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Production and Characterization of Bioflocculant by Freshwater Bacteria Recovered from Surface Water and Sediment Matrix

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ABSTRACT

The development of flocculants that are biodegradable, safe and subsequently reduce health and environmental risks is highly required. The present study was designed to evaluate bioflocculant production by freshwater bacteria recovered from surface water and sediment milieu. The flocculating activity (FA) was ascertained using kaolin clay as the suspended solids. The resultant effects of diverse sources of nitrogen and carbon on bioflocculant activity and the bioflocculant characterization were assessed using spectrophotometric method. Among the bacteria isolates screened for bioflocculant activity, three bacteria exhibited significant bioflocculant activity. The API-20NE identification system assigned the bacteria to *Ralstonia pickettii* (>99%); *Stenotrophomonas maltophilia* (>98%) and *Alcaligenes* sp. (>99%). Among the diverse carbon sources analyzed for *R. pickettii*, fructose and glucose showed effectiveness for bioflocculant production. An increase in the FA was observed as the pH increases and it attains optimum at neutral pH**,** and subsequently drops as the increase in pH reached pH 11 for *R. pickettii* and *S. maltophilia*. The FA was noticed to rise with culture age with a peak of activity attained after 9 days of cultivation. The purified bioflocculant yield was 0.263, 0.32 and 0.341 g for *S. maltophilia*, *R. pickettii* and *Alcaligenes* sp., respectively. Chemical analysis of the purified bioflocculant from *R. pickettii* and *Alcaligenes* sp. revealed that it contained both protein and carbohydrate while that of *S. maltophilia* contained only carbohydrate. Findings from this study revealed that purified bioflocculant bacteria from freshwater milieus can find application in the establishment of process condition for large scale effluent treatment process.

Keywords: Flocculation, Glycoprotein, Bioflocculant, Kaolin, Nitrogen, Carbon.

Introduction

Bioflocculants are polymers produced by microorganisms in the course of their growth.[1] Bioflocculants are extracellular microbial products that mainly consist of polysaccharides. The exopolymeric substances are commonly produced by bacteria, yeasts and fungi during their development, playing a crucial role in the flocculation process.[2] Various microorganisms that secrete biopolymer flocculants have been evaluated and isolated from samples that originated from the soil, sediments, activated sludge, rivers and deep seawater.[3-4] Generally, the screened and isolated groups of microorganism include bacteria, fungi, algae and actinomycetes.[5] Bioflocculants are eco-friendly and can substitute inorganic flocculants.[6] Inorganic flocculants used in wastewater treatment can compromise the sustainability of the process by producing secondary pollution of metal concentrations in water and sludge resulting in final disposal problems.[7]
Aluminium (which can be found in inorganic flocculants) has shown to enhance Alzheimer's disease.[8] Therefore the large quantity of flocculants used worldwide is of great concern because of the health problems caused by flocculants.[8] This makes it crucial to find a more environmentally friendly and economical alternative flocculant.[7] Bioflocculants have an advantage over synthetic flocculants with attributes of being non-toxic, biodegradable, and free of secondary pollution due to degradation.[9] The major setback to industrial applications and the large scale production of bioflocculants is yet to be established as there are several inhibitors such as cost of production, scale up challenges and effectiveness or reliability of the flocculant produced. The importance of producing and characterizing efficient bioflocculants with improved industrial performances has triggered the search in harsh environments in a bid to find microbial species that have high flocculation efficiencies and improved bioflocculant potential. The uniqueness and environmentally friendliness of bacterium flocculants have encouraged further investigations into screening, isolation, and characterization of a polymeric flocculant-producing bacterium.[10] Given this, the need for utilizing alternative cost-effective substrates in the screening and characterization of more microorganisms with bioflocculant production potentials, improved culture conditions for better yields of bioflocculant and lesser production costs is essential. This study aimed at investigating bioflocculant characteristics and production by bacteria isolated from surface water and sediments of freshwater habitat.

