

**EFFECT OF OPTIMIZATION OF CULTURAL PARAMETERS ON
EXOBIOPOLYMER PRODUCTION BY MICROBIAL ISOLATES AND THEIR
APPLICATION IN WASTEWATER TREATMENT**

ABSTRACT

The optimization of culture media and conditions for efficient production of exopolymers by microorganisms cannot be overemphasized. As such, this work investigated the effect of optimizing culture media on bioflocculant production using *Alcaligenes aquatilis* AP4 and *Bacillus clausii* NB2 isolated from wastewater samples. It also studied the application of the resulting bioflocculants for treatment of wastewater. The basal medium used for the batch fermentation was supplemented with different concentrations of carbon and nitrogen sources. The bioflocculants produced were tested on kaolin solution, brewery and palm-oil effluents in concentrations ranging from 100-1000mg/L. It was observed that optimization of culture media by the two strains had a significant effect ($P \leq 0.05$) on bioflocculant production. At 72 hours of incubation, 15.0 and 20.0g/l glucose concentration gave the best conditions for bioflocculant production by isolates AP4 and NB2 respectively. In addition, 0.50 and 0.30 g/l of inorganic nitrogen source ($(\text{NH}_4)_2\text{SO}_4$) and 0.70g/L of organic nitrogen (Urea) were required by both isolates. 10 and 15ml/L of bioflocculant dosage respectively gave the best flocculating activities. The ability of the bioflocculants to flocculate kaolin solution, brewery wastewater and palm-oil effluent were confirmed. In addition, the bioflocculants also showed a tremendous ability to reduce COD and turbidity of wastewater.

Keywords: bioflocculant, biopolymer, optimization, COD, turbidity, flocculating activity.

INTRODUCTION

Biopolymers could be grouped into either intracellular or extracellular (Nwodo *et al.*, 2012). Extracellular polymers (exobiopolymers) are high-molecular weight mixtures of polymers consisting of long-chains of repeating unit of sugar residues which are produced and released into the external environment. Some naturally occurring biopolymers are chitosan, cellulose, guar gum, sodium alginate, starch, tannin, and microbial polymers which sometimes secrete their biopolymer into their surrounding environment (Vijayabaskar *et al.*, 2011). Some of these microbes synthesize their biopolymer in form of bioflocculant which could be polysaccharides (Yimet *et al.*, 2007; Gong *et al.*, 2008; Lian *et al.*, 2008; Liu *et al.*, 2010), glycoprotein (Okaiyeto *et al.*, 2013; Abdel-Aziz *et al.*, 2015; Ogunsade *et al.*, 2015) or functional proteins (Xia *et al.*, 2008; Zhenget *et al.*, 2008).

These bioflocculant producing organisms are generally present in environmental samples such as soil (Arafa *et al.*, 2014; Jiang *et al.*, 2015), activated sludge (Zhang *et al.*, 2012; Li *et al.*, 2015),

crude oil (Zaki *et al.*, 2011) wastewaters (Dlangamandla *et al.*, 2016; Nurul-Adela *et al.*, 2016), and lignocellulosic biomass (Guo *et al.*, 2016). Generally, microbial flocculants can be produced by eubacteria, actinomycetes and fungi during their growth and it is preferred to the conventional inorganic and organic flocculants because of their biodegradability, harmless to humans and the environment, safety for ecosystems, high flocculating capability and lack secondary pollution (Gao *et al.*, 2006; Azmi *et al.*, 2015). This bioflocculant can be used in industrial and wastewaters treatment processes such as pharmaceutical, fermentation, food industries dredging and downstream processing (Zhang *et al.*, 1999; Salehizadeh and Shojaosadati, 2001). Specifically, bioflocculants have been applied in various ways and found to be potent in the removal of heavy metal ions, turbidity and COD from effluents (Zhang *et al.*, 2012; Zhao *et al.*, 2017), removal of dye and precipitation of inorganic solid particles from suspension (Bender *et al.*, 1994; Penget *et al.*, 2014), treatment of drinking water (Zhang *et al.*, 2015), treatment of oil-polluted wastewaters and landfill leachate (Liet *et al.*, 2015).

Moreover, the high cost of bioflocculant has been one of the limiting factors towards its application (Zhang *et al.*, 2010), as such, different culture media have been experimented as substrates for bioflocculant production to cushion this effect such as dairy and sauce wastewaters, effluent from a hydrogen-producing bioreactor (Zhou *et al.*, 2003; Li *et al.*, 2004; Dong *et al.*, 2008); Brewery, Palm-oil and Sewage effluents (Adebayo-tayo and Adebami, 2014); lignocellulosic biomass (Liu *et al.*, 2017), soybean juice and fishmeal wastewaters (Huang *et al.*, 2001; Zhou *et al.*, 2003). Alternatively, optimization of culture conditions such as carbon and nitrogen sources, bioflocculant dosage concentration, pH and temperature are important to obtain effective and optimum bioflocculants. Effective optimization will also reduce the problem created by high cost of production. As such, this research work is carried out to determine the effect of optimizing the concentration of culture parameters on the bioflocculants production and the application of the produced bioflocculants in the treatment of wastewater samples.

MATERIAL AND METHODS

Culture preparation

Isolation and characterization of bioflocculants producing isolates AP4 and NB2 obtained from palm oil mill effluent and brewery wastewater respectively in south-western part of Nigeria had been previously reported. The strains were preserved in 20% glycerol stock at -80°C in the Department of Microbiology University of Ibadan, Ibadan Nigeria. 16S rDNA Sequence determination and Phylogenetic Analysis of the two strains showed that AP4 and NB2 were *A. aquatilis* and *B. clausii* respectively.

The stock cultures were re-isolated and maintained on Nutrient agar, incubated at 30°C for 72h and stored at 4°C . The seed culture was grown in a 250ml flask containing: Nutrient broth, 10.0g; Potato dextrose broth, 5.0g; Glycerol, 3ml; Yeast Extract, 6.5g; Sodium chloride, 1.0g in

1litre of distilled water. The pH of the medium was adjusted to 7.0 and the medium was autoclaved and inoculated with pure culture from the stock culture and incubated for 24hrs.

Biofloculant production

The isolates were used for biofloculant production using Biofloculant Production Broth medium (BPB). The BPB composition include: 10g glucose, 2g KH₂PO₄, 0.2g MgSO₄.7H₂O, 0.1NaCl, 0.5g CaCO₃, and 0.5g yeast extract. The mixture was dissolved in 1 liter deionized water with the initial pH adjusted to 7.0. The medium was sterilized, inoculated with pure culture of the isolates and incubated on a rotary shaker at 120 rpm and 37°C for 3 days. Kaolin suspensions at a concentration of 5,000mg/l were used to evaluate the flocculating abilities of the produced exopolysaccharides (Zhenget *al.*, 2008).

Single and consortium culture

The two isolates were used separately and as consortium. The inoculum of the two isolates were first grown aerobically in 250ml Erlenmeyer flasks at 25°C on a rotary shaker at 120rpm for 72 hrs. 1ml of each strain were then inoculated into a fresh 250ml Erlenmeyer flask containing culture broth. The medium was incubated at 25°C on a rotary shaker at 120rpm for 72 hrs, after which the fermentation broth was centrifuged at 4000 × g for 30 minutes at 4°C and the supernatant was used for determination of flocculating activity using Kaolin suspension at concentration of 5,000mg/l. The optical density (OD) of the clarifying upper phase solution was measured at 550nm with a UV spectrophotometer (Malieheet *al.*, 2016).

