Production of bioflocculant by the marine actinomycete *Nocardiopsis aegyptia* sp. nov.

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Abstract: Bioflocculants are biopolymers produced by microrganisms and used for the removal of colloidal particles from solution. This research aimed to explore the potentiality of marine actinobacteria to produce biofloculants. *Nocardiopsis aegyptia* sp. nov., an actinobacterium isolated from marine sediment of Alexandria was assessed for its bioflocculant-producing potential. Some factors affecting its production were investigated. The favorable carbon and nitrogen sources were glucose and peptone, with an optimal initial pH of 7. The highest flocculating efficiency achieved for kaolin suspension was 89 %, after 96 h of cultivation. The high activity of *Nocardiopsis aegyptia* bioflocculant suggests its potential application on a commercial scale. It has the advantage of good performance under high salinity and alkaline condition. The bioflocculant was purified to homogeneity by ethanol precipitation. The chemical analysis of the purified product revealed that it contained mainly polysaccharide (85%) and protein (7%). FTIR analysis indicated the presence of carboxyl, hydroxyl and amino functional groups. Scanning electron microscopy (SEM) of the purified biofocculant showed a porous crystal-linear flute-like structure. [Mona E. M. Mabrouk. **Production of bioflocculant by the marine actinomycete *Nocardiopsis aegyptia* sp. nov.** *Life Sci J* 2014;11(12):27-35]. (ISSN:1097-8135). http://www.lifesciencesite.com. 5

Keywords: Bioflocculant; *Nocardiopsis aegyptia* sp. nov. DSM 44442^T; Flocculating activity; SEM.

1. Introduction

In a broad sense, bioflocculation is the removal of colloidal particles from solution by flocculating substances of biological origins. Bioflocculants are biodegradable macromolecular flocculant secreted by microorganisms (Luvuyo et al., 2011). Flocculating agents are widely applied in treatment of water and wastewater, downstream processing, food fermentation processes, and removal of heavy metals and dyes (Mabinya et al., 2011; Nwodo et al, 2014). The use of synthetic chemical flocculants, namely, inorganic coagulants such as aluminium (aluminum sulfate and poly-aluminum chloride), and synthetic organic flocculants such as polyacrylamide derivatives and polyethylene imine, has resulted in some health and environmental problems (Nwodo et al, 2014). Aluminum has been found to induce Alzheimer's disease (Campbell, 2002). It is evident that acrylamide (the monomer of polyacrylamide), which still remains in the flocculant products, is not only neurotoxic and carcinogenic but also non biodegradable in nature (Pruser and Flynn, 2011). Conversely, flocculants of microbial origin referred to as bioflocculants are innocuous, environmentally friendly and attractive alternatives to substitute existing chemical flocculants, because of their nontoxic, harmless characters, biodegradability; lack of secondary pollution from degradation intermediates and their environmentally inert nature (Li et al., 2009; Yang et al., 2012).

The enormous advantages associated with bioflocculants have attracted considerable scientific attention (Nwodo et al., 2012). Continual exploration for microbes with high bioflocculant yield and high flocculation efficiency has therefore, become a subject of intensive investigations globally (Ugbenyen et al., 2012). Indeed, novel efficient bioflocculants from microorganisms inhabiting unusual environments such as the marine environments are beginning to be of great interest.

The marine environment is not yet fully explored, it is a huge treasure trove of marine actinomycetes resources (Williams, 2009). Nowadays, marine microorganisms are good candidates for bioremediation and have been recognized as a rich source of biological macromolecules that are of potential interest to various industries (Zhang and Kim, 2010). Marine actinobacteria are one of the most efficient groups of novel secondary metabolite producers and are very important from an industrial point of view (Manivasagan et al., 2014). Though members of actinobacteria are virtually unlimited sources of novel compounds and hold a prominent position due to their diversity and proven ability to produce novel bioactive compounds (Subramani and Aalbersberg, 2012).