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Materials and Methods

Sample collection and bacteria isolation

Surface water and sediment samples were collected from Ikpoba River (Longitude 6°13'35.53, Latitude 5°46'33.54) in Benin City Nigeria using sterile plastic containers. The samples were immediately transported on ice to the Applied Microbial Processes &

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Environmental Health Research Group Laboratory, University of Benin, Nigeria for analysis not later than 4 h after collection. Samples (water and sediment) were serially diluted (10^1 - 10^5) and platted using the spread plate method on tryptone soy agar (Lab M, Lancashire, United Kingdom). Plates were incubated for 18 h at 30°C. Thereafter, distinct colonies were purified on nutrient agar, incubated for 18 h at 30°C and thereafter preserved on nutrient agar slants at 4°C until ready for use.

Cultivation of bioflocculant producing bacteria

The aquatic bacteria for bioflocculant screening was cultivated using peptone (1.0 g); glucose (10 g); $MgSO_4.7H_2O$ (0.3 g); KH_2PO_4 (0.2 g) and K_2HPO_4 (5 g) in 1 L filtered natural river water as the medium. Using NaOH (0.1M) and HCI (0.1M) the initial pH was modified to 7.0.[11] A loopful of the bacteria were introduced into 5 mL of the prepared medium and incubated for 5 days at 120 rpm at 30°C.[11] After incubation, 2 mL of the cultivated medium was centrifuged for 30 min at 4000 rpm and analyzed for its flocculating activity.

Assay of flocculating activity

The FA was ascertained using the procedure earlier described by Kurane *et al*.,[12] with kaolin clay (4 g/L) as suspended solids. A 2 mL of the culture supernatant + 3 mL 1% $CaCI_2$ were gently mixed with 100 mL of kaolin clay suspension, vortexed and left for 5 min undisturbed. The same procedure was employed in preparing the control by replacing the bioflocculant with sterile tryptone soy broth. The turbidity of the uppermost phase of the flask was determined at 550 nm using a UV-VIS spectrophotometer and the FA was evaluated as follows:

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

Where A is the control's optical density (OD) at 550 nm; and B is the sample's OD at 550 nm. All procedures were carried out in three biological replicates.

Influence of various carbon and nitrogen sources

The influence of diverse nitrogen and carbon sources on the activity of flocculation in the test bacteria were evaluated following the procedure of Cosa and Okoh.[4] The carbon sources were fructose, glucose, starch and sucrose, while the nitrogen sources were ammonium chloride, inorganic nitrogen (ammonium sulphate) and organic nitrogen (urea) in place of peptone.

Influence of pH and cations on flocculating activity

The effects of salts and pH on FA were determined using the method illustrated above. However, various metal salt solutions were used in replacing the $CaCl_2$ solution with the FA measured. $MgCl_2$, $FeSO_4$, and KCl solutions were used. To evaluate pH influence on the FA, the culture medium was modified using NaOH and HCl to modify the pH range from 12-3.[13]

Time course of bioflocculant activity

For this experiment, the medium composed of 5 g of KH_2PO_4; 0.3 g of $MgSO_4$; 10 g of glucose; 1g of ammonium chloride and $7H_2O$ per litre of water.[11] The isolates were cultivated under optimal proliferation conditions. Physiological saline solution was formulated by dissolving 0.45 g NaCl into 50 mL of sterile distilled H_2O to respective isolates to standardize the inocula. Their $OD_{(660nm)}$ was determined by mixing 100 µL of vortexed culture supernatant with 1 mL of saline solution in curvets and adjusted to 0.1. The time course assays were carried out using the procedure demonstrated.[10] Seed culture (1% v/v inoculated saline solution) was gently mixed with 150 mL of medium and incubated at 160 rpm at 30°C. The sample was collected for a period of 10 days at the appropriate interval of time (every 24 h). Two millilitres of incubated culture broth was spinned for 30 min at 4000 rpm with the cell-free supernatant used to ascertain the FA. The pH of each sample broth was equally determined. All procedures were carried out in three independent biological replicates.

