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RESEARCH ARTICLE

PRODUCTION AND CHARACTERIZATION OF BIOFLOCCULANT PRODUCED BY *BACILLUS CLAUSII* NB2

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Abstract

Molecular and bioflocculant properties characterization of a bacterium strain isolated from brewery wastewater was investigated. Effect of biotechnological parameters (pH, temperature, carbon source, incubation time, stationary and with agitation cultivation and agitation speed) was studied and obtained bioflocculant was characterized. Compositional analysis of the purified bioflocculant was determined. The new isolate strain was identified by 16S rRNA gene nucleotide sequence to have 91% similarity to *Bacillus clausii*. Cultivation at pH 5.0 and 30^{o}C were the best conditions for bioflocculant production by *Bacillus clausii* NB2 strain with flocculating activity ranged from 28.83 – 81.05% and 42.68 – 79.68%. Glucose as carbon source was the best for bioflocculant production by *Bacillus clausii* NB2 strain with flocculating activity ranged from 4.76 – 84.23%, followed in order by abattoir effluent (flocculating activity 78.84%), palm oil effluent (flocculating activity 77.70%), sewage (flocculating activity 73.44%) and brewery effluent (flocculating activity 63.14%) respectively. Incubation time, agitation condition and agitation speed had a significant effect (P≥0.05) on bioflocculant production by this strain. The highest yield of bioflocculant production was obtained by submerged cultivation, in aerobe conditions for 72h at 160 rpm agitation speed. Functional group analyses using Fourier Transform Infrared (FT-IR) spectrophotometer shows the presence of -OH, -NH2, -CONH2 CO and -COO as functional groups and chemical analyses of the purified bioflocculant revealed it to be a glycoprotein with 88.71% of carbohydrate and 11.29% of protein.

Key words: *Bacillus clausii*, bioflocculant, flocculating activity, compositional analysis

Introduction

Microbial flocculants or bioflocculant are biodegradable polymeric compounds produced by many microorganisms (eubacteria, actinomycetes and fungi) during their growth. Bioflocculation is a dynamic bioprocess resulting from the synthesis of extracellular polymers by microorganisms during their growth. The term flocculation is the process of conglomeration of microbial cells to form flocs with other compounds present in fermentative medium (Gao *et al*., 2006).

Bioflocculant have some advantages over conventional synthetic organic flocculants; this includes biodegradability, non-toxicity to humans and the environment, safety for ecosystems,

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highest flocculating capability. As a result of these it can be applied in wastewater treatment, downstream processing, fermentation processes (Salehizadeh and Shojaosadati, 2001; Seo, 1993). Flocculants are usually high molecular weight compounds that bring about settling through two processes: neutralization of the charges on the particles and bridging the particles in suspension, which result in aggregation and settling of particles (Weber, 1985).

Fermentative conditions and nutritive compounds of cultivation medium have been reported to have influence on bioflocculant production (He *et al*., 2004). Organic flocculants have been used extensively to enhance the flocculation of suspended solids in the treatment of process water; wastewater and effluents, important among this type are polyacrylamide and polyacrylic acid (Kurane *et al*., 1994). Organic flocculants are especially recommended for flocculating suspended solids and in non-potable raw water clarification, primary and secondary waste water effluents clarification and oil waste clarification (Chen, 2000). Bioflocculants have been reported to be capable of removing inorganic/ organic particles through their flocculating activity. It has been investigated that bioflocculant is effective in removing suspended solids, heavy metals and microorganisms, and in reducing the turbidity of different types of industrial wastewater effluents (Kurane *et al*., 1994; Gao *et al*., 2009; Lin and Harichund, 2011). Comparative effect of medium composition on bioflocculant production by microorganisms isolated from wastewater samples has been reported (Adebami and Adebayo-Tayo, 2013)

In spite of various advantages that synthetic or chemical flocculants provide, their status has not been favorable currently and there have been concerns on their safety. Most of high molecular weight is recalcitrant. It is a non-disputed evident that the acrylamide monomer is not only neurotoxic and carcinogenic but also non-biodegradable in nature (Vanhorick and Moens, 1983; Dearfield *et al*., 1988). They have detrimental effect both on flora and fauna. Aluminium compounds used as floculants have been shown to cause Alzheimer's disease (Master *et al*., 1985; Kowall *et al*., 1989).

Due to low flocculating capability and high cost of production, industrial production of bioflocculants has not been established. Therefore there is a need to search for microorganisms with great capability for bioflocculant production with reduction in cost of production (Gao *et al*., 2006).

In this study, molecular and chemical bioflocculant characterization of a producing bacterium isolated from brewery wastewater was studied, effect of biotechnological parameters (pH, temperature, carbon source, incubation time, stationary and with agitation cultivation, agitation speed on bioflocculant production and compositional analysis evaluation of the purified bioflocculant were determined.

Materials and Method

Microorganism and culture preparation

Bioflocculant producing strain *Bacillus clausii* NB2 was isolated from brewery wastewater in the previous work and in collection of the microorganisms of Department of Microbiology University of Ibadan, Ibadan Nigeria (Adebami and Adebayo-Tayo, 2013). The stock cultures were maintained on tryptone soy broth agar containing (g/L): tryptone 15.0, soy peptone 5.0, sodium chloride 5.0, agar 15.0, pH 7.3, incubated at 30^{o}C for 72h and stored at 4^{o}C.