It is anticipated that marine environment may be a reservoir of novel bioflocculant producing actinobacteria due to its uniqueness conditions which is very different from terrestrial environment. Very scanty information exists implicating the actinobacteria group as bioflocculant producers (Nwodo et al., 2013). Furthermore, our literature survey revealed that no reports are available on bioflocculant production by marine actinobacteria.

Therefore, to our best knowledge, this work is one of the early attempts to screen diverse marine

actinobacteria for bioflocculant production. In the present study, we report for the first time the production of bioflocculant by the marine *Nocardiopsis aegyptia* sp. nov., DSM 44442^T. A series of experiments were conducted to examine some factors affecting its activity and the characteristics of the product.

2. Materials and methods

Microorganism

Nocardiopsis aegyptia sp. nov., DSM 44442^T (AJ539401) is a novel species isolated from the marine sediment of the Meditteranean Sea, Alexandria, Egypt (Sabry et al., 2004). The bacterium was grown on ISP medium and maintained as spore suspension.

Screening and culturing of bioflocculant-producing actinobacteria

A screening experiment was first conducted on 35 marine actinobacteria strains to select the most potent organism in bioflocculant production. The production medium was as follows (g/ l): glucose 10; peptone, 1; KH_2PO_4, 2; K_2HPO_4, 5 ; $MgSO_4 \cdot 7H_2O$ 0.2; NaCl, 20. The initial pH was adjusted to 7.0 with NaOH or HCI (Zhang et al., 2007). Seed culture was prepared in 100 ml Erlenmeyer flask containing 20 ml of ISP2 medium (Shirling and Gottlieb, 1966) by inoculating 2.0 ml of spore suspension containing 2.5–3.0 10^6 CFU/ml and incubation at 30 °C with agitation at 150 rpm for 3 days. Sterilized portions (50 ml) of production medium in a 250ml Erlenmeyer flasks were inoculated with 4% (v/v) of the seed culture and incubated at 30 °C in a rotary shaker at 150 rpm for 72 h. At the end of the incubation, the cell-free culture supernatants were used to determine the bioflocculant produced against kaolin clay (Kurane et al., 1994). The strain with the highest flocculating activity was selected for further investigation.

Bioflocculant production by *Nocardiopsis aegyptia*

Production of the bioflocculant by *N. aegyptia* was performed in 250 ml Erlenmeyer flasks containing 50 ml of the production medium previously described. pH value of the medium was adjusted to 7. Each flask was inoculated with 4% (v/v) of the seed culture and incubated at 30 °C with shaking at 150 rpm. Samples were withdrawn at different time intervals and monitored for cell growth and flocculating activity. Culture broth was centrifuged at 10,000 rpm for 20 min to separate the cells which were washed twice with distilled water and dried at 65°C to constant weight as a measurement of cell growth (Muthulakshmi et al., 2013).The cell-free supernatant was used to study flocculating activity. Uninoculated medium was used as a control. All experiments were performed in triplicates for the mean calculation.

Measurement of flocculating activity

Flocculation activity was measured according to the method of Kurane et al. (1994) with slight modifications. Briefly, 0.3 ml of 1% $CaCl_2$ and 0.2 ml of sample were added into 10 ml of Kaolin suspension (4.0 g/L, pH 7.0) in a test tube. The mixture was vortexed using a vortex mixer for 1 min and allowed to still for 5 min at room temperature (30°C) and the formation of visible flocs was observed. Afterwards, 2 ml of the clarifying upper phase layer were carefully withdrawn and its optical density (OD) was measured at 550 nm with a spectrophotometer. A control was conducted by repeating same method except that cell free culture filtrate was replaced with sterile cell free fermentation medium. All assays including the control were performed in triplicates and the flocculation activity was calculated according to the following equation (Kurane and Matsuyama, 1994).

Flocculating activity % = [(A –B) /A] × 100%

where A and B are the optical density of the control and the culture sample respectively at 550nm.

Distribution of the flocculating activity

To find out whether the bioflocculant was secreted extracellularly or attached to the bacterial cell surface, the activity was assayed separately in the whole fermentation broth, in supernatant and in washed cells. Culture broth of 4 ml was centrifuged at 10,000 rpm for 20 min. The supernatant was collected. The precipitated cells were washed twice with distilled water, and resuspended in 4 ml distilled water.