Extraction and bioflocculant characterization

Bioflocculant purification and characterization were done using the procedures previously illustrated.[14,15] Briefly, the culture was spinned for 30 min at 4,500 rpm after five days of fermentation. A 1 mL of sterile distilled H_2O was introduced into the upper phase and spinned at 4,500rpm for 15 min. A 2 mL of ethanol was mixed with the supernatant, vortexed and left undisturbed for 12 h at 4°C. The precipitate was vacuum dried to get the biopolymer and was dissolved directly in sterile distilled H_2O supplemented with 1 mL of mixed n-butyl alcohol and chloroform solution (2:5 v/v). After vortexing, the mixture was left undisturbed for 12 h at 20°C. The upper part of the medium was withdrawn, spinned (4,500 rpm) for 15 min with supernatant concentrated at 40°C, and dissolved in 2 mL of ethanol. The folin-Lowry method was used in measuring the protein content of the purified bioflocculant while phenol-sulphuric acid was used in measuring the total sugar content.

Identification of the bioflocculant-producing bacteria

The species of bioflocculant-producing bacteria were confirmed using Analytical Profile Index 20NE (API 20NE) according to the manufacturer's instructions (bioMerieux, Marcy-l'Étoile, France). API lab plus software (bioMerieux, Marcy l'Etoile, France) was used for the final identification. Strain identification at the species level was categorized into 4 sub-groups following the manufacturer's instructions: (i) acceptable species identification, per cent identification of ≥80.0% and *T* value of ≥0.0.; (ii) good species identification, per cent identification of ≥90.0% and *T* value of ≥0.25; (iii) very good species identification, per cent identification of ≥99.0% and *T* value of ≥0.5; (iv) excellent species identification, per cent identification of ≥99.9% and *T* value of ≥0.75. Only excellent and very good identification reports were accepted.

Statistical analysis

The pH of the medium as it affects bioflocculant's FA was analyzed using descriptive statistics. Bioflocculant production time course, with bioflocculant activity were analyzed using analysis of variance (ANOVA). The p-values ($p<0.05$) were considered statistically significant.

Results and Discussion

Identification and screening of bioflocculant-producing bacteria

The attention of major scientific and biotechnological research has been drawn towards bio-flocculation as a result of its biodegradability and safety for ecosystems.[16] The development of flocculants that are biodegradable, safe and subsequently reduce health and environmental risks is highly required. Screening for bioflocculant production was carried out on over one hundred previously isolated and characterized freshwater bacteria from the Ikpoba River in Benin City Nigeria. Amongst these are three test bacteria which significantly demonstrated bioflocculant activity against kaolin suspension respectively. The analytical profile index 20 NE (API-20NE) identification system assigned the bacteria isolates to *Ralstonia pickettii* (>99%); *Stenotrophomonas maltophilia* (>98%) and *Alcaligenes* sp. (>99%). The *R. pickettii* and *S. maltophilia* were recovered from water samples, while *Alcaligenes* sp. was recovered from the sediments of the Ikpoba River. This is an indication that surface water and sediment from Ikpoba River is a reservoir of bacteria with bioflocculant potential. This is in line with the report of Piyo *et al*.[17] who described that surface water and sediment were good sources for isolating flocculant-producing microorganisms**.**

Influence of nitrogen and carbon sources

Among the diverse sources of carbon analysed for *R. pickettii*, fructose and glucose effectively produced bioflocculant. Since culture conditions affect productivity and distribution of bioflocculant, it is, therefore, essential to optimize these factors.[18] However, glucose yielded higher flocculating activity (76.1%) (Figure 1a). It was observed in this study that glucose and fructose effectively produce

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bioflocculant. Glucose promoted the highest production of bioflocculant with an optimum FA of 76.1%. This agrees with the report of Luvuyo *et al*.[19] that glucose promoted the most significant production of bioflocculant with an optimum FA of 72% when compared to sucrose (31%), lactose (42%) and starch (11%). In this study, it was observed that an optimum source of carbon for bioflocculant production by *R. pickettii* was glucose.