Determination of Flocculating Activity

The flocculating activity was determined according to the method of Kuraneet *al.* (1986) as modified by Gaoet *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% CaCl₂ (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 10mL 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 550nm with a UV spectrophotometer and the flocculating activity determined as follows:

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

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

Optimization of cultural parameters for biofloculant production

Biofloculant production by the isolates was carried out under optimized conditions. The basal medium was supplemented with different concentrations of the best carbon source (5.0-30.0 g/L), nitrogen sources (0.05-1.0 g/L) and dosage (5.0-30.0 ml/L). The medium was inoculated and incubated under shaking (120rpm) condition. After incubation the Kaolin assay was carried out to check the maximum flocculating activity of the isolates after which the absorbance was then read using a spectrophotometer at a wavelength of 550nm.

Application of biofloculants in the treatment of wastewaters

Wastewaters used for this analysis were collected from two different states in south-western part of Nigeria. The Brewing effluent was collected from International Breweries Plc Omi-asoro, Ilesha, Osun State while Palm-oil effluent was collected from Fiditi village, Oyo State. The Dissolved Oxygen (DO), Chemical dissolved oxygen (COD), Turbidity, pH, Conductivity, Salinity and Temperature of the wastewater samples were determined. All wastewaters were stored at 4°C before flocculation. Varied concentrations (100-1000mg/L) i.e. (0.1-1.0ml/l) of biofloculant and 1 ml of 1% CaCl₂ solution was added to the 100 ml wastewaters at pH 7.0. The compound was mixed thoroughly and allowed to settle for 5 minutes, after which the optical density (OD) of the clarifying upper phase solution was measured at 550nm with a UV spectrophotometer and the flocculating activity determined. The dissolved oxygen, chemical oxygen demand (COD) and turbidity of the wastewaters were measured using Dissolved Oxygen meter (Extech Instrument), Multi-parameter Photometer (Hanna) and Microprocessor turbidity meter (Hanna) respectively. The removal efficiency of COD and turbidity of the Brewery and Palm-oil wastewaters were determined using the method of Gong *et al.* (2008). The results obtained were compared with chemically synthesized flocculants in which the biofloculant was replaced with Aluminium sulphate (Alum).

Statistical Analysis

The Data obtained were subjected to one-way analysis of variance (ANOVA) to determine their significance at $P \leq 0.05$. Tukey-Kramer test method was used. All data were treated in replicates, the standard deviation of the mean values was taken (Kao and Green, 2008).

RESULTS AND DISCUSSION

BLAST (Basic Local Alignment Search Tool) analyses of the 16SrRNA gene nucleotide sequence of the bacterial strain, coded AP4 and NB2, showed 95 and 97% similarities to *A.aquatis*(accession number KT748636) and *B.clausii*(accession number HM560954) respectively. A phylogenetic tree was constructed between them and similar sequences found in GenBank as shown in Figure 1.

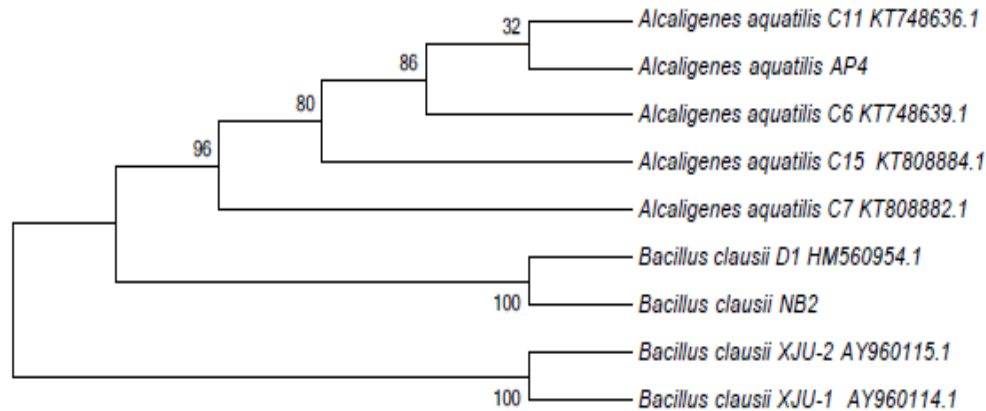


Figure 1: Phylogenetic tree showing the relationships between *A. aquatilis* AP4 as well as *B. clausii* NB2 strains and other closely related species collected from the Gene Bank. The dendrogram was generated by the neighbor-joining method. Bootstrap values per 100 bootstrap analysis presented for values greater than 50 %

Effect of single starter/consortium on bioflocculant production by the isolates

The production of bioflocculant and its activity by single starter and consortium isolates are shown in Figure 2. It was observed that there was a significant difference ($P \leq 0.05$) in the flocculating activity of using the strains singly and in consortium. The flocculating activity of the single and combined starter ranged from 54.31^c - 82.66^a %, 58.10^c - 79.84^a % and 50.15^c - 80.66^a% for *A. aquatilis* AP4, *B.clausii* NB2 and Consortium respectively. At the end of 72 hrs of incubation, the highest flocculating activity was 82.66^a % in *A. aquatilis* AP4, 79.84^a % in *B.clausii* NB2 and 80.66^a% in their consortium. This finding was similar to the report of Gül and Dönmez, (2013) on the decolourization of Remazol Blue dye by *Aspergillus versicolor* and *Rhizopus arrhizus* both as individual and mixed cultured. At the end of incubation period, 46.3, 89.4 and 86.5% activities were observed for *R. arrhizus*, *A.versicolor* and their mixed culture respectively. Malieheet *al.* (2016) observed an increased yield when the consortium of *B. pumilus* JX860616 and *A. faecalis* HCB2 were used for bioflocculant production. Ntsalubaet *al.* (2013) reported 89% flocculating activity for the consortium of *Methylobacterium* sp. Obi and *Actinobacterium* sp. Mayor. However, Menetrez, (2012) and Wang *et al.* (2016) reported that a lower activity of consortium may be sometimes noticed because synthetic microbial consortia are often fragile and unstable which limit their applications in the industrial biotechnology level in contrast to what is obtained in their environment. Kim *et al.* (2008) observed that mixed cultures created by arbitrarily combining different species, population compositions are often unstable and prone to domination by a single species or extinction.

Recently, there has been growing interest in the biotechnological consortia synthesis of isolates for their industrial applications. Natural microbial consortia hold many appealing properties in a bioprocessing context, such as stability, functional robustness, and the ability to perform complex tasks (Zuroff and Curtis, 2012). For instance, Microbial consortium have been earmarked as a reliable and efficient alternative to single strains for lignocellulose degradation (Brossiet *al.*, 2015). Cortes-Tolalpa *et al.* (2016) used an interesting approach by varying environmental sources of microbial inocula in the lignocellulose-degrading of wheat plant by using up to six organisms as consortia. These approach gave rise to the fact that microbes sometimes live in synergistic communities in most natural environments in which individual species with specialized roles cooperate to survive and thrive together (Mee and Wang, 2012). Gül and Dönmez. (2013) reported that addition of surfactants such as dodecyl trimethyl ammonium bromide (DTAB) in small concentration increases the activity of consortium organisms.

