured against time. The results are represented as mean value of triplicate ± Standard deviation. Error bars represent the standard deviation of mean value of triplicate.*

Hence the size of inoculum 1% (v/v) was then used for subsequent inoculation because it is reasonable to use a small volume of cells to produce bioflocculant and achieve a high flocculation. An increase in inoculum size could leads to bioflocculant production inhibition due to nutrient limitations as the microorganisms have to adapt to the environment thus it might not be conducive enough for survival leading to nutrient deprivation hence hindering bioflocculant production and activity (Cosa *et al*., 2013). Small inoculation size will lengthen the lag phase, while a large inoculation will make an excessive overlap of the niche and hinder the flocculant production by the organism (Salehizadeh and Shojaosadati, 2001).

The presence of carbon source in the medium plays a vital role in promoting cell growth especially in microorganisms with extracellular polysaccharide production potential. In the current study several sugars (carbon sources) were investigated to determine which sugars promoted bioflocculant production in *A. faecalis* (Figure 2). Amongst the sugars, maltose is the most preferred carbon source used by the bacterium to produce bioflocculant, with a flocculating activity of 91%. Most of the carbon sources were well used by *Alcaligenes faecalis* to produce bioflocculant as shown by the high flocculating activities but molasses was poorly utilized as a carbon source by the bacterium. In contrast to our findings, Toeda and Kurane, (1991), reported that glucose, galactose and sucrose were better carbon sources for *A. cupidus* KT201 compared to maltose. Whereas other literatures revealed that various microorganisms could utilizes different carbon sources. Ugbenyen *et al*. (2014) stated that *Bacillus* sp. Gilbert utilizes sodium carbonate as the only source of carbon with the highest flocculating activity of 95.2%. Cosa *et al*. (2011), indicated that the *Virgibacillus* species produced the highest bioflocculant (91.8%) when glucose was used as a sole source of carbon.

The impact of different sources of nitrogen was also studied while glucose was used as the only source of carbon for bioflocculant production (Figure 3). Urea showed to be the nitrogen source most preferred by the bacteria with a flocculation of 97%. Sheng *et al*. (2006) stated that *Klebsiella sp.* showed an increase in the flocculating activity by 94.54% when urea was used as a nitrogen source and Kurane and Nohata, (1991), reported that *Alcaligenes latus* had maximum flocculating activity when urea were used as sole nitrogen sources thus supporting the statement by Cosa *et al*. (2013), which reported that nitrogen sources of organic origins were most preferred sources in bioflocculant production since they are more easily absorbed by the cells thus enhancing cell activity of the microorganisms. In the case of *A. faecalis* nitrogen sources of organic origins like yeast extract (22%) and casein (34%) showed poor flocculating activity. Interestingly, this bacterium could also utilized ammonium sulphate, an inorganic nitrogen source for bioflocculant production with a flocculating activity up to 80%. Piyo *et al*. (2011) reported a similar finding where *Bacillus sp. Gilbert* utilized ammonium chloride (an inorganic nitrogen source) effectively, to produce microbial flocculant with a flocculation of 91%.

The pH of the environment contributes greatly to the activity of microorganisms. Changes in pH may affect how the microorganism functions. The pH stimulate flocculation by affecting the stability of pensile particles and flocculants formation (Ugbenyen and Okoh, 2014). Every microorganism has its own tolerant pH, where it reproduce and functions. *A. faecalis* by nature survives at alkaline pH, of 7-11. The bioflocculant was produced mostly at alkaline conditions, where the maximum flocculation was observed at pH 9. *A. faecalis* produced bioflocculant at a pH range that is wide apart as shown by the flocculating activity in Figure 4. Similar results were observed by, Ugbenyen *et al*. (2014), that reported that *Bacillus sp. Gilbert* had the highest flocculating activity of 93.77% at pH 9. In another study, Ugbenyen and Okoh, (2014), reported that a consortium of *Cobetia* and *Bacillus* species produced flocculant at pH range that is wide apart and the highest was observed at pH 8 with a flocculating activity of 88%. Luvuyo *et al*. (2013) also reported that the mixed culture of *Methylobacterium* sp. Obi and *Actinobacterium* sp. Mayor produced flocculant at pH range of 3-12 and the highest flocculating activity observed at both alkaline pH 11 and acid pH of 3.