The inoculums was grown in a 250 mL flask containing 50 mL of Lab-lenco powder 1.0; yeast extract 2.0; peptone 5.0; sodium chloride 5.0 per litre of de-ionized water. The pH of the medium was adjusted to 7.0 and then medium was autoclaved and inoculated with pure culture from the stock culture and incubated for 24 h submerged conditions of cultivation.

Bioflocculant production by Bacillus clausii NB2 strain

For bioflocculant production the cultivation took place on bioflocculant production broth (BPB) containing (g/L): glucose 20, urea 0.5, yeast extract 0.5, $(NH_4)_2SO_4$ 0.2, K_2HPO_4 5.0, KH_2PO_4 2.0, NaCl 0.1 and $MgSO_4$ .7 H_2O 0.2. The initial pH of

the medium was adjusted to 7.0. The medium was sterilized, inoculated with pure culture of the isolate and incubated on a rotary shaker at 120 rpm and 37oC for 3 days. Kaolin suspensions at a concentration of 5,000 mg/L were used to evaluate the flocculating capability of the cultures (Zheng *et al*., 2008).

Determination of flocculating activity

The flocculating activity was determined according to the method of Kurane *et al*. (1986), modified by Gao *et al.* (2006). A suspension of kaolin clay was used as test material for flocculating activity determination. The kaolin clay was suspended in distilled water at a concentration of 5 g/L at pH 7.0 and used as a stock solution for the subsequent assays. The following solutions were mixed in a test tube: kaolin clay suspension (9 mL), culture supernatant (0.1 mL) and 1% $CaCl_2$ (0.25 mL). A reference tube in which the culture supernatant was replaced with distilled water was also included and measured under the same conditions. The final volume of all mixtures was made up to 10 mL with distilled water. After mixing gently, the solutions were allowed to settle for 5 min. at room temperature. The optical density (OD) of the clarifying upper phase solution was measured at wavelength of 550 nm with a UV/Vissible spectrophotometer (Jenway, UK.) and the flocculating activity determined as follows:

Flocculating rate (%) = [(B − A) / B] × 100%

Where A and B are optical densities at wavelength of 550 nm of the sample and control respectively.

16S rDNA sequence determination and phylogenetic analysis of the bioflocculant producing strain

The bacterial strain was identified using molecular technique based on the 16S rRNA gene amplification by polymerase chain reaction (PCR) followed by sequencing of the amplified gene as designed according to Gupta *et al.* (1987) and Xiong *et al.* (2010).

Cells were incubated in 250-mL flasks containing 50 mL fresh Luria Bertani broth (LB) for 16 h at 37°C with shaking at 120 rpm. The genomic DNA of the strain was then extracted using CTAB method of DNA extraction from microorganisms. PCR amplification was carried out to determine the partial 16S rRNA gene. The PCR program was 30 cycles of 94°C (1 min), 55°C (30 s), and 72°C (1.5 min) (7). The PCR primers were 5-CCAGCAGCCGCGGTAATACG-3 (forward) and 5-TACCAGGGTATCTAATCC-3 (reverse). Purification of the PCR products and the determination of sequences were performed by Macrogen USA (9700 Great Seneca Highway, Rockville, MD 20850, USA). The 16S rRNA gene sequence of strain obtained was compared with the NCBI database

(http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Effects of biotechnological parameters on bioflocculant production by Bacillus clausii NB2 strain

Effect of pH on flocculating activity of bioflocculant produced by *Bacillus clausii* NB2 strain was investigated. The initial pH of the culture media was varied between the pH range of 3.0–12.0 by adjusting with either 1.0 N HCl or NaOH solutions (Yim *et al*., 2007).

The effect of incubation temperature on bioflocculant production by *Bacillus clausii* NB2 strain was carried out by varying the incubation temperature between 25 – 45^{o}C.

Effect of carbon sources on bioflocculant production by *Bacillus clausii* NB2 strain was also investigated. Carbon sources such as wastewaters effluents (palm-oil, abattoir, and brewery), sewage and glucose were used according to the method proposed by Lachhwani (2005).

Effect of incubation time on bioflocculant production by *Bacillus clausii* NB2 strain was investigated. The fermentative medium was inoculated and incubated at different time interval of 12 h -72h respectively.

Effect of incubation under stationary and agitation condition of cultivation was investigated. Inoculated broth was incubated under stationary cultivation conditions and also under agitation cultivation conditions by varied the agitation speeds between 80 – 160 rpm. Kaolin assay was carried out to check the maximum flocculating activity.

Extraction and purification of the bioflocculant

The purification and characterization of the bioflocculant was performed using the method described by Chang *et al.* (1998) and Chen *et al.* (2002). Fermentation culture broth was prepared based on the optimal culture conditions determined earlier. After three days of submerged cultivation, the culture was centrifuged at 4 600 rpm for 30 min and at 4°C to separation of the biomass. One volume of distilled water in ratio with wet biomass quantity was added to the supernatant and centrifuged again for 15 min to remove insoluble solutes. Two volumes of cold ethanol were then added to the supernatant, mixed and left standing at 4°C for 12 h. The precipitate was vacuum dried to obtain the crude bioflocculant. The crude product was weighed and dissolved in a small volume of distilled water and one volume of mixture of chloroform and n-butyl alcohol (5:2 v/v) was added. After mixing, the mixture was left at room temperature for 12 h. The upper phase was centrifuged at 3 000 x g for 15 min and the supernatant was dialyzed against distilled water. Thereafter, the dialysate was vacuum dried to obtain a pure bioflocculant.