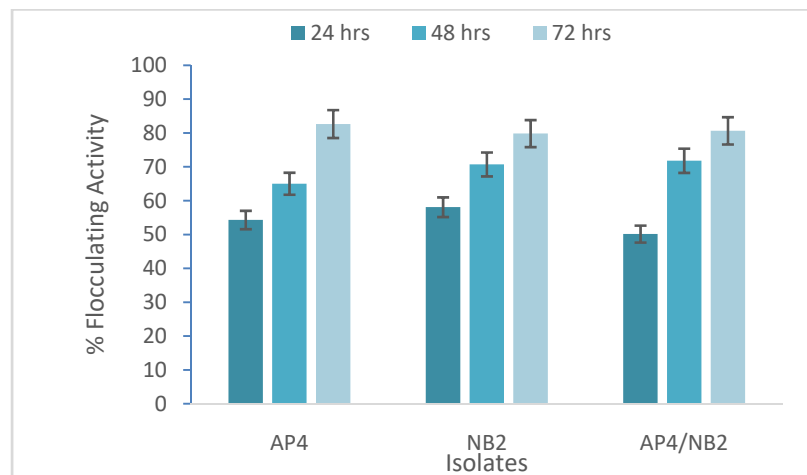


Figure 2: Effect of single and combined starter on the bioflocculation productions

Key: AP4= *A. aquatilis* AP4; NB2= *B. clausii* NB2 and AP4/NB2= Consortium by *A. aquatilis* AP4 and *B. clausii* NB2

Effect of glucose optimization on the flocculating activities of the isolates

Figure 3 shows the effect of varied concentrations of glucose on bioflocculant production by AP4 and NB2. At 72 hrs of incubation, there was a significant difference ($P \leq 0.05$) in the flocculating activity of different glucose concentration, which ranged from 77.44^f - 89.66^a % and 58.79^f - 80.23^a % for isolates AP4 and NB2 respectively. It was discovered that isolate AP4 had its highest flocculating activity at 15.0 g/l of glucose concentration while NB2 had its highest at 20.0 g/l. This finding was similar to the report of Okeiyato *et al.* (2016a) that 20 g/L of glucose was required by *Bacillus* sp. AEMREG7 to obtain the best flocculating activity of 88.03% among several carbon sources tested. Luo *et al.* (2016) reported that 25 g/L of glucose

concentration was required by bioflocculant-producing *Bacillus megaterium* SP1 to obtained 90.6% flocculating activity. Liu *et al.* (2016) report on alkali-tolerant strain of *Microbacterium esteraromaticum* C26 showed that glucose gave the best among the carbon sources tested at concentration 3g/L which is in contrast to the present study.

According to Minty *et al.* (2013) and Wang *et al.* (2016) who reported that the backbone of functional biological molecules is carbon; which could occur as polysaccharides, disaccharides, monosaccharides and alcohol sugar. Glucose is a monosaccharide, which therefore provides faster growth than other sugars, and is mostly consumed first in sugar mixtures (Bren *et al.*, 2016). Moreover, when added to nutrient broth, it may increase the overall growth rates and biomass of bacteria over time (Cosa *et al.*, 2013). In addition, there have been various reports of complex sugars being used for bioflocculant production. For instance, Li *et al.* (2017) reported that starch was found the most favourable by *Oceanobacillus polygona* in the production of bioflocculant MBF-HG6. Alternatively, glucose may be inhibitory to cell growth if the concentration is too high (Dalhoff, 1979; Borralho *et al.*, 2002; Schmidt-Glenewinkel and Barkai, 2014). Increasing the amount of glucose have been shown to limit microbial growth by inhibiting proteinaceous enzymes (Boyd and Lichstein, 1951; Wang *et al.*, 2015).

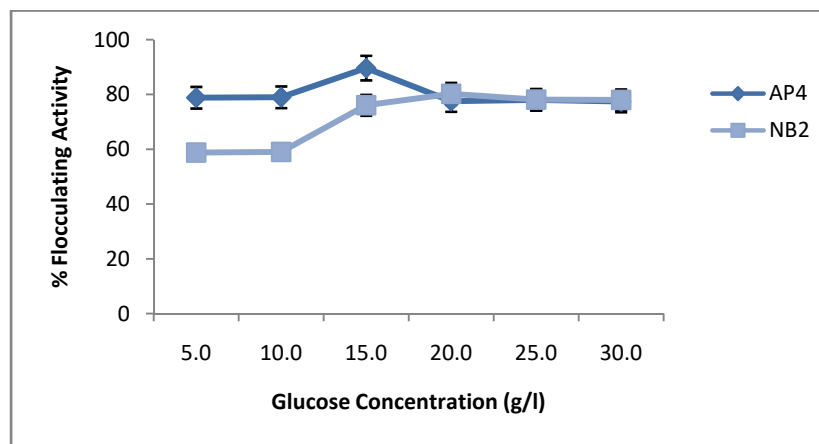


Figure 3: Effect of different conc. of glucose on the flocculating activities of AP4 and NB2

Effect of optimization of Nitrogen sources on the flocculating activities of the isolates

Different concentration of organic (Urea) and inorganic (Ammonium sulphate) nitrogen sources on the flocculating ability of the isolates were investigated. Figure 4 shows the effect of varied concentrations of Ammonium sulphate on bioflocculant production by AP4 and NB2. There was a significant difference ($P \leq 0.05$) in the flocculating activity of the isolates at different Ammonium sulphate concentration. At 72 hrs of incubation, the flocculating activities ranged from 75.44^f - 83.55^a % and 77.12^f - 86.22^a % respectively. It was discovered that isolate AP4 had its highest flocculating activity at 0.50 g/l of Ammonium sulphate concentration while NB2 had its highest at 0.30 g/l.

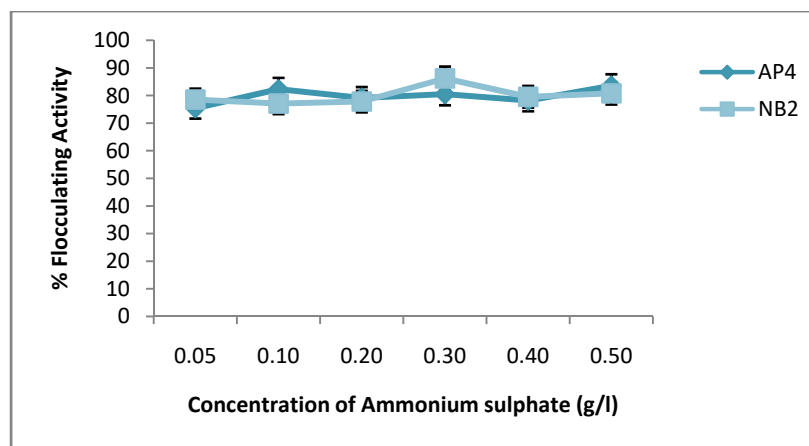


Figure 4: Effect of different conc. of Ammonium sulphate on the flocculating activities of AP4 and NB2

Figure 5 shows the effect of varied concentrations of urea on bioflocculant production by AP4 and NB2. There was also a significant difference ($P \leq 0.05$) in the flocculating activity of these isolates at different urea concentrations. At 72 hrs of incubation, the flocculating activities ranged from 60.63^e - 79.22^a % and 65.51^d - 83.44^a % respectively in which the highest flocculating activity of urea for both isolates was observed at 0.70g/L.