Cation dependency is one vital factor which indicates whether the cation supplied may assist in charge destabilization during the flocculation process by the flocculant and influences adsorption of the bioflocculant onto suspended particles (Wang *et al*., 2011.). In Figure 5, the highest flocculating activity of 78% was observed when monovalent cation K^+ was used. Virtually both cations with one valent electron and two valent electrons aided the flocculation of the produced bioflocculant except Fe^{2+} and trivalent cation (Al^{3+}) which showed poor flocculating activity. These findings contrast the work of Wu and Ye, (2007), who reported that cations with one valent electron reduce the strength of the bonds and result in a loose arrangement of flocs, thus decreasing the flocculating activity. Trivalent cations Al^{3+} inhibited flocculating activity of the bioflocculant of *A. faecalis*, whereas Cosa and Okoh, (2014), reported that only Fe^{3+} inhibited flocculation and Al^{3+}enhanced the flocculating activity of a consortium constructed from *Halobacillus* and *Oceanobacillus* genera by 86.6% and in another study Al^{3+} showed stronger synergistic effects than divalent cations on *A. cupidus* KT201 (Toeda and Kurane, 1991). Ogunsade *et al*. (2015) reported that cations with two valent electrons (specifically Ca^{2+} and Mg^{2+}) enhanced flocculation of *Bacillus amyloliquefaciens* ABL 19 by 94% and 87% respectively; while Li^+ and Na^+ (cations with one valent electron) caused a decline by 66% and 67% respectively thus supporting the statement by Wu and Ye, (2007).

Several studies have been done to indicate the relationship between cell

growth, pH and the flocculating activity of extracellular biopolymeric flocculants (EBFs) production (Mabinya *et al*., 2012). In Figure 6, *Alcaligenes feacalis* showed an increase in cell growth with an increase in flocculating activity. The flocculating activity was poor during the first 36 hours in the lag phase and peaked during the exponential phase above 50% between 48-84 hours. However the optimal flocculating activity was observed between 60-72 hours with a flocculating activity of 68% and 69% respectively. Gradual decline was observed during 96 and 108 hours respectively. The decline in flocculation activity might be as a result of autolysis of cell or the presence of enzymes that are capable of digesting the bioflocculant (Ugbenyen *et al*., 2014). Studies by (Kurane and Nohata, 1991), have reported that *A. latus* showed maximum flocculant production during the middle and late exponential phase (2-3 days) which is 48-72 hours and rapid decrease during stationery phase. Whereas other literature shows that other microorganism such as *Bacillus sp.Gilbert* peaked after 12 hours with a flocculating activity of 82.7% (Ugbenyen and Okoh, 2013). The increase in cell growth during the first 3 days might be indicative that the microbial flocculant was produced by biosynthesis during growth of the bacterium (Mabinya *et al*., 2012).

5.0 Conclusion

In this study *A. faecalis* has shown potential to be a producer of bioflocculant. Optimization has resulted in improved flocculating efficiency. Optimal flocculating activity was observed when maltose, urea were used as sole carbon and nitrogen sources, respectively, in the presence of K^+ under alkaline pH of 9. Further studies on the extraction of the bioflocculant are important for treatment of water and wastewater.

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Conflict of Interest

The authors declare that there is no conflict of interest

Authors' contributions

Conception: AMU, JJS and AKB
Design: AMU
Execution: TAM and AMU
Interpretation: AMU, TAM, JJS and AKB
Writing the paper: AMU
Proofreading the paper: JJS and AKB

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