Factors affecting bioflocculant production

Carbon and nitrogen sources are considered to influence bioflocculant production (Sheng et al., 2006). Hence, we assessed the role of these factors by replacing glucose (1%, wv^{-1}), the original carbon source in the production media with 10 g/l of one of the following carbohydrates; galactose, rhamnose, fructose, maltose, lactose, sucrose, starch or dextrose. Similarly, peptone was substituted with organic as well as inorganic nitrogen sources. The concentration of each nitrogen compound was fixed at 0.09 g N/ l. The effect of pH was evaluated by adjusting the initial pH value of the fermentation medium from 4 to 11 using 1N of HCl or NaOH.

Bioflocculant purification

Bioflocculant was purified according to the method of Sathiyanarayanan et al. (2013). The fermentation broth was centrifuged at 10,000 rpm for 20 min to remove cells and cell-debris. To the obtained supernatant, three volumes of cold ethanol (4°C) were added, instantly, till white cotton-like flocs were formed and then left to stand at 4 °C overnight. The resulting precipitate was collected by centrifugation at 10,000 rpm for 15 min, washed with ethanol and redissolved in distilled water. After repeating the purification process twice, the bioflocculant was

dialyzed against distilled water at 4°C overnight. Two volumes of cold ethanol were added to obtain the precipitate which was further washed twice with ethanol. The precipitate (bioflocculant) was dried and recovered as an off-white powder to be used for chemical analysis.

Structural characterization

Composition analysis

The total sugar was determined by the phenol–sulphuric acid method (Dubois et al., 1956) using glucose as the standard solution. The protein content was measured using the Folin-Lowry method with bovine serum albumin as the standard (Lowry et al., 1956).

Fourier-Transform Infrared Spectroscopy

The major functional groups were detected using Fourier transform infrared spectra (FT-IR). Purified bioflocculant was ground with potassium bromide (KBr) powder and pressed into pellets for (FT-IR) with spectral range 4000–400 cm^{-1} by Bruker Tensor 37 FTIR, (Germany) spectrometer.

Ultra -Violet spectrum

The UV absorption spectrum was recorded using UV-Visible Spectrophotometer (Lambda 4B UV/Vis, Perkin-Elmer, Japan) between 190 and 500 nm, in order to examine the existence of proteins and nucleic acids qualitatively (Yang et al., 2012).

Scanning electron microscopic observation of surface structure

Purified bioflocculant, flocculated and unflocculated Kaolin samples were placed on a slide carbon coated stub and fixed by air drying. The fixed specimens were coated with gold in JEOL-JFG1100E ion-sputter-coater and images were examined with Scanning electron microscopic (SEM) (JEOL JEM-5300, USA), equipped with an Energy Dispersive X-ray (EDX) Analyzer, which was used to obtain the elemental composition of the bioflocculant.

3. Results and discussion

Screening and identification of bioflocculant-producing actinobacteria

Fig. 1. A photograph showing Koline clay particles flocculated with *Nocardiopsis aegyptia* culture filtrate(B) and control without bioflocculant(A).

The marine environment provides a real untapped resource for novel microorganisms. Totally 35 different marine actinomycetes were screened for their capability to produce bioflocculant. Among them, strain *Nocardiopsis aegyptia* sp. nov., DSM 44442^T exhibited the highest flocculating activity (87%) (Figure 1), and was thus selected for this study.