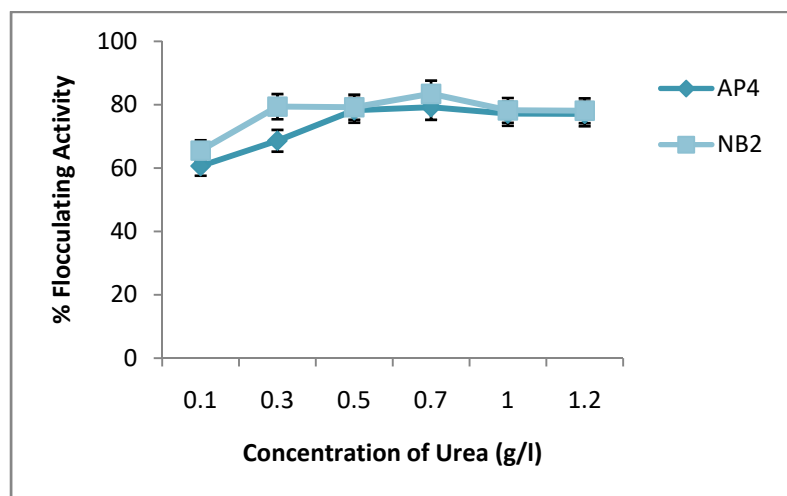


Figure 5: Effect of different conc. of Urea on the flocculating activities of AP4 and NB2

This finding was in contrast to the report of Li *et al.* (2015) on the optimization of nitrogen sources for bioflocculant production using *Bacillus methylotrophicus* C412 strain, he observed that Urea did not support the growth of the isolate, while, 1.0g/L of beef extract and 3.0g/L of ammonium sulphate gave the best flocculating activity for the strain. deKokeret *al.*, 2004 reported that high concentration of ammonium sulphate can possibly precipitate protein components in the bioflocculant which could inhibit the extracellular flocculating activity and biomass. Lin and Harichund, (2012) made use of yeast extract and peptone as the source of

nitrogen, and observed that organic nitrogen supported the growth of the isolate than the inorganic. His view was also supported by Xia *et al.* (2008) and Zhenget *al.* (2008). Kuraneet *al.* (1994) and Lin and Harichund(2012) were of the opinion that increase in organic nitrogen sources improved the bioflocculant productions by the bacterial isolates.

Effect of dosage optimization on the flocculating activities

Figure 6 shows the effect different concentration of bioflocculants dosage produced by isolates AP4 and NB2 for the flocculation of kaolin solution. There was a significant difference ($P \leq 0.05$) in the flocculating activity of the isolates at different dosage concentration. At 72 hrs of incubation, the flocculating activities ranged from 75.78^e - 86.02^d % and 76.98^e - 82.74^a % and in which the highest flocculating activity of dosage was 10ml/L and 15ml/L respectively.

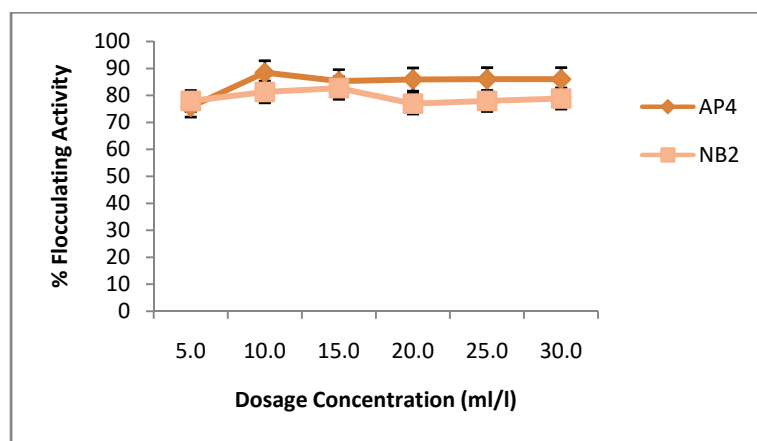


Figure 6: Effect of dosage conc. on the flocculating activities of AP4 and NB2

This finding was in contrast to the report of Husam and Nisreen, (2013) on *Azotobacter chroococcum* in which 0.25 mL of bioflocculant dosage was required to obtain the highest flocculating activity of 60.8%. Tang *et al.* (2014) reported that 2.0mg/L of the purified bioflocculant obtained from *Paenibacillus mucilaginosus* gave the best flocculating activity of 98.4%. Malieheet *al.* (2016) report on the bioflocculants produced by the consortium of *Bacillus pumilus* JX860616 and *Alcaligenes faecalis* HCB2 noted that 0.8mg/ml of the produced bioflocculant gave the best flocculating activity of 85%. Tang *et al.* (2014) observed that an increase in dosage led to decrease in flocculation. This phenomenon was clarified by Yokoi *et al.* (1997) as incomplete dispersion of excess polysaccharide in which only the kaolin particles around the polysaccharides participate in the flocculation reaction. Lee *et al.* (1995) and Kwon *et al.* (1996) explained that the increase in dosage made the excess polysaccharide to be oversaturated on many binding sites of the surface of kaolin particles, as such the force of attraction of the other particles was reduced which made the flocculating activity to decrease.

Application of biofloculants produced by the selected strains on Kaolin solution, Brewery wastewater and Palm-oil effluent

The amount of dissolved oxygen, COD, Turbidity, pH, conductivity and salinity of the wastewaters used for this analysis were determined. Table 1 showed the characteristics of the wastewaters.

Table 1: Characteristics of the wastewaters used for the analysis

Wastewater	Dissolve Oxygen (mg/L)	COD (mg/L)	Turbidity (FTU)	pH	Conductivity (S/cm)	Salinity (mg/L)
Brewery	9.2m at 25.4°C	3682	995	9.0	1.22	842
Palm-oil	2.3 at 26.4°C	6988	1530	4.7	0.86	620

Table 2 shows the flocculating activities of biofloculants and Alum in Kaolin solution, Brewery wastewater and Palm-oil effluent. It was observed that as the concentration of biofloculants increases, the flocculating activity of biofloculants also increases to a certain level. At concentration of 400mg/L, *A. aquatilis* AP4 and *B. clausii* NB2 show the highest flocculating activity of 88.32% and 86.74% respectively in kaolin solution (table 2) while 600mg/L was the best concentration required by Alum (84.44%).