Chemical characterization of bioflocculant

Determination of the effect of pH, temperature and cationic/metal salts on the flocculating activity

The effect of pH on flocculating activity of the bioflocculant produced by *Bacillus clausii* NB2 strain was investigated by adjusting the pH (3.0 and 12.0) of the kaolin suspension using 2 N HCl or NaOH.

The effect of temperature on flocculating activity of the bioflocculant produced by *Bacillus clausii* NB2 strain was also determined. The flocculation experiment was conducted between 30°C and 100°C (10°C interval) by carrying out the experiment in water bath. The kaolin solutions were subjected to heat prior to the determination of OD in the presence of bioflocculant for 10 min (Nakata and Kurane, 1999).

Flocculation inhibition assay

The flocculation inhibition assay was determined by taken 100 µL of different concentrations of potential inhibitors (K_2HPO_4, $NaNO_3$, CH_3COONa, Na_2CO_3, and D-GLU), 100µL of bioflocculant solution (1 g/L). 100 µ L of $CaCl_2.2H_2O$ (30 g/ L) were added to 10 m L of kaolin suspension (5 g/ L) in a test tube. The mixture was vortexes for 5 sec and left for 5 min. The OD was measured with UV/Visible spectrophotometer (Jenway, UK.) at wavelength of 550 nm. Flocculation activity was compared to the control without the salts.

Chemical analyses of the purified bioflocculants

The functional groups of the bioflocculant were characterized using a Fourier transform infrared spectrophotometer Shimadzu (Japan). The bioflocculant was ground with KBr salt at 25°C and pressed into a pellet for FT-IR spectroscopy analysis over a wave number range of 4 000-370 cm^{-1}.

Total sugar content of the purified bioflocculant was determined by the phenol-sulphuric acid method using glucose as the standard solution as described by Chaplin and Kennedy (1986). Total protein content was measured by the Lowry *et al.* (1951) method using bovine serum albumin as the standard solution.

Statistical analysis

The results of the analysis are expressed by ANOVA using SAS. Sequential differences among means were calculated at the level of $P \leq 0.05$, using Duncan Multiple Range Test (Duncan, 1956)

Results and discussion

BLAST (Basic Local Alignment Search Tool) analyses of the 16SrRNA gene nucleotide sequence of the bacterial strain, coded NB2, showed a 97% similarity to *Bacillus clausii* species (accession number GQ848482.1). A phylogenetic tree was constructed between it and similar sequences found in GenBank as shown in Figure 1.