Bioflocculant production

The correlation between bioflocculant production and culturing time may differ among different organisms. It was observed that extention in cultivation period resulted in an increase in cell growth with concomitant increase in flocculating acivity (Figure 2). The production of the flocculant was associated with cell growth, increased during the logarithmic growth and reached its maximum flocculating efficiency (89%) in stationary phase (4 days). Thus, the corresponding steady increase in cell growth is possibly an indication that the bioflocculant was produced by biosynthesis during growth and not by cell autolysis (Ugbenyen et al., 2012). Further increase in cultivation period resulted in a slight decrease in both flocculating activity and cell growth; this could be attributed to cell autolysis and/or the presence of a bioflocculant-degrading enzyme (Li et al., 2009). Similar to our finding, bioflocculant production by *Rhodococcus erythropolis* (Kurane et al., 1994); freshwater *Streptomyces* sp. Gansen (Nwodo et al., 2012) and *Streptomyces* xn17 (Zhang et al., 2013) which were all synchronous with cell growth and reached the maximum concentration in the stationary phase of cell. On the contrary, the bioflocculant production by *Streptomyces griseus* was not parallel to the cell growth and was released into the culture in death phase (Shimofuruya et al., 1996). It is evident that the flocculant biosynthesis occurred during different phases of the microbial growth for different organisms (Mabinya et al., 2012).

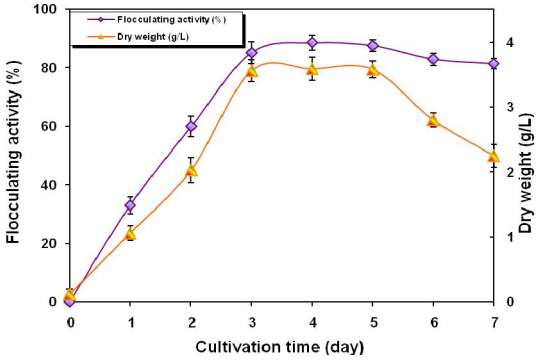

Fig. 2. Growth and flocculating activity of *N. aegyptia*. The cultures were grown in 50 ml of production medium, pH 7 and incubated at 30 °C in a rotary shaker at 150 rpm for 7 days.

Localization of *Nocardiopsis aegyptia* flocculating activity

The distribution of flocculating activity in the tested strain (Figure 3) shows that both fermentation broth and cell free supernatant possessed highest activity, whereas the washed cells had very low value. This indicates that the bioflocculant expressed by *N. aegyptia* is an extracellular product. The biopolymer located on the cell surface is anticipated to bind the kaolin particles and lead to flocculation. These results are in agreement with previous findings with *Klebsiella* sp.; *Streptomyces* xn17 and *Rhizopus* sp. (Yang et al., 2012; Zhang et al., 2013; Pu et al., 2014). As a result, the culture supernatant was used for further purification and practical applications.

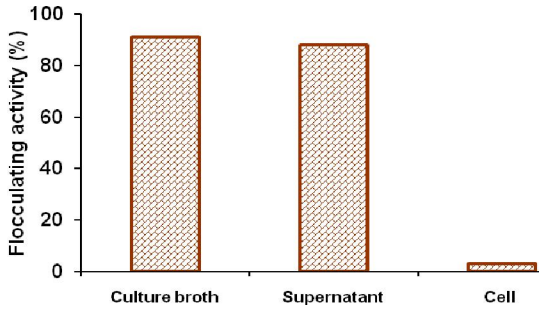

Fig. 3. Distribution of flocculating components in culture of *N. aegyptia*. The cultures were grown in production medium, pH 7 and incubated at 30 °C in a rotary shaker at 150 rpm for 4 days.

Factors affecting bioflocculant production

The production of bioflocculant may be affected by constituents of the culture medium and cultivation conditions (Zhang et al., 2007). To optimize cultivation conditions, some critical factors affecting the process were investigated. As noted in previous studies, the optimum pH for bioflocculant accumulation varied with different organisms (Ugbenyen et al., 2012). The Initial pH of the production medium determines the electric charge of the cells and the oxidation-reduction potential, which can affect absorption of nutrients and enzymatic reaction (Salehizadeh and Shojaosadati, 2001; Zhang et al., 2007). *Nocardiopsis aegyptia* sp. nov. bioflocculant was produced at a pH range of 4–11, with the highest flocculating at neutral pH (Figure 4). The flocculation efficiency was low at acidulous, but active in alkalescent conditions. Similarly, *Arthrobacter* sp.; *Arthrobacter* sp. Raats; *Streptomyces* sp. Gansen and *Brachybacterium* sp. produced bioflocculant optimally under neutral pH (Su et al., 2011; Mabinya et al., 2012; Nwodo et al., 2012,2013). While *Streptomyces griseus* (Shimofuruya et al., 1996) and *Streptomyces* xn17 (Zhang et al., 2013) produced bioflocculant under acidic conditions. The activity observed at pHs 8 and 9 recommend its potential application under alkaline condition.