Table 2: Flocculating activity of biofloculants and Alum in Kaolin solution

Test Materials	Flocculants	% Flocculating Activity					
		100 mg/L	200 mg/L	400 mg/L	600 mg/L	800 mg/L	1000 mg/L
Kaolin Solution	AP4	75.78 ^e	83.45 ^d	88.32 ^a	85.93 ^b	85.02 ^c	85.02 ^c
	NB2	77.98 ^d	81.31 ^b	86.74 ^a	78.98 ^c	77.98 ^d	77.88 ^d
	Alum	72.56 ^c	76.87 ^d	82.61 ^c	84.44 ^a	86.44 ^a	87.32 ^b
Brewery Effluent	AP4	76.65 ^f	78.11 ^e	87.86 ^b	89.41 ^a	82.78 ^c	79.97 ^d
	NB2	66.52 ^e	75.33 ^d	85.81 ^a	80.33 ^c	80.34 ^c	81.02 ^b
	Alum	60.67 ^c	63.88 ^d	71.44 ^c	80.56 ^b	80.66 ^b	82.23 ^a
Palm-Oil Effluent	AP4	67.34 ^f	70.22 ^e	75.44 ^b	79.49 ^a	74.98 ^c	73.01 ^d
	NB2	55.22 ^c	63.45 ^d	70.76 ^b	70.88 ^b	74.43 ^a	68.04 ^c
	Alum	50.22 ^f	68.33 ^e	69.50 ^d	72.12 ^c	74.44 ^b	77.32 ^a

Mean followed by different superscript within a column are significantly different ($P \leq 0.05$).

Biofloculants from the two selected strains also show their ability to flocculate both brewery wastewater and palm-oil effluent. Concentration of 600 mg/L was required by *A. aquatilis* AP4 with percentage activity of 89.41% while 400 mg/L was needed by *B. clausii* NB2 to produced the best flocculating activity (85.81%) in Brewery wastewater. Alum has its best flocculation of 82.23% at a concentration of 1000mg/L.

On testing the two bioflocculants on Palm-oil effluent, it was observed that both showed the ability to flocculate this effluent but with lower percentage of activity when compared with that of Kaolin solution and Brewery wastewater. This is could be as a result of higher turbidity of the palm-oil wastewater compared to that of Brewery wastewater (table 1). In addition, palm-oil wastewater contained very high amount of fatty-acids which could be a factor in the observed flocculation reduction. The concentration of 600 mg/L was needed by *A. aquatilis*AP4 to produce best flocculating activity of 79.49% and 800 mg/L by *B. clausii* NB2 to carry out the same task (74.43%), whereas 1000mg/L was required by Alum to produce its best flocculating activity of 77.32%.

Lee *et al.* (2014), stated that production industries normally generates different kinds of wastewaters which are made of a wide range of different particles such as organic and inorganic particles, dissolved solids, soluble and insoluble metals, very fine suspended solids, and other impurities. Moreover, the industrial application potential of bioflocculants in the removal of the above mentioned impurities cannot be questioned, as theseexo-polysaccharides have been used extensively in the purification of drinking water, treatment of domestic, brewery, pharmaceutical wastewaters, turbidity reduction, BOD, COD, and toxic heavy metals removal, ability to decolorize cationic dyes in wastewaters, harvesting and removal of pathogens of micro-algae from water (Lei *et al.*, 2015; Okeiyatoet *al.*, 2016b; Li *et al.*, 2017; Zhao *et al.*, 2017).

Table 3: Effect of bioflocculants on COD and turbidity content of brewery and palm-oil wastewaters

Flocculant		COD (mg/L)			Turbidity (FTU)		
		Initial	Final	% Reduction	Initial	Final	% Reduction
AP4	Brewery	3682	460	87.5 ^a	995	113	88.6 ^b
	Palm-oil	6988	1509	78.4 ^c	1530	470	69.3 ^e
NB2	Brewery	3682	615	83.3 ^b	995	135	86.4 ^c
	Palm-oil	6988	1565	77.6 ^d	1530	708	53.7 ^f
ALUM	Brewery	3682	1020	72.3 ^e	995	195	80.4 ^a
	Palm-oil	6988	2579	63.1 ^f	1530	738	51.8 ^d

There was a significant difference ($P \leq 0.05$) in the percentage reduction of the COD and turbidity of the bioflocculants produced by AP4 and NB2 as illustrated in table 3. In brewery wastewater, AP4, NB2 and Alum were observed to lower the concentration of COD by 87.5, 83.3 and 72.3% respectively, while 78.4, 77.6 and 63.1% reduction were recorded for palm-oil effluent. When the bioflocculants were tested for their ability to flocculate highly turbid brewery and palm oil effluents, 88.6, 86.4 and 80.4% reductions in brewery wastewaters were recorded, while, 69.3, 53.7 and 51.5% reduction of palm-oil effluent were observed respectively. According to Choudharyet *al.* (2011), COD is primarily required to oxidize organic matter and is considered as a reference for the organic load of wastewaters, both in the industrial and municipal sectors.

Chemical oxygen demand (COD) describes the amount of oxygen that is needed to chemically oxidizes organic compounds in water, using an environmentally oxidant (Ajayiet *al.*, 2016). Bioflocclulants produced by both *A. aquatilis*AP4 and *B. clausii* NB2 demonstrated that they could both flocculate Kaolin solution and reduced COD and turbidity level of Brewery wastewater and Palm-oil effluent. Ugbenyen and Okoh, (2014) and Cosa&Okoh, (2014) reported that the bioflocclulants produced by the consortium of *Oceanobacillus*&*Halobacillus*and the consortium of *Cobetia*&*Bacillus*sp were able to cause reduction in the COD of brewery wastewater up to 99.9 and 95.4% respectively, whereasGong *et al.*(2008), obtained a lower reduction of 80.4% by a bioflocclulant produced by *Serratiaficaria*. However, it should be noted that the initial amount of COD and turbidity of the wastewaters is a very important factor in the determination of reduction percentages.Li *et al.* (2017) observed 91.08% reduction in turbidity of tannery wastewater by the bioflocclulant produced by salt-tolerant alkaliphilic*Oceanobacilluspolygoni*.

As shown in table 3, both AP4 and NB2 bioflocclulantswere more effective in reducing COD and turbidity of brewery and palm-oil wastewaters in comparison to inorganic flocculant (Alum). These results are in accordance with the finding of Ugbenyen and Okoh, 2014 who revealed thatthe bioflocclulants produced by the consortium of *Cobetia andBacillus sp. gave a higher* flocculation of kaolin clay of 90%, compared to alum's 66.82%. MoreoverCosa and Okoh, (2014) observed that the bioflocclulant produced by the consortium of *Oceanobacillus and Halobacillus*gave a better result than both organic and inorganic bioflocclulants in reducing COD and turbidity of brewery and dairy wastewaters.Furthermore, Gong *et al.* (2008) demonstrated that bioflocclulants produced by *S.ficaria* were able to remove the colour and chemical oxygen demand (COD) of pulp effluent more effectively than traditional chemical flocculants. Pan *et al.*(2009) obtained 50.6 and 47.5% COD removal rate respectively when bioflocclulant PG.a21 Ca and PAC were used to treat domestic wastewater.