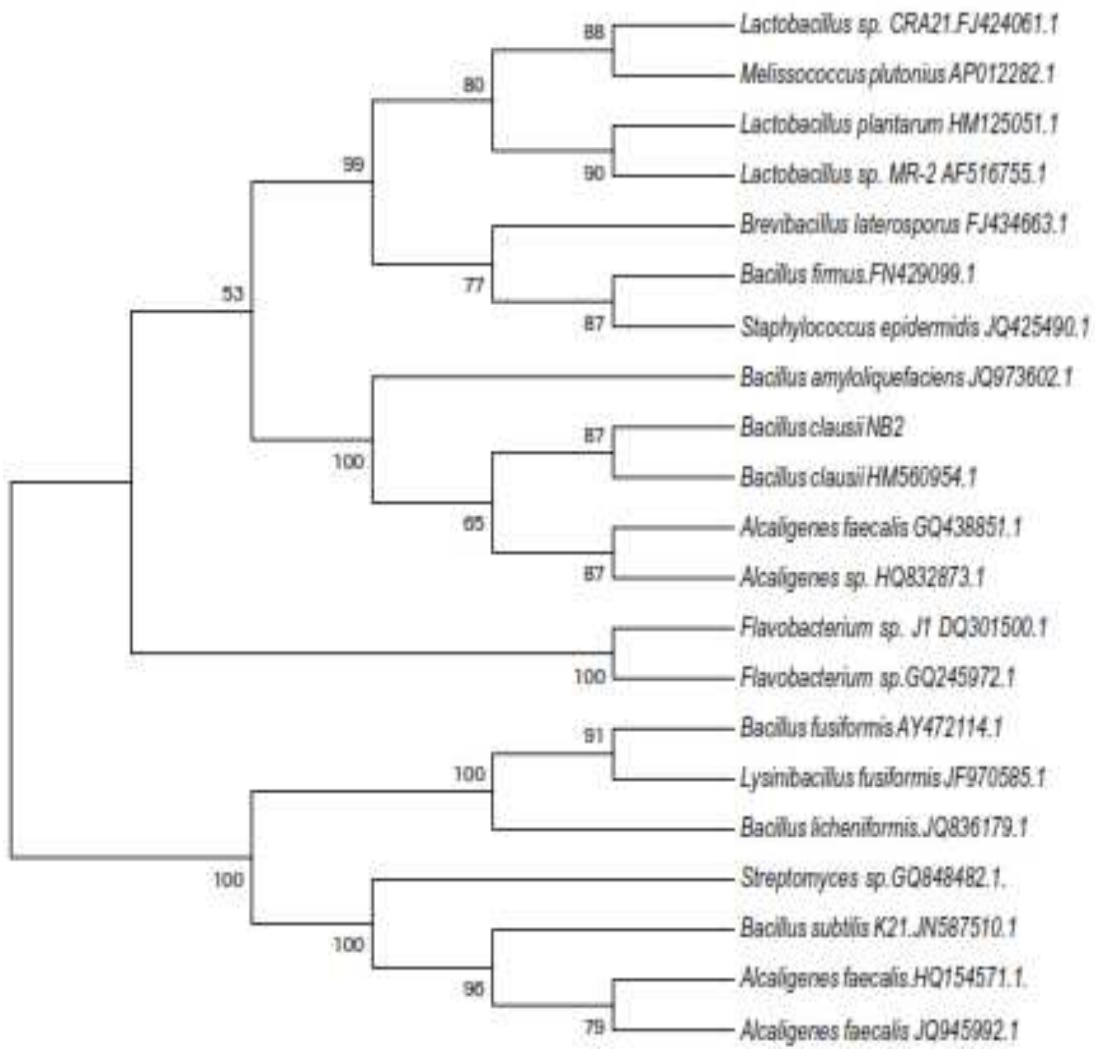

Figure 1. *Phylogenetic tree showing the relationships between NB2 strain and Bacillus clausii species (in pointed arrow) and other closely related sequences collected from the Gene Bank. The dendogram was generated by the neighbor-joining method. Bootstrap values per 100 bootstrap analysis presented for values greater than 50 %*

Figure 2 shows the effect of pH on bioflocculant production by *Bacillus clausii* NB2 strain. There was a significant difference (P≥0.05) in the flocculating activity of *Bacillus clausii* NB2 strain at different pH. After 24, 48 and 72 h of incubation the flocculating activity ranged from 28.83e - 74.00a %, 30.31e - 78.60a % and 36.55e - 81.05a % in which the highest flocculating activity was recorded at pH 5.0.

Figure 3 shows the effect of temperature on bioflocculant production by *Bacillus clausii* NB2 strain. There was also a significant difference (P≥0.05) in the flocculating activity of *Bacillus clausii* NB2 strain at different incubation temperature. At 24, 48 and 72 h of incubation, the flocculating activity ranged from 42.68 - 58.67 %, 59.81 - 68.22 % and 69.03 - 79.68 % respectively, in which the highest flocculating activity was recorded at temperature of 30oC after 72 h of incubation.

Figure 4 shows the effect of different carbon sources on bioflocculant production by *Bacillus clausii* NB2 strain. There was also a significant difference (P≥0.05) in the flocculating activity of *Bacillus clausii* NB2 strain at different carbon source. At 24, 48 and 72 h of incubation the flocculating activity ranged from 44.76 - 71.22 %, 46.28 - 78.84 % and 63.14 - 84.23 % in which the

highest flocculating activity was recorded by cultivation on medium containing abattoir effluent at 24 and 48 h and glucose at 72 h of incubation respectively.

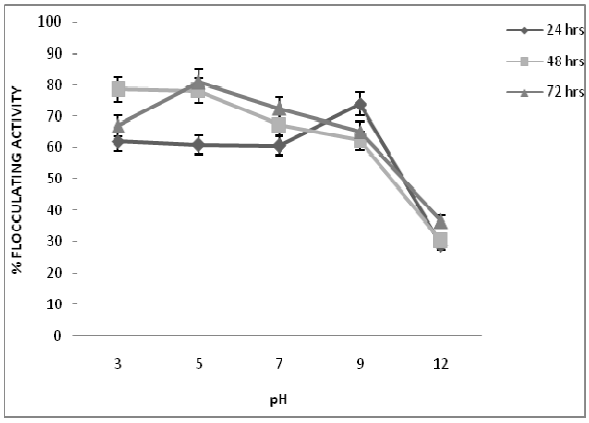

Figure 2. *Effect of initial pH on the biomass flocculation of Bacillus clausii NB2 strain*

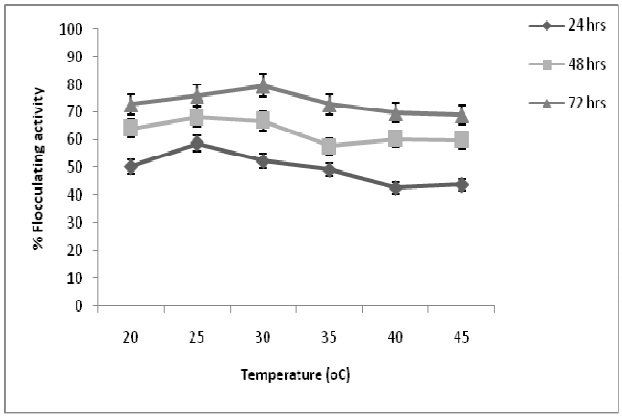

Figure 3. *Effect of temperature on the biomass flocculation of Bacillus clausii NB2 strain*