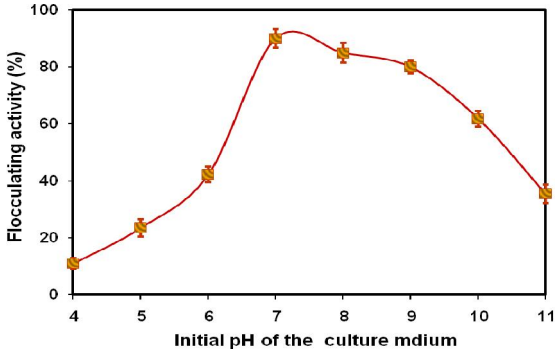

Fig. 4. Effect of initial pH of the production medium on the flocculating activity of *N. aegyptia* cultivated at 30 °C in a rotary shaker at 150 rpm for 4 days.

The importance of carbon and nitrogen sources has been reported to have a crucial effect on the production of bioflocculant (Salehizadeh and Shojaosadati, 2001), which may differ with different bioflocculant-producing microorganisms (Cosa et al., 2011). Our data suggest that *Nocardiopsis aegyptia* sp. nov. could grow on all tested carbon sources and could utilize a relatively wide range of tested carbon sources for the bioflocculant production (Figure 5). It is evident that glucose and rhammnose were the most suitable carbon sources, with the flocculating efficiency exceeding 80% after 96 h cultivation. Overall, glucose had the most pronounced effect because it gets utilized readily compared to other sugars. Glucose has been reported as a preferred carbon source in previous studies for bioflocculant production by various microorganisms (Ugbenyen et al., 2012; Nwodo et al., 2012; Cosa et al., 2013).

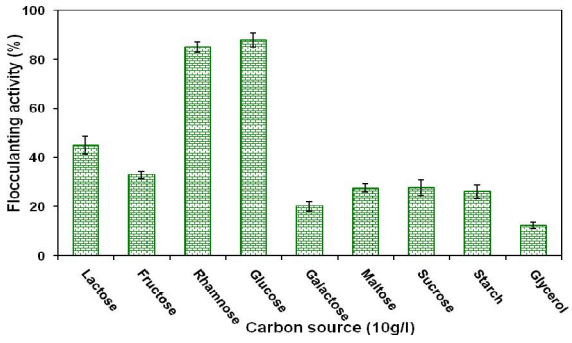

Fig. 5. Effect of different carbon sources on flocculating efficiency of *N. aegyptia* cultivated at 30 °C in a rotary shaker at 150 rpm for 4 days

It has been well documented that nitrogen source(s) are important nutrient factor(s) that enhance bioflocculant production (Cosa et al., 2011; Ugbenyen

et al., 2012). Among the nitrogenous compounds investigated in this study, peptone and potassium nitrate provided the best nitrogen source for bioflocculant production (Figure 6).

The preference of this bacterium for glucose and peptone as sole sources of carbon and nitrogen is similar to the results obtained for bioflocculant production by *Proteus mirabilis* TJ-1(Xia et al., 2008);and *Virgibacillus* sp. Rob (Cosa et al., 2011).

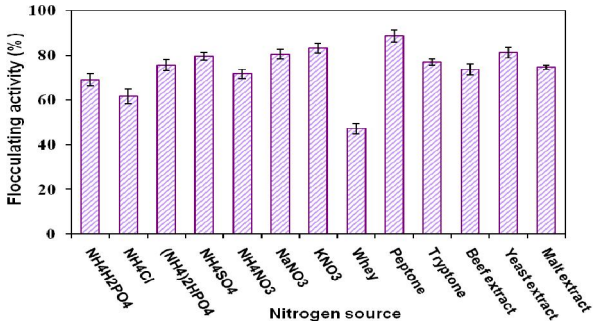

Fig. 6. Effect of different nitrogen sources on flocculating activity by *N. aegyptia* cultivated at 30 °C in a rotary shaker at 150 rpm for 4 days. The nitrogen concentration was 0.09 g N/ l.