Glucose and fructose were effective for the production of bioflocculant from the carbon sources examined for *S. maltophilia*. However, glucose yielded higher flocculating activity (71.3%) (Figure 1a). Patil *et al*.[20] stated that sucrose and glucose as carbon sources promoted bioflocculant production by *Bacillus subtilis*. Kurane and Nohata[21] also reported that glucose and fructose enhanced bioflocculant production and cell elongation. In the case of *Alcaligenes* sp., glucose and sucrose effectively promoted bioflocculant production with higher flocculating activity yield by sucrose (78%) (Figure 1a). *Alcaligenes* sp. favoured sucrose as the optimum carbon source for bioflocculant production which confirmed the report of Gong *et al.*[22] For *Alcaligenes* sp., production of flocculant and cell elongation was boosted by peptone. Cosa *et al*.[23] previously reported that *Virgibacillus* sp. produced bioflocculant best with peptone and glucose as the sole nitrogen and carbon sources respectively.

Similarly to all the nitrogen sources tested, ammonium chloride appeared to be more favourable for bioflocculant production with the highest flocculation activity of 92% (Figure 1b). Ammonium chloride was more favourable among the profiled nitrogen sources for the production of bioflocculant with the utmost FA of 92% which is in line with the report of Cosa *et al*.[24] that the best nitrogen source was ammonium chloride and it demonstrated FA of 93%. Xia *et al*.[25] elucidated the significance of nitrogen and carbon sources for the production of bioflocculant. Based on findings by Kurane and Nohata,[21] glucose and peptone demonstrated the highest effectiveness for bioflocculant production from *S. maltophilia*. Similar reports by Xia *et al*.[25] were observed for *Proteus mirabilis*, where glucose as carbon source enhanced the optimal production of bioflocculant. Likewise, among the tested sources of nitrogen, peptone appeared to be more favourable for the production of bioflocculant with the utmost flocculation activity of 71.2% for *S. maltophilia* (Figure 1b). Also, peptone appeared to be more favourable among the nitrogen sources studied for the production of bioflocculant with the highest FA of 72.4% for *Alcaligenes* sp. (Figure 1b). In a previous study by Zayed *et al*.,[18] peptone and ammonium chloride as nitrogen sources were used effectively for the production of the bioflocculant by the bacterium. Hence, the optimum bioflocculant production was demonstrated using ammonium chloride as the sole source of nitrogen.

Influence of cations and pH on the flocculating activity

The use of cations as supplement neutralize and stabilize functional groups' residual negative charge resulting in bridges formed between particles, this subsequently stimulates flocculating activity.[19] Cations decrease the negative charge on the particle and polymer. This decrease promotes primary adsorption of flocculants on particles that are suspended thereby enhancing the process of bioflocculation.[26] Calcium chloride appeared more favourable for the production of bioflocculant by demonstrating the highest flocculation activity of 74.2% of all the tested cations sources for *R. picketti* (Figure 2a). Iron sulphate appeared more favourable for bioflocculant production among the tested cations sources by demonstrating the highest flocculation activity of 73% for *S. maltophilia* (Figure 2a). $CaCI_2$ appeared to be more favourable for bioflocculant production with the highest flocculation activity of 72.6% among the tested cations sources for *Alcaligenes* sp. (Figure 2a). There is an increase in the FA as the pH increase and attain optimum activity at neutral pH after which a decrease in the flocculating activities occurred as the pH increased to pH 11 for *R. pickettii* and *S. maltophilia*. However, it continued to increase for *Alcaligenes* sp. at pH 9 and started decreasing at pH 11 (Figure 2b). The ionic potential of kaolin suspension increases while the electrostatic force decreases when supplemented with salt (Calcium chloride). Its effect on the ionic potential rises with the molar concentration and charge of the ions. Divalent ions can absorb on the anionic surface of the kaolin clay particles and function as the bioflocculant chains' cleavage points, thereby resulting in increased FA. Hence, they could react with anionic charged yet diverse parts of the polysaccharide chain which make it extend lesser, with decreased bridges forming capacity. However, these two effects are unlikely observed with KCl. There was an upsurge in the FA of *R. pickettii* and *S. maltophilia* at an increase in pH close to neutral. In this study, the initial pH of the medium for production affected the activities of the studied bacteria strains and production of bioflocculant, and by extension affected the flocculating activity. Similarly, Okaiyeto *et al*.[16] also described that the initial pH of the medium affected bioflocculant production by *Halobacillus* sp.