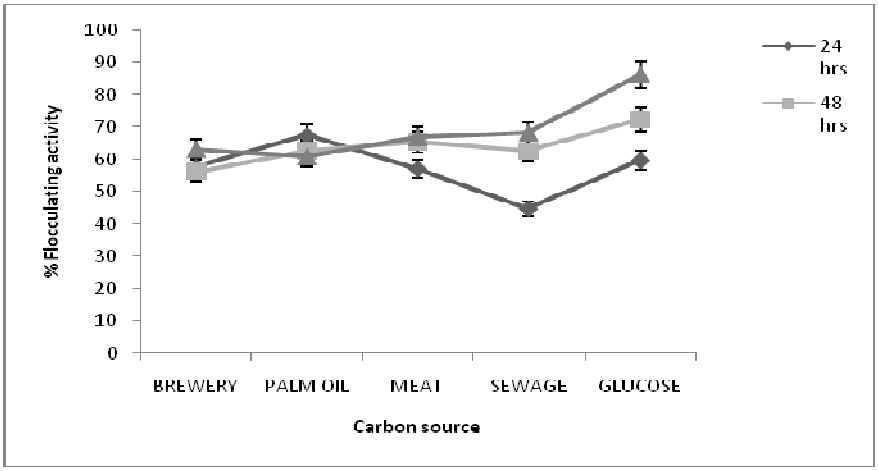

Figure 4. *Effect of carbon sources on the biomass flocculation of Bacillus clausii NB2 strain*

Flocculation is the process of conglomeration of destabilized particles into larger aggregates for proper separation from wastewater (Droste, 1997). The initial pH of the fermentative medium affected the bioflocculant production by *Bacillus clausii* NB2 strain. It has been reported that the initial pH of the fermentative medium is one of the factors affecting the production and flocculating activity of the bioflocculant (Salehizadeh and Shojaosadati, 2001). The flocculating activity of bioflocculant produced by *Bacillus clausii* NB2 strain was stable within pH 3.0 – 9.0 at 72 h of incubation with highest activity at pH 5.0. This result contradicted the reports presented by Lachhwani (2005) and Cosa *et al.* (2011) using strains RDL1 and RDL2, and *Virgibacillus Rob.* spp. strain that have their maximum yield of flocculation at alkaline pH of 7.5 and 12.0 respectively. *Gyrodium impudicum* KG03 bioflocculant was reported to have maximum activity at acidic pH range (4.0) (Yim *et al.*, 2007). Leonard *et al.* (2012) reported that *Arthrobacter* sp. 5J12A strain had optimum bioflocculant production activity at neutral pH range (7.0).

Bacillus clausii NB2 strain preferred glucose (86.23%) than other carbon sources used as

substrate for their cultivation for 72 h in medium containing brewery wastewater, sewage, palm oil effluent or abattoir effluent. The results reveal that glucose was the best carbon sources for bioflocculant production by the used strain at 72 h. These results are in accordance with the report of Cosa *et al.* (2011) on *Virgibacillus Rob*. spp. strain who reported glucose as a preferred carbon source. Kurane *et al.* (1991) also reported that *Rhodococcus erythropolis* strain showed better bioflocculant production when glucose and fructose are present in the fermentative medium. Patil *et al.* (2010) reported that the bioflocculant produced by *Bacillus subtilis* strain is enhanced by glucose and sucrose as carbon sources.

Figure 5 shows the effect of different incubation time on bioflocculant production by *Bacillus clausii* NB2 strain. There was a significant difference (P≥0.05) in the flocculating activity of *Bacillus clausii* NB2 strain in different incubation time. The flocculating activity ranged from 28.45f - 86.84a % in which the highest flocculating activity was recorded at 72 h of submerged cultivation with agitation at 120 rpm rpm.

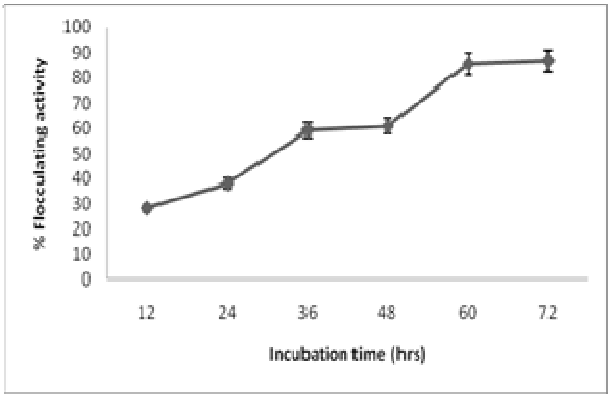

Figure 5. *Effect of incubation time on the flocculating activity of Bacillus clausii NB2 strain*

Figure 6 shows the effect of stationary and with agitation cultivation on bioflocculant production by *Bacillus clausii* NB2 strain. There was a significant difference (P≥0.05) in the flocculating activity of *Bacillus clausii* NB2 strain under stationary and with agitation cultivation. In stationary cultivation system, the flocculating activity ranged from 33.51 - 63.37 % while in agitation condition of cultivation, the activity ranged from 60.48 - 86.22 % in which the highest flocculating activity was recorded in agitation at 72 h of cultivation.

Figure 7 shows the effect of agitation speed on bioflocculant production by *Bacillus clausii* NB2 strain. There was also a significant difference (P≥0.05) in the flocculating activity of *Bacillus clausii* NB2 strain at different agitation speed during cultivation. At 24, 48 and 72 h of incubation the flocculating activity ranged from 48.02 - 61.10 %, 59.67 - 77.44 % and 63.53 - 88.67 % in which the highest flocculating activity was recorded at 140, 160 and 140 rpm speeds, respectively.