Characteristics of *Nocardiopsis aegyptia* bioflocculant

Composition analysis

The extracted dried flocculate looked as off white powder. About 1.3 g of the purified bioflocculant could be recovered from 1.0 L of fermentation broth. Its content of total sugar and total protein were 85% and 7% (w/w), respectively, indicating that the *Nocardiopsis aegyptia* bioflocculant was mainly polysaccharides.

Functional group analysis

The FTIR spectrum of *N. aegyptia* bioflocculant was consistent with the results of most bioflocculants produced by different microorganisms (Kumar et al., 2004; Sathiyanarayanan et al., 2013). It displayed clear absorption peaks at a broad stretching intense; peak at 3450 cm^{-1}(Figure 7), which is a common characteristic of hydroxyl and amino groups (Li et al., 2009), stretching peak observed at 1638 cm^{-1} suggestive of the presence of carboxyl groups with C=O in an amide group (Cosa et al., 2013) and a peak at 1410 cm^{-1} is the result of COO^- symmetric vibration (Zhang et al., 2013). The absorption peaks around 1,000-1,100 cm^{-1} are generally known to be characteristic for all sugar derivatives (Yadav et al., 2012). Furthermore, wave numbers 1242.45–1085.72cm^{-1} are typical of phenol and tertiary alcohol OH bend indicative of the presence of carboxylic groups, carboxylate ions, aromatic ring stretch and C - O and C- O - C from polysaccharides (Kurane et al., 1994; Yadav et al., 2012). The peaks at 616.59 and 532.83 cm^{-1} are the absorption peaks for the aromatics–CH bending vibrations (Zhang et al., 2013). These functional groups serve as the binding sites for suspended particles hence, causing floc formation (Luvuyo et al., 2013).