The previous report by Xia *et al*.;[25] Cosa *et al*.[23] and Zhang *et al*.[11] stated that an important factor that influences bioflocculant production and FA is initial pH of the production medium. The pH of the natural habitat of the test bacteria, however, appears to have no bearing on its potential to produce bioflocculant as the habitat had a pH of 8.42 which is alkaline. The pH of the production medium reportedly influences and/or affects the production of bioflocculant.[9] It ascertains the oxidation-reduction potential and electric charge of cells, which may consequently affect nutrients absorption and enzymatic reaction.[25]

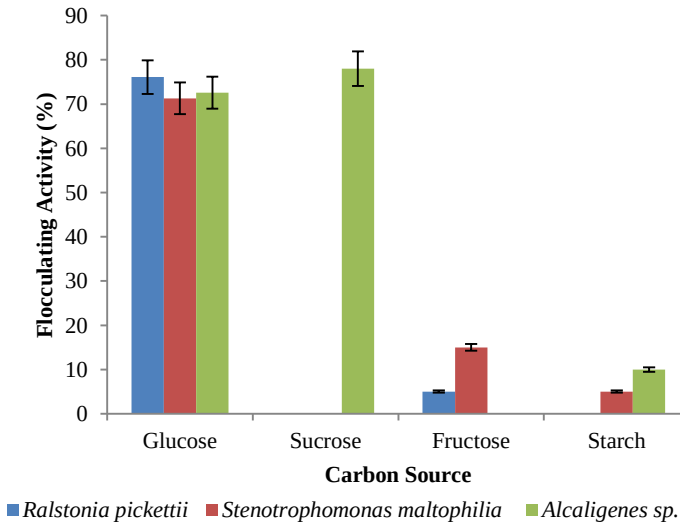

Figure 1a: Influence of carbon sources on the bioflocculant activity of the bacterial isolates

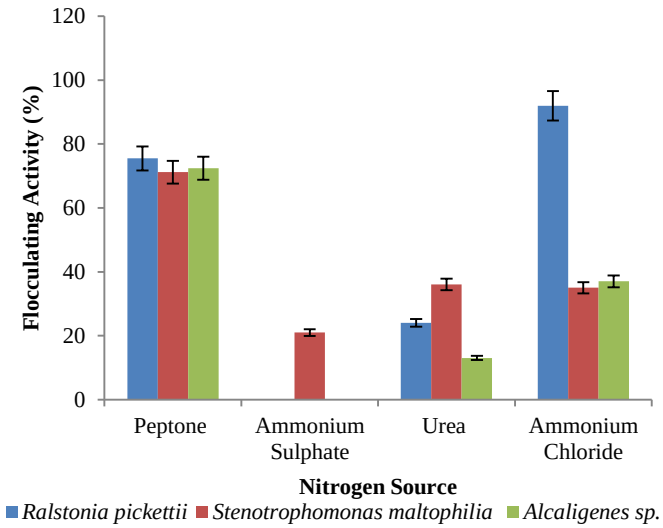

Figure 1b: Effects of nitrogen sources on the bioflocculant activity of the bacterial isolates