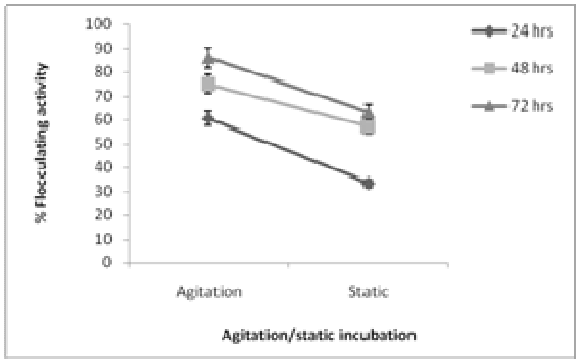

Figure 6. *Effect of stationary and with agitation cultivation on the flocculation of Bacillus clausii NB2 biomass*

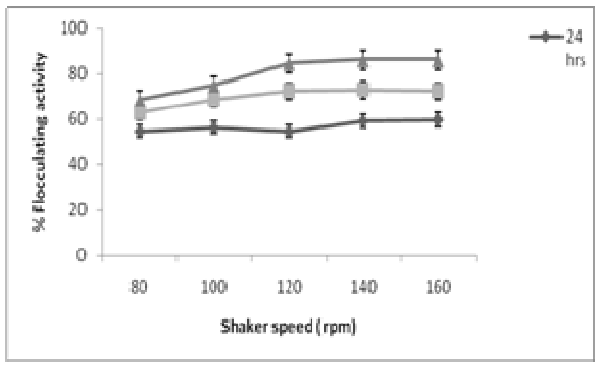

Figure 7. *Effect of agitation speed on the flocculating activity of Bacillus clausii NB2 strain*

The flocculating activities of bioflocculants produced by *Bacillus clausii* NB2 strain was observed to increase with an increase of the incubation time (72 h). Liu *et al.* (2010) reported that bioflocculant production was almost in parallel with cell multiplication where the produced bioflocculant reached its maximum flocculating efficiency in the early stationary phase (at 24 h), while the cell production reached its maximum in

the stationary phase (at 72 h). It was y reported that the bioflocculant was produced by biosynthesis during its growth, not by cell autolysis (Lu et al., 2005). The fact that the highest flocculating activity was recorded during trophophase and not in idiophase stage indicated that bioflocculant was produced by biosynthesis during growth of the bacterium strain and not by cell autolysis (Bu'Lock *et al*., 1965; Gao *et al*., 2006). Fugita *et al*. (2001) reported that increased bioflocculant production with incubation time may significantly justify the fact that bioflocculants compounds are produced by biosynthesis. This result was in contrast to the finding of Cosa *et al.* (2011) on *Halobacillus Mvuyo* spp. strain reported a maximum production yield after eight days of cultivation; highest yield was also reported for *Virgibacillus Rob*. spp. strain after four days and six days for *Oceanobacillus* spp. respectively.

Incubation under shaking condition is effective for maximum bioflocculant production by *Bacillus clausii* NB2 strain. This report was in contrast to the report of Salehizadeh and Shojaosadati (2001) who reported that sometimes due to agitation of the culture medium, yield of polymeric flocculant production by bacterial cells might be greatly reduced.

The effect of agitation speed on the bioflocculant production by the NB2 strain showed that the shaking speed of 140 rpm was the most preferred having 88.67% flocculating activity. Decrease in flocculating activity was observed when shaking below or above 140 rpm. This may be as a result of the fact that shaking speed determines the concentration of the dissolved oxygen, which can affect the biochemical transformation of the nutrients and enzymatic activity of the two strains (Salehizadeh and Shojaosadati, 2001). This study contracted the work of Zhang *et al.* (2007) on consortium of *Staphylococcus* spp. and *Pseudomonas* spp. strains with 160 rpm being the best agitation speed for highest flocculating activity.

Figure 8 shows the effect of temperature on the activity of bioflocculant produced by *Bacillus clausii* NB2 strain. There was a significant difference (P≥0.05) in the flocculating activity of bioflocculant when it was subjected to treatment at different temperature ranging from 30° – 100°C. At 24, 48 and 72 h of incubation, the effect of temperature on the flocculating activities of bioflocculant produced by *Bacillus clausii* NB2 strain ranged from 29.67- 56.40 %, 43.01 - 74.43 % and 30.44 - 82.92% respectively. At 24 hrs of incubation, the highest activity was recorded at 30°C and at 48 and 72 h of incubation; the highest activity was recorded at 40°C, while the lowest was observed at 100°C in all the incubation time.

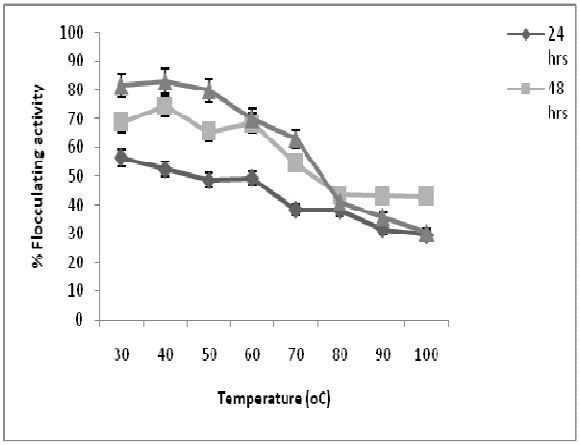

Figure 8. *Effect of temperature on the bioflocculant produced by Bacillus clausii NB2 strain*

Figure 9 shows the effect of hydrogen ion concentration on the bioflocculants produced by *Bacillus clausii* NB2 strain.