Table 4: Bioflocclulant applications, isolates, COD and turbidity removal percentage, dosage and reaction pH of some researched bioflocclulants

Applications	Isolate(s)	Bioflocclulant	Initial COD (mg/L)	COD removal (%)	Initial turbidity (NTU)	Turbidity removal (%)	Bioflocclulant Dosage (mg/L)	Reaction pH	Author(s)
Chinese medicine wastewater	<i>n.r</i>	F2-F6	n.r	62.6	<i>n.r</i>	<i>n.r</i>	8, AlCl ₃ , 5*	7.0	Dong <i>et al.</i> , 2008
River water	<i>Serratiaficaria</i>	S-1	205	87.1	19.0	84.2	4ml/L*	5-7	Gong <i>et al.</i> , 2008
Pulp and paper mill effluent	“	S-1	6535	72.1	0.652	98.2	4.0	5-7	“
Brewery wastewater,	“	S-1	784	80.7	88.8	91.8	4.0	5-7	“
Meat processing wastewater	“	S-1	556	76.3	121.6	93.7	4.0	5-7	“
Soy Sauce brewing wastewater	“	S-1	1207	64.1	79.0	93.7	4.0	5-7	“
Domestic wastewater	<i>n.r</i>	PG.a21 Ca	143	50.6	<i>n.r</i>	<i>n.r</i>	<i>n.r</i>	<i>n.r</i>	Pan <i>et al.</i> , 2009
Swine wastewater	<i>n.r</i>	xn11 + xn7	1372-3025	42%	230-800	91,	50-80 *	8.0	Zhang <i>et al.</i> , 2012

Brewery Wastewater	Consortium of <i>Oceanobacillus</i> & <i>Halobacillus</i>	<i>n.r</i>	8213	99.7	750	93.9	0.2	5.62	Cosa&Okoh, 2014
Dairy Wastewater	“	<i>n.r</i>	4813	99.9	1382	88.3	0.2	7.5	“
River Water	“	<i>n.r</i>	92	70.8	174	98.6	0.2	7.2	“
Livestock wastewater	<i>Rhodococcus erythropolis</i>	RSF	1,400 – 1,600	63	170–220	93.9	40; CuSO ₄ 80	11	Penget <i>et al.</i> , 2014
Brewery Wastewater	Consortium of <i>Cobetia</i> & <i>Bacillus</i> sp	-	821	95.4	750	94.8	<i>n.r</i>	8.0	Ugbenyen and Okoh, 2014
Dairy Wastewater	“	-	4813	99.1	1382	81.2	<i>n.r</i>	8.0	“
River Water	“	-	92	73.9	174	98.9	<i>n.r</i>	8.0	“
Algae-laden lake water	“	-	132	72.0	18.2	93.4	<i>n.r</i>	8.0	“
Starch wastewater,	Consortium of <i>R. radiobacter</i> & <i>B. sphaericus</i>	CBF	<i>n.r</i>	88	<i>n.r</i>	<i>n.r</i>	14; CaCl ₂ , 1.5*	7.5	Li <i>et al.</i> , 2015
Printing & dyeing wastewater	“	CBF	<i>n.r</i>	66	<i>n.r</i>	<i>n.r</i>	14; CaCl ₂ , 1.5*	7.5	“
Landfill leachate	“	CBF	<i>n.r</i>	58	<i>n.r</i>	<i>n.r</i>	14; CaCl ₂ , 1.5*	7.5	“
Aquaculture wastewater	<i>B. megaterium</i>	SP1	35.6	64	27.1	83.7	<i>n.r</i>	7.0	Luo <i>et al.</i> , 2016
Dairy wastewater	<i>B. subtilis</i>	-	45600**	43.8	50.50**	19.6	<i>n.r</i>		Didar & Ferdosi-Makan, 2016
Domestic wastewater	<i>n.r</i>	-	317	89.7%	83	91.8%	40.0	7.5	Guo <i>et al.</i> , 2016
Brewery wastewater	<i>B. clausii</i>	AP4	3682	87.5	995	88.6	10 & 1% CaCl ₂	7.0	Present study
Brewery wastewater	<i>A. aquatilis</i>	NB2	3682	83.3	995	86.4	10 & 1% CaCl ₂	7.0	Present study
Palm oil wastewater	<i>B. clausii</i>	AP4	6988	78.4	1530	69.3	10 & 1% CaCl ₂	7.0	Present study
Palm oil wastewater	<i>A. aquatilis</i>	NB2	6988	77.6	1530	53.7	10 & 1% CaCl ₂	7.0	Present study

n.r means not reported.

* reported in ml/L

** reported in mg/L

CONCLUSION

Optimization of Carbon and Nitrogen sources as well as dosage of biofloculants producing *A. aquatilis* AP4 and *B. clausii* NB2 were done. 15.0, 0.50, 0.70g/L and 10ml/L of glucose, ammonium sulphate, urea and dosage respectively were required by AP4 to have the best flocculating activity in kaolin solution, while 20.0, 0.30, 0.70g/L and 10ml/L of glucose, ammonium sulphate, urea and dosage were required by NB2. The consortium of the two isolates have a significant influence ($p \leq 0.05$). The biofloculants were able to flocculate kaolin solution, brewery wastewater and palm-oil effluent at a lower dosage and effectively reduce the COD and turbidity of wastewaters.

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370 REFERENCES

- 371 Abdel-Aziz, S.M., Hamed, H.A., Fadel, M. and Moharam, M.E. (2015). Properties and Role of
372 Exopolysaccharides Produced by *Paenibacillus.alvei* NRC14 for Cell Protection. Journal
373 of Applied Science, 1(8):35-47.
- 374 Adebayo-Tayo, B.C. and Adebami, G.E. (2014). Production and characterization of
375 bioflocculant produced by *Bacillusclausii* NB2. Innovative. Romanian. Food
376 Biotechnology, 14:13–25.
- 377 Ajayi, A.A., Peter-Albert, C.F., Ajojesu, T.P., Bishop, S.A., Olasehinde, G. and Siyanbola, T.O.
378 (2016). Biochemical Oxygen Demand and Carbonaceous Oxygen Demand of the
379 Covenant University Sewage Oxidation Pond. Covenant Journal of Physical and Life
380 Sciences (CJPL), 4(1):11-19.
- 381 Azmi, M.A., Norli, I., Farehah, Z.A., Ishak, S.A., Siti, M.N. and Norfariha, A.T. (2015). Crude
382 and Pure Bioflocculants Produced from *Bacillussubtilis* for Low Concentration of
383 Copper (Cu²⁺) removal. Iranica Journal of Energy and Environment, 6(2):103-110.
- 384 Bender, J., Rodriguez-Eaton, S., Ekanemesang U.M. and Phillips P. (1994). Characterization of
385 metal-binding bioflocculants produced by the cyanobacterial component of mixed
386 microbial mats. Applied Environmental Microbiology, 60:2311-2315.
- 387 Borralho, T., Chang, Y., Jain, P., Lalani, M. and Parghi, K. (2002). Lactose Induction of the lac
388 operon in *Escherichia coli* B23 and its effect on the o-nitrophenyl β-galactoside Assay.
389 Journal of Experimental Microbiology and Immunology, 2:117-123.
- 390 Boyd, W.L., and Lichstein, H.C. (1951). The Inhibitory Effect of Glucose on Certain Amino
391 Acid Deaminases. University of Minnesota. Journal of Bacteriology, 62 (6):711-715.
- 392 Bren, A., Park, J.O., Towbin, B.D., Dekel, E., Rabinowitz, J.D. and Alon, U. (2016). Glucose
393 becomes one of the worst carbon sources for *E.coli* on poor nitrogen sources due to
394 suboptimal levels of Camp. Scientific Reports, 6:1-10.