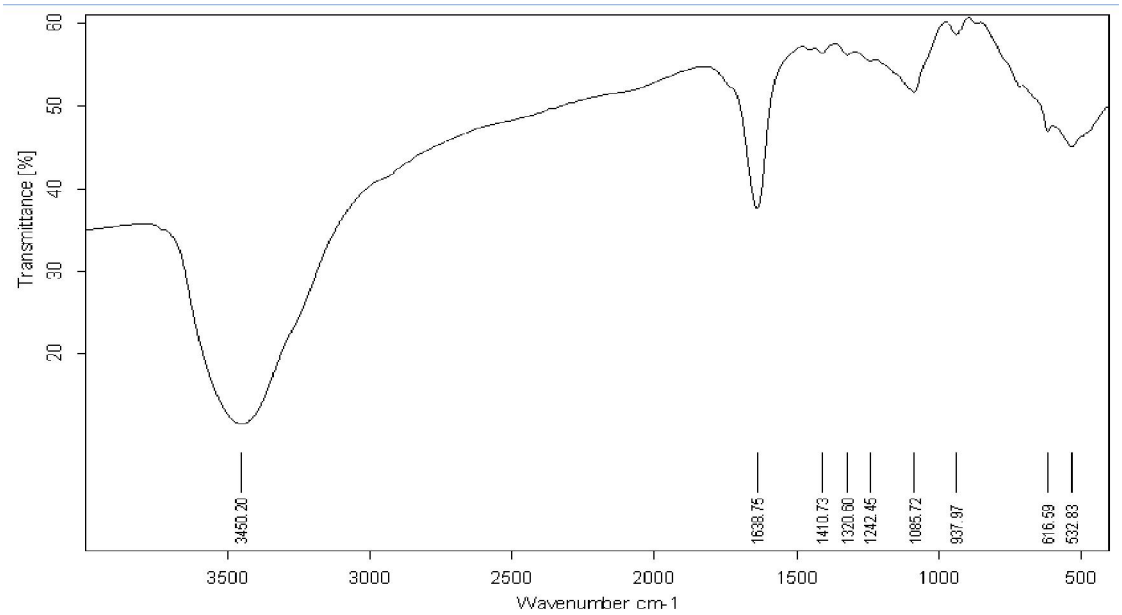

Fig. 7. FTIR spectrum of the purified bioflocculant produced by *N. aegyptia*

Purity analysis of bioflocculant

The ultraviolet (UV) scan chromatography of *N. aegyptia* sp. nov**.** bioflocculant (Figure 8) displayed an intense peak at 210 nm characteristic for aldehydes polysaccharide, and a peak at 267 nm which suggests the presence of an aromatic ring. The aromatic rings in bioflocculant could originate from aromatic amino acids. Based on the composition analysis, the purified bioflocculant was predicted as a polysaccharide molecule.

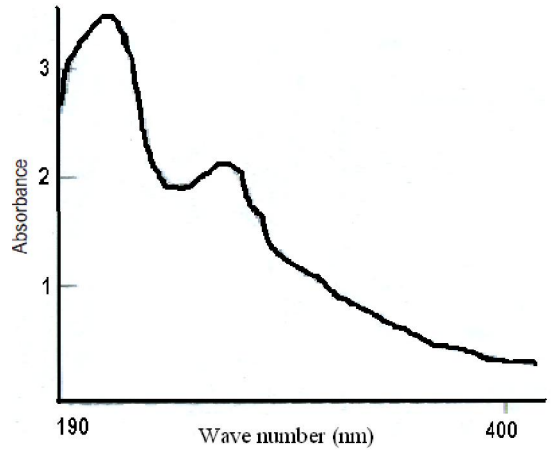

Fig. 8. The ultraviolet scan chromatography of *N. aegyptia* bioflocculant

Elemental analysis

The EDX analysis of the purified biofocculant revealed its elemental composition as represented in Table1. The presence of elements may be an indication that the bioflocculant is organo-metallic, or perhaps the metals could have originated from the fermentation medium.

Table 1. Elemental analysis of the purified bioflocculant produced by *N. aegyptia*

Element	Concentration (wt %)
Na	7
P	6.9
S	1.9
Cl	66.3
K	16.9
Cu	0.5
Zn	0.6

Analysis of the morphology structure of the bioflocculant

Surface morphology structure of bioflocculant as revealed by Scanning Electron Microscopy (SEM) shows a crystal linear porous stacked flute-like structure in the compact nature (Figure 9 A, B). This structural configuration of the bioflocculant molecule may contribute to its high flocculating efficiency. On the other hand, the Kaolin particles before flocculation were fine and scattered (Figure 9 C, D). However, during the process of flocculation as a result of the interaction between the biofocculant and suspended Kaolin particles, the scattered kaolin connected together, appeared as a tightly knit continuous stretch, adsorbed onto the binding sites of the bioflocculant and separated from water, which thus aggregated, forming larger flocs (Figure 9 E, F). Consequently the floc settled down from suspension which reveals the excellent flocculating performance of *N. aegyptia* to Kaolin clay. These findings were similar to that reported by Nwodo et al., (2014) and Cosa et al., (2013).

Conclusion

Literature evidenced that marine actinomycetes are untapped resource for unusual biomolecules. Therefore, explorations of marine environment for bioflocculant producers will have wider applications in bioremediation and exploring the chemical ecology of marine organisms. This research adds an important piece of information on the application of marine actinobacteria as bioflocculants. To our knowledge, this is the first report on the bioflocculant formed by *N. aegyptia* sp nov**.** The data obtained appears to hold promise as a source of new bioflocculant that can find possible application as a polymer for environmental bioremediation and other biotechnological processes. Of special interest is its possible production under high pH. Further characterization of the produced bioflocculant, as well as optimization cost of production will be the subject of further research.