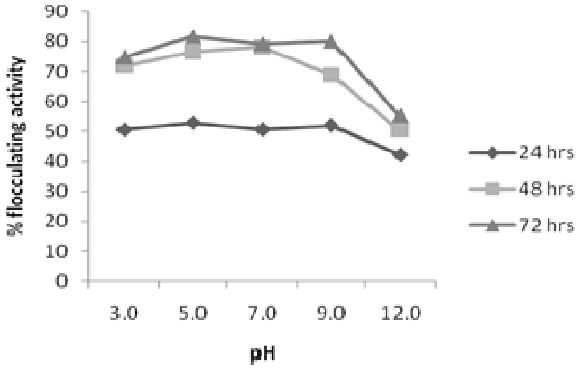

Figure 9. *Effect of pH on the activity of the bioflocculant produced by Bacillus clausii NB2 strain*

There was a significant difference (P≥0.05) in the flocculating activity of bioflocculant when it was subjected to different pH, ranging from 3.0 – 12.0. At 24, 48 and 72 h of incubation, the effect of pH on the flocculating activity of bioflocculant

produced by *Bacillus clausii* NB2 strain ranged from 42.21 - 52.86 %, 50.41 - 78.09 % and 55.22 - 81.66 % respectively. At 24, 48 and 72 h of incubation, highest flocculating activity was recorded at pH 5.0, 7.0 and 5.0 respectively, while the lowest was observed at pH 12.0 in all the incubation time.

Figure 10 shows the result obtained for the effect of different concentration on cationic inhibition on the bioflocculant, at low concentration of the cationic (10 – 500 ppm), there was no dra 20 reduction in the flocculating activity of ... bioflocculant, but as the concentration of these mono cationic increases, the flocculating activity of bioflocculant decreases. It was observed that Na^+ (from $NaNO_3$) and D-glucose do not have inhibitory effect on the bioflocculant activity.

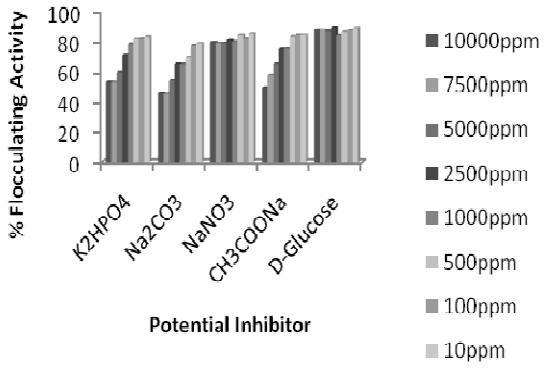

Figure 10. *Effect of potential inhibitors on the flocculating activity of bioflocculant produced by Bacillus clausii NB2 strain*

Bioflocculants produced by *Bacillus clausii* NB2 strain were not affected by increasing the reaction temperatures in the range of 30-70°C while a sharp decrease was observed after these temperature ranges. This result also indicates that too high clay suspension temperature is unfavorable for the flocculation performance of the biopolymer. This can be explained by chemical kinetics according to Lu *et al.* (2005) and Gong *et al.* (2008) that both the rate of biopolymer diffusion and suspended particle collision frequency were improved at higher temperatures which contribute to the increase in rate of reaction. Also, denaturalization of proteins in the bioflocculant and an increase in hot movement of Kaolin particles can be responsible for this sharp decrease at high temperature (Liu *et al*., 2009).

The effect of pH on the activity of bioflocculants produced by *Bacillus clausii* NB2strain under different pH range of 3.0-12.0 indicated that the bioflocculant showed stability to flocculate kaolin suspension within pH 3.0-9.0 with highest activity at pH 5.0. It can be explained that the biopolymer solution is suitable to be applied in neutral, weakly acid and weakly alkaline circumstances. This report is in contrast to that of Yokoi *et al.* (1995) that the pH range for polyglutamate from *Bacillus subtilis* PY-90 strain was pH 3.0–5.0 while Takagi and Kadowaki (1985) also reported pH 4.0–8.0 for the cationic polysaccharide from *Paecilomyces* spp. I-1 strain. The effect of potential inhibitors on the flocculating activity of the bioflocculant produced by *Bacillus clausii* NB2 strain shows that high concentrations of K_2HPO_4 and Na_2CO_3 (>1000 ppm) and -CH_3COONa (>2500 ppm) inhibited flocculation. D-GLU and $NaNO_3$ have no significant effect on the flocculating activity at both high (10 000 ppm) and low (10 ppm) concentrations. These observations were similar with the finding of Buthelezi *et al.* (2009) on the bioflocculants produced by strains *Staphylococcus aureus*, (A22), *Pseudomonas plecoglossicida* (A14), *Pseudomonas pseudoalcaligenes* (A17), *Exiguobacterium acetylicum* (D1), *Bacillus subtilis* (E1), *Klebsiella terrigena* (R2). Figure 11 shows the FT-IR spectroscopy performed on purified bioflocculant produced by *Bacillus clausii* NB2 strain. The spectrum showed a sharp, intense absorption peak at 3489.34 – 3385.18 cm-1 is characteristic of a hydroxyl and amino group for the bioflocculant produced by *Bacillus clausii* NB2 strain. A weak peak at 2,949.26 cm-1 known to be typical of carbohydrates, indicating -COH asymmetrical stretching vibration. A weak peak at 2312.73 cm-1 is typical of aliphatic band, while that of 1859.08 – 1791.93 cm-1 is typical of -C=O acid chloride. Sharp peak at 1653.05 is typical of Carboxyl, -CO-NH or -NH2 group. Weak asymmetrical stretching peak observed from 1541.18 – 1338.64 cm-1 is characteristic of -NH band vibration -CONH. A very sharp stretching peak at 1070.53 cm-1 indicated asymmetrical stretching vibration of a -C-O-C- ester linkage.