395 Brossi, M.J., Jiménez, D.J., Cortes-Tolalpa, L. and van Elsas, J.D. (2015) Soil derived microbial
396 consortia enriched with different plant biomass reveal distinct players acting in
397 lignocellulose degradation. *Microbial Ecology*, 71:616–627.

398 Choudhary, R., Rawtani, P. and Vishwakarma, M. (2011). Comparative study of Drinking Water
399 Quality Parameters of three Manmade Reservoirs i.e. Kolar, Kaliasote and Kerwa Dam.
400 *Current World Environment Journal*, 6(1);145-149.

401 Cortes-Tolalpa, L., Jiménez, D.J., Brossi, M.J., Salles, J.F. and van Elsas, J.D. (2016). Different
402 inocula produce distinctive microbial consortia with similar lignocellulose degradation
403 capacity. *Applied Microbiology and Biotechnology*, 100:7713–7725.

404 Cosa, S., and Okoh. A.I. (2014). Biofloculant production by a consortium of two bacterial
405 species and its potential application in industrial wastewater and river water treatment.
406 *Polish Journal of Environmental Studies*, 23:689–696.

407 Dalhoff, A. (1979). The "glucose effect" on growth inhibition of *Escherichia coli* by
408 streptomycin, trimethoprim and sulfamethoxazole, respectively. *FEMS Microbiology*
409 *Letters*. 6 123-127.

410 deKoker, T.H., Mozuch, M.D., Cullen, D., Gaskell J. and Kersten, P.J. (2004). Isolation and
411 purification of Pyranose 2-Oxidase from *Phanerochaete chrysosporium* and
412 characterization of gene structure and regulation. *Applied Microbiology and*
413 *Biotechnology*, 70(10), 5794–5800.

414 Didar, Z. and Ferdosi-Makan, A. (2016). Biofloculant production by different microbial species
415 and their potential application in dairy wastewater treatment. *Journal of Advanced*
416 *Environmental Health Research*, 4(1):18-24.

417 Dlangamandla, C., Dyantyi, S. A., Mpentshu, Y. P., Ntwampe, S. K. O. and Basitere, M.
418 (2016). Optimisation of biofloculant production by a biofilm forming microorganism
419 from poultry slaughterhouse wastewater for use in poultry wastewater treatment. *Water*
420 *Science & Technology*, 73(8):1963-1968.

421 Dong, S., Ren, N., Wang, A., Ma, F. And Zhou, D. (2008). Production of bioflocculant using the
 422 effluent from a hydrogen-producing bioreactor and its capacity of wastewater treatment.
 423 Journal of Water Resource and Protection, 1:1-65.

424 Gao, J., H. Bao, M. Xin, Y. Liu, Q. Li, and Y. Zhang. (2006). Characterization of a bioflocculant
 425 from a newly isolated *Vagococcus* sp. W31. Journal of Zhejiang University Science B.
 426 7:186–192.

427 Gong, W., S. Wang, X. Sun, X. Liu, Q. Yue, and B. Gao. (2008). Bioflocculant production by
 428 culture of *Serratia ficaria* and its application in wastewater treatment. Bioresource
 429 Technology, 99:4668–4674.

430 Gül, U.D. and Dönmez, G. (2013). Application of mixed fungal biomass for effective reactive
 431 dye removal from textile effluents. Desalination and Water Treatment, 51:3597-3603.

432 Guo, J., Du, J., Chen, P., Tan, X., Huang, X., Gan, P. and Fu, L. (2016). Enhanced efficiencies of
 433 sludge dewatering and domestic wastewater treatment by using the bioflocculant from
 434 rice stover. Water and Environment Journal, ISSN 1747-6585, 1-7.

435 Huang, M.S., Shen, R.H. and Xia, J. (2001). Microbial flocculates: Production and application
 436 on wastewater treatment. Journal of Shanghai University (Natural Science Edition), 7:
 437 244–248.

438 Husam S.A. and Nisreen H.O. (2013). Optimization of flocculation conditions of
 439 exopolysaccharide bioflocculant from *Azotobacter chroococcum* and its potential for river
 440 water treatment. Journal of Microbiology and Biotechnology Research, 3 (3):93-99.

441 Kao, L. S. and Green, C.E. (2008). Analysis of Variance: Is there a difference in means and what
 442 does it mean? Journal of. Surgical Research, 144(1):158–170.

443 Kim, C.H., Jung, B.O., Choi, K.S. and Kim, J.J. (1996). Synthesis of Water Soluble Chitosan
 444 Derivatives with Quaternary Ammonium Salt and Their Flocculating Behaviour,
 445 KongopHwahack, 7(1):118.

446 Kurane, R., Takeda, K. and Suzuki, T. 1986. Screening for and characteristics of microbial
 447 flocculants. Agric, Biology and Chemistry, 50: 2301-2307.

448 Kurane, R.; Hatamochi, K.; Kakuno, T.; Kiyohara, M.; Hirano, M.; Taniguchi, Y. (1994).
 449 Production of a bioflocculant by *Rhodococcus erythropolis* S-1 grown on alcohols.
 450 Bioscience Biotechnology and Biochemistry, 2:428–429.

451 Kwon, G.S., Moon, S.H., Hong, S.D., Lee, H.M., Kim, H.S., Oh, H.M. and Yoon, B.D. (1996).
 452 A novel flocculant biopolymer produced by *Pestalotiopsis* KCTC 8637P.
 453 Biotechnology Letter, 18:1459-1464.

454 Lee, S., R. John, F. Mei, and C. Chong. (2014). A review on application of flocculants in
 455 wastewater treatment. Process Safety and Environmental Protection, 92:489–508.

456 Lee, S.H., Lee, S.O., Jang, K.L. and Lee, T.H. (1995). Microbial flocculant from *Arcuadendron*
 457 *sp.* TS-49. Biotechnology Letters, 17: 95-100.

458 Lei, X., Chen, Y., Shao, Z., Chen, Z., Li, Y., Zhu, H., Zhang, J., Zheng, W. Zheng, T. (2015).
 459 Effective harvesting of the microalgae *Chlorella vulgaris* via flocculation-flotation with
 460 bioflocculant. Bioresource Technology, 198: 922-925.

461 Li, J., Yun, Y., Xing, L. and Song, L. (2017). Novel bioflocculant produced by salt-tolerant,
 462 alkaliphilic strain *Oceanobacillus polygoni* HG6 and its application in tannery wastewater
 463 treatment. Japan Society for Bioscience, Biotechnology, and Agrochemistry, 1-9.

464 Li, L., Ma, F., and Pan, T. (2015). Research development of compound bioflocculant.
 465 International Power, Electronics and Materials Engineering Conference (IPEMEC 2015).
 466 Atlantis Press. Pg 1002-1006.