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Bioflocculant production at pH 7 saves large quantities of alkali and acid required for pH adjustment.[9]

Also, pH changes might cause variation in the bioflocculant's charge status and the surface properties of suspended materials, thus resulting in varying flocculating activity. The bioflocculant produced in this case was polysaccharide and since the production occured at acidic pH, it is not expected that the COO^- and H^+ of the polysaccharide should breakdown but rather bond together ($COOH^+$), and also bond easily with the anionic particles of the kaolin clay suspension, consequently leading to increased flocculation. Bioflocculants production by different organisms had been affected variously by the initial pH of culture media. Yim *et al.*[13] reported that maximum activity was observed by the bioflocculant produced by *Gyrodium impudicum* KG03 at acidic pH 4.0. Furthermore, the report of Zhang *et al.*[11] stated that an alkaline pH of 7.5, the production of bioflocculant and its activity were significantly stimulated. However, at neutral pH, the produced bioflocculant by *R. pickettii* was active. This is similar to the report of Kurane *et al.*[12] that *Rhodococcus erythropolis* S-1 was active at neutral pH, while alkaline conditions were preferred by bioflocculant produced by *Virgibacillus* sp. Rob.[23]

Time course of bioflocculant activity

It was initially observed that FA steadily increases with culture age. The activity attains peak (71%) after 9 days of cultivation and afterwards, a sudden and significant decrease was observed in the flocculating activity for *R. pickettii* (Figure 3a). For *S. maltophilia* peak activity (64%) was attained after 6 days of cultivation and afterwards, the flocculating activity decreased dramatically (Figure 3b). For *Alcaligenes* sp. its 72% peak activity was attained after 7 days of cultivation and subsequently, a significant decrease in FA was observed (Figure 3c). Microorganisms have shown to notably differ as a result of culture times needed for their bioflocculant production. Flocculating activity attains maximum level after six to nine days and then declined linearly with cultivation time respectively. This is similar to the findings of Shimforuya *et al.*[27] in which the bioflocculant produced by *Streptomycetes griseus* demonstrated an increase in flocculating activity as the cultivation time increased. Cosa *et al.*,[23] elucidated that the production of bioflocculant by *Virgibacillus* sp. Rob demonstrated maximum flocculating activity within the sixth day of incubation. The result of this study is contrary to the report of Deng *et al.*[28] that highest FA was attained in 96 h by a bioflocculant produced by *Aspergillus parasiticus*. A decrease in the FA of *R. pickettii* was observed after 9 days of cultivation which disagrees with the report of Fujita *et al.*[29] and Gong *et al.*[22] that on the third day of cultivation, bioflocculant produced by *Serratia ficaria* attained its maximum FA and *Citrobacter* sp. TKF04 bioflocculant demonstrated maximum FA within a day respectively.

Characterization of the bioflocculant produced by the bacterial isolates

Partially purified bioflocculant yielded 0.263, 0.32 and 0.341 g for *S. maltophilia*, *R. pickettii* and *Alcaligenes* sp., respectively as shown in Table 1. The analysis of bioflocculant purified from *R. pickettii* and *Alcaligenes* sp. revealed that it contained both protein (3.4 and 4.5 mg/mL) and carbohydrate (4.3 and 3.1 mg/mL), hence insinuating the biomolecule to be a glycoprotein. This is in agreement with the study of Zaki *et al.*[30] where their purified bioflocculant contained sugars and protein indicating the composition of the bioflocculants as glycoproteins. The purified bioflocculant from *S. maltophilia* when analyzed revealed carbohydrate content of 8 mg/mL and that it lacks protein (0 mg/mL). Hence, the bioflocculant is chiefly made up of polysaccharide. This is similar to the report of Wang *et al.*[31] that their purified bioflocculant is predominantly made up of polysaccharide (with monosaccharide units of mannose, rhamnose, galactose and glucose respectively).