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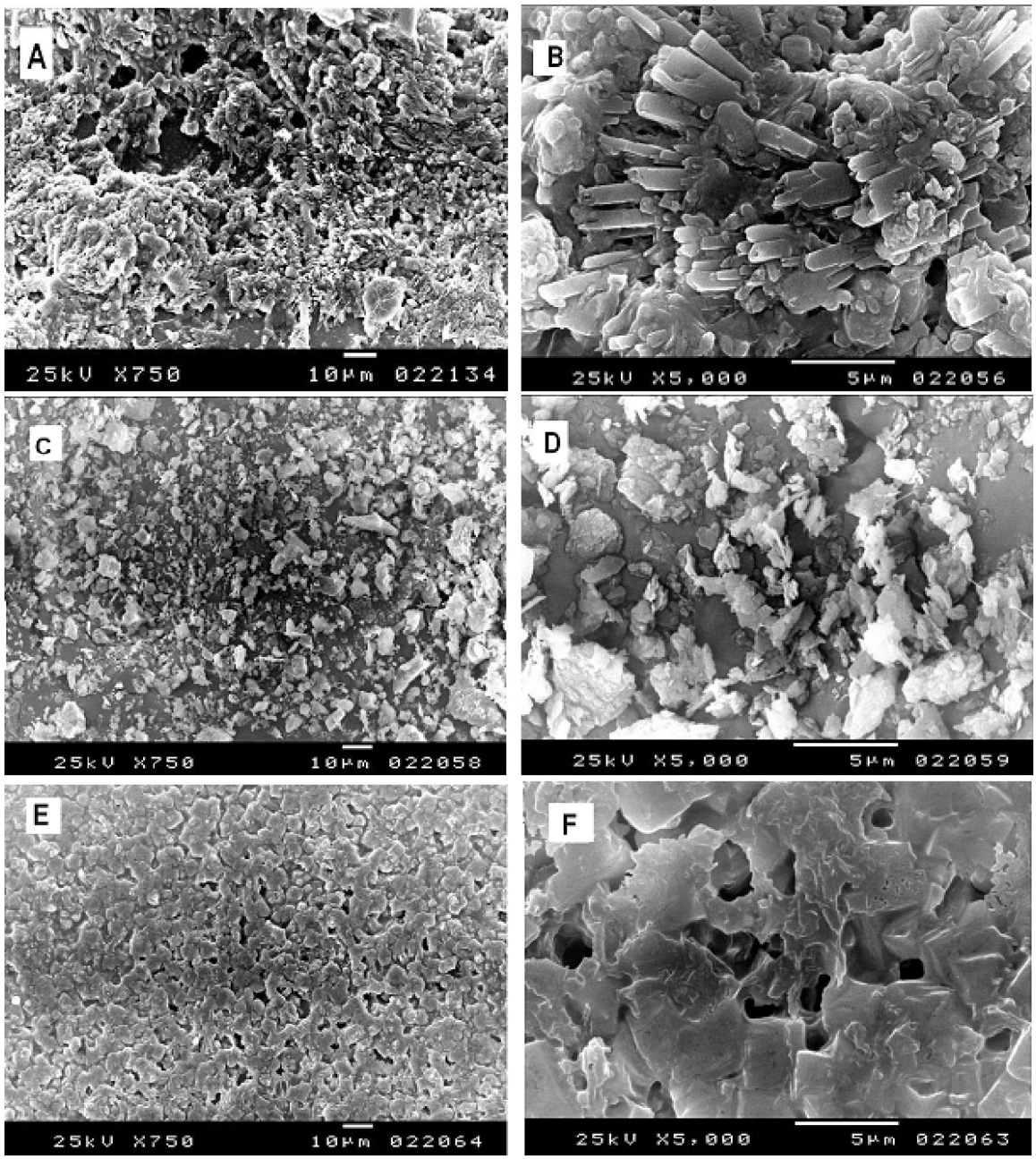

Fig. 9. SEM of *N. aegyptia* purified bioflocculant (A, B), Kaolin particles(C, D) and Kaolin clay suspension treated with bioflocculant (E, F).

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