Sharp peak at 887.59 cm-1 could be associated with glycosidic linkages between the sugar monomers. The weak peak at 773.48 – 609.53 cm-1 is typical of Benzene rings. The presence of characteristic peak for carbohydrate and amide shown by infrared spectral indicated that the bioflocculants produced by *Bacillus clausii* NB2 strain is a glycoprotein.

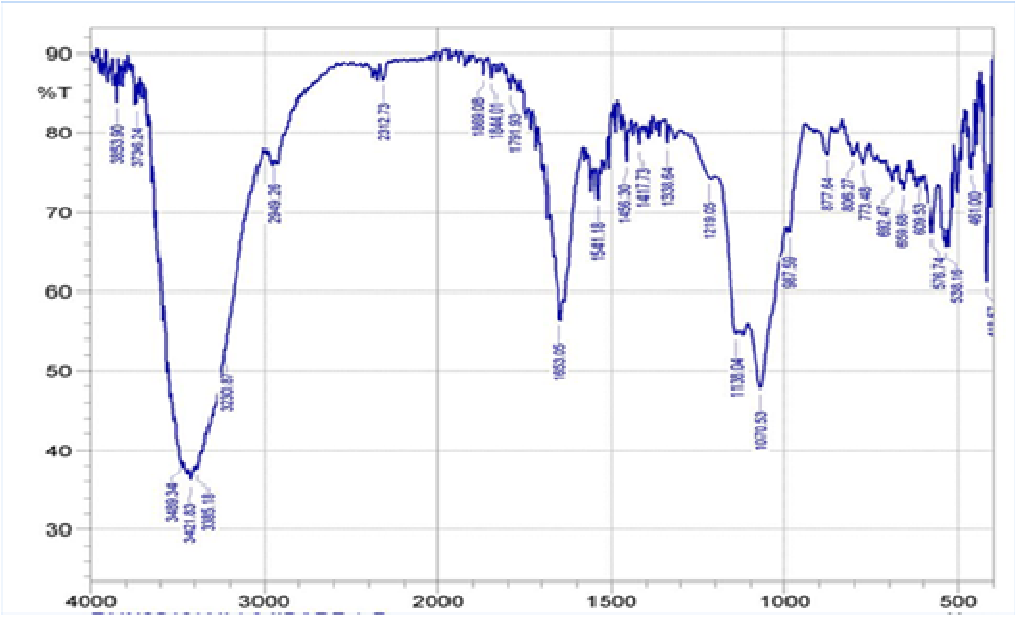

Figure 11. *Fourier transform infrared (FT-IR) spectrum of the partially purified bioflocculant produced by Bacillus clausii NB2 strain*

It was observed that bioflocculants produced by NB2 strains of *Bacillus clausii* was glycoproteins when the carbohydrate and protein components of the purified bioflocculant produced were analyzed. Carbohydrate happened to be the major components contained 88.71% to 11.29% of proteins.

Table 1. *Chemical analysis of the purified bioflocculant*

Components	OD	%
Sugar[1]	0.4401	88.71
Protein [2]	0.0562	11.29
Dry weight (g/L)	1.1	

[1]Optical Density (OD) at wavelength of 490 nm;

[2]Optical Density (OD) at wavelength of 750nm

The presence of characteristic peak for carbohydrate and amide shown by infrared spectrum indicated that the bioflocculant produced by *Bacillus clausii* NB2 strain was glycoprotein. The FT-IR spectrum of the polymer proved the presence of carboxyl group (COO-), hydroxyl group (OH-) and amino group (NH2) which may work as functional moieties to generate new or modified polymer (Satish *et al*., 2009). The Van der Waal forces may first be the attractive force, then OH, COO- groups of bioflocculant and H+, OH- groups on the surface of particles may form hydrogen bonds as the bioflocculant chains approach the surface of particles. The presence of Ca^{2+}, Al^{3+} on the surface of the particles and carboxyl group of the bioflocculant may therefore leads to the formation of chemical bonds

Conclusions

A new bacterial strain phylogenetic determined as *Bacillus clausii* strain was studied for their bioflocculant activity. Some biotechnological parameters as: temperature, pH, time, agitation and agitation speed during submerged cultivation were studied in order to establish the optimum conditions for biomass flocculation. Also it was establish that some cations can serves as potential inhibitors on bioflocculant activity. The fact that bioflocculant can be produced relatively inexpensively from a variety of bacteria, the

microbial flocculation is a promising alternative to present treatment processes for wastewater and drinking water treatment. However, more research still needs to be conducted to optimize the conditions for maximum yield of flocculation process.

Unlike some synthetic flocculants, bioflocculant are generally non-toxic and benign to the environment (Buthelezi, 2008). They have a potential to improve productivities and product quality in bio-processing, wastewater treatment, and many other industrial operations (Sanghi *et al*., 2006).

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