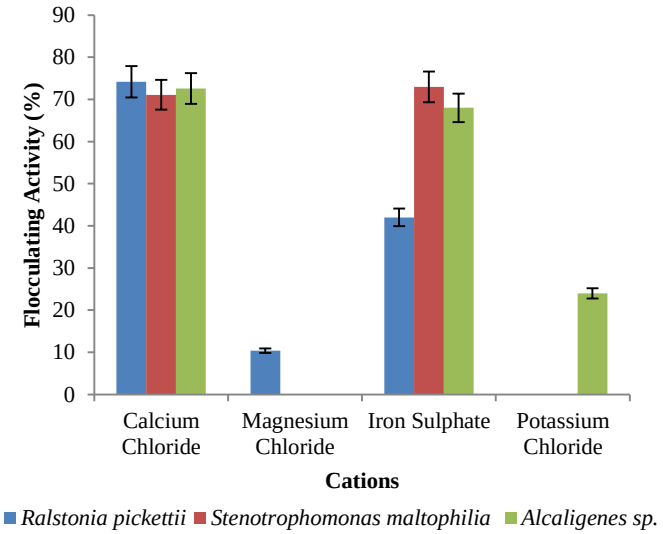

Figure 2a: Effects of cations on the bioflocculant activity of the bacterial isolates

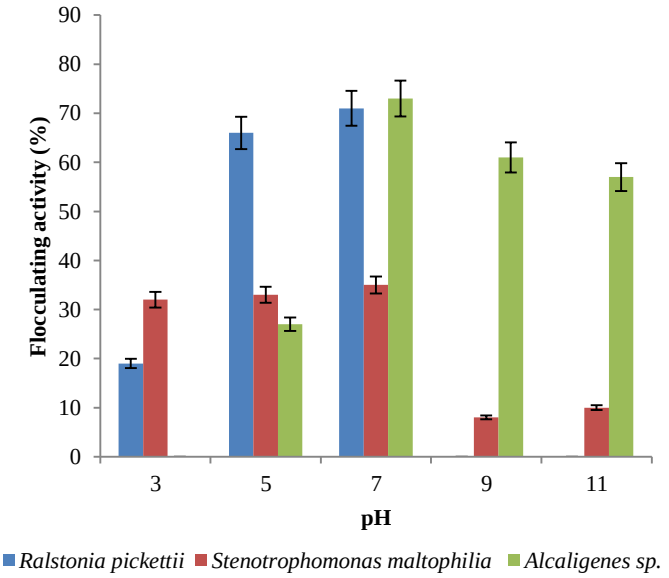

Figure 2b: Effects of pH on the bioflocculant activity of the bacterial isolates

Table 1: Properties of the partially purified bioflocculant produced by the bacterial isolates

Species of bacteria	Composition		
	Dry weight (g/L)	Total protein (mg/mL)	Total carbohydrate (mg/mL)
Ralstonia pickettii	0.32	3.4	4.3
Stenotrophomonas maltophilia	0.263	0	8
Alcaligenes sp.	0.341	4.5	3.1

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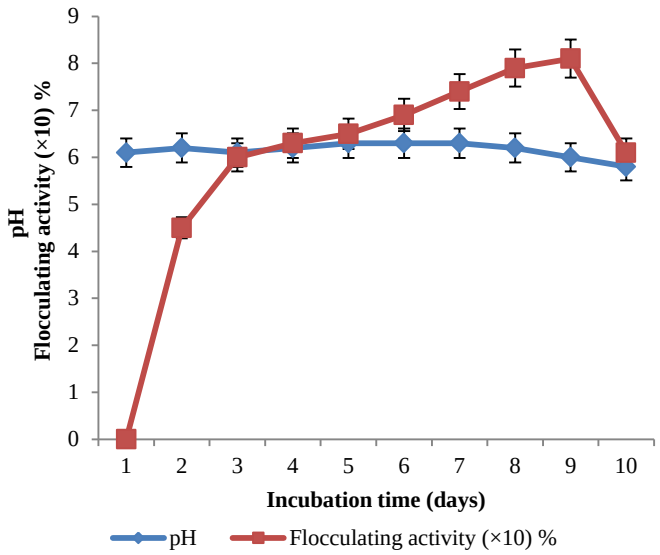

Figure 3a: Time course of bioflocculant activity by *Ralstonia pickettii*

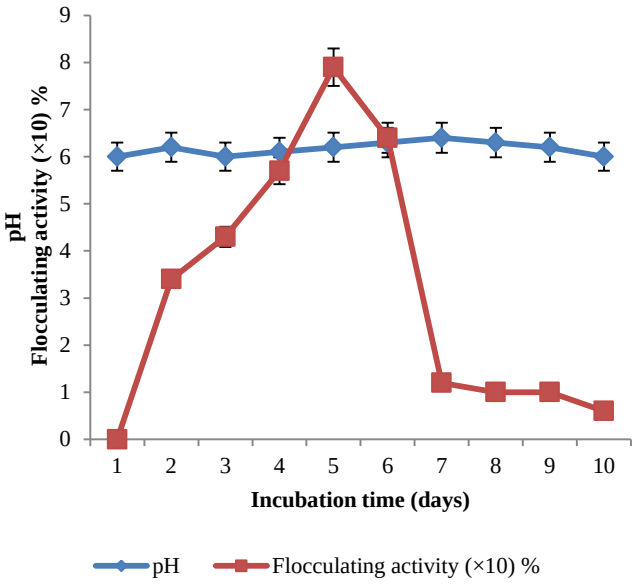

Figure 3b: Time course of bioflocculant activity by *Stenotrophomonas maltophilia*

Conclusion

The study indicated that surface water and sediment samples collected from the freshwater habitat is a reservoir with the potential to produce bioflocculants bacteria. The results showed that the isolates selected demonstrated preference for organic nitrogen source and diverse carbon source for maximum flocculating activity. The bioflocculant possessed strong FA over a wide range of pH, with low dosage requisites. The high flocculating efficiency of bioflocculant could be due to the presence of amino, carboxyl and hydroxyl groups as the major functional groups in its molecular chain. This bioflocculant could serve as alternative for non-degradable chemical flocculants. This can have wide application in wastewater treatment, therefore making it a prospect for further research and process conditions for feasible advancement on industrial-scale application.

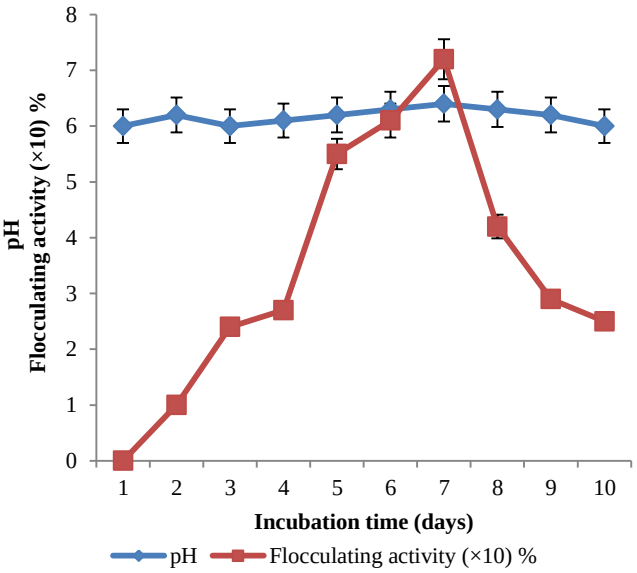

Figure 3c: Time course of bioflocculant activity by *Alcaligenes* sp.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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