467 Lian, B., Ye, C., Jin, Z., Teng, H.H., Zhu, L. and Yua, S. (2008). Microbial flocculation by
 468 *B. mucilaginosus*: applications and mechanisms. Bioresource Technology, 99:4825–4831.

469 Lin, J., and C. Harichund. (2012). Production and characterization of heavy-metal removing
 470 bacterial bioflocculants. African Journal of Biotechnology, 11:9619–9629.

471 Liu, C., Lu, J., Lu, L., Liu, Y., Wang, F. and Xiao, M. (2010). Isolation, structural
 472 characterization and immunological activity of an exopolysaccharide produced by
 473 *Bacillus licheniformis*. Bioresource Technology, 101:5528–5533.

474 Liu, W., He, R. and Liu, C. (2016). An alkali-tolerant strain of *Microbacterium esteraromaticum*
475 C26 produces a high yield of cation-independent bioflocculant. AIMS Environmental
476 Science, 3(3):408-419.

477 Liu, C., Hao, Y., Jiang, J. and Liu, W. (2017). Valorization of untreated rice bran towards
478 bioflocculant using a lignocellulose-degrading strain and its use in microalgal biomass
479 harvest. Biotechnology Biofuels, 10(90):1-12.

480 Luo, L., Zhao, Z., Huang, X., Du, X., Wang, C., Li, J., Wang, L. and Xu, Q. (2016). Isolation,
481 Identification, and Optimization of Culture Conditions of a Bioflocculant-Producing
482 Bacterium *Bacillus megaterium* SP1 and Its Application in Aquaculture Wastewater
483 Treatment. BioMed Research International Article ID 2758168, 1-9.

484 Maliehe, T.S., Selepe, N.T., Ntombela, G., Simonis, J., Basson, A.K., Ngema, S., Xaba P.S. and
485 Mpanza, F. (2016). Production and characteristics of bioflocculant TPT-1 from a
486 consortium of *B. pumilus* JX860616 and *Alcaligenes faecalis* HCB2. African Journal of
487 Microbiology Research, 10(37):1561-1575.

488 Mee, M.T. and Wang, H.H. (2012). Engineering ecosystems and synthetic ecologies. Molecular
489 BioSystem. 8(10):2470–2483.

490 Menetrez, M.Y. (2012). An Overview of Algae Biofuel Production and Potential Environmental
491 Impact. Environmental Science and Technology, 46(13):7073–7085.

492 Minty, J.J., Singer, M.E., Scholz, S.A., Bae, C., Ahn, J., Foster, C.E., Liao, J.C., and Lin, X.N
493 (2013). Design and characterization of synthetic fungal-bacterial consortia for direct
494 production of isobutanol from cellulosic biomass. Applied Biological Sciences,
495 Engineering, 110(36):14592–14597.

496 Ntsaluba, L., Nwodo, U.U., Mabinya, L.V. and Okoh. A.I. (2013). Studies on bioflocculant
497 production by a mixed culture of *Methylobacterium* sp. Obi and *Actinobacterium* sp.
498 Mayor. BMC Biotechnology, 13(62):1–7.

499 Nurul-Adela, B., Nasrin, A. and Loh, S. (2016). Palm oil mill effluent as a low-cost substrate for
500 bioflocculant production by *Bacillus marisflavi* NA8. Bioresource Bioprocess, 3(20): 1-8.

501 Nwodo, U. U., Green, E. and Okoh. A.I. (2012). Bacterial exopolysaccharides: functionality and
502 prospects. *International Journal of Molecular Sciences*, 13:14002–14015.

503 Ogunsade, O.O., Bakare, M.K. and Adewale, I.O. (2015). Purification and Characterization of
504 Biofloculant produced by *Bacillus amyloliquefaciens* ABL 19 Isolated from Adeti
505 Stream, Ilesa, Osun State, Nigeria. *Nature and Science*, 13(2):54-64.

506 Okaiyeto, K., Nwodo, U.U., Okoli, A.S. Mabinya, L.V. and Okoh, A.I. (2016a). Studies on
507 Biofloculant Production by *Bacillus* sp. AEMREG7. *Polish Journal of Environmental*
508 *Studies*, 25(1): 241-250.

509 Okaiyeto, K., Nwodo, U.U., Okoli, S.A., Mabinya, L.V. and Okoh, A.I. (2016b). Implications
510 for public health demands alternatives to inorganic and synthetic flocculants:
511 biofloculants as important candidates. *Microbiology Open*. 5(2): 177–211

512 Okaiyeto, K., Nwodo, U.U. Mabinya, L.V. and Okoh. A.I. (2013). Characterization of a
513 biofloculant produced by a consortium of *Halomonas* sp. Okoh and *Micrococcus* sp.
514 Leo. *International Journal of Environmental Research and Public Health*. 10:5097–5110.

515 Peng, L., Yang, C., Zeng, G., Wang, L., Dai, C., Long, Z., Liu, H. and Zhong, Y. (2014)
516 Characterization and application of biofloculant prepared by *Rhodococcus erythropolis*
517 using sludge and livestock wastewater as cheap culture media. *Applied Microbiology and*
518 *Biotechnology*, 98: 6847–6858.

519 Salehizadeh, H. and shojaosadati, S. (2001). Extracelullarbiopolymeric flocculants. Resent trends
520 and biotechnological importance. *Biotechnology Advances*, 19: 371-385.

521 Schmidt-Glenewinkel, H and Barkai, N. (2014). Loss of growth homeostasis by genetic
522 decoupling of cell division from biomass growth: implication for size control
523 mechanisms. *Molecular Systems Biology*, 10: 769.

524 Seo, H. (1993). Biofloculant production from *Bacillus* sp. A56.
525 *SanopMisaengmulHakhoechi*. 21:486-493.

526 Suryani, L.A., Artika, I.M. and Susanti, H.E. (2011). Characterization of Bioflocculant
 527 Producing-Bacteria Isolated from Tapioca Waste Water. HAYATI Journal of
 528 Biosciences, 18(4):193-196.

529 Tang, J., Suijian, Q., Zhigang, L., Qun, A., Mingquan, X. and Bo, Y. (2014). Production,
 530 purification and application of polysaccharide-based bioflocculant by
 531 *Paenibacillus mucilaginosus*. Carbohydrate Polymer, 113:463–470.

532 Ugbenyen, A.M., and A.I. Okoh. (2014). Characteristics of a bioflocculant produced by a
 533 consortium of *Cobetia* and *Bacillus* species and its application in the treatment of
 534 wastewaters. Water SA 40:139–144.

535 Vijayabaskar, P., Babinastarlin, S., Shankar, T., Sivakumar, T. and Anandapandian, K.T.K.
 536 (2011). Quantification and Characterization of Exopolysaccharides from *Bacillus subtilis*
 537 (MTCC 121). Advances in Biological Research, 5 (2): 71-76,

538 Wang, E.X., Ding, M.Z., Ma, Q., Dong, X.T. and Yuan, Y.J. (2016) Reorganization of a
 539 synthetic microbial consortium for one-step vitamin C fermentation. Microbial Cell
 540 Factories, 15(21):1-11.

541 Wang, J., Wen, B., Xu, Q., Xie, X. and Chen, N. (2015). Optimization of carbon source and
 542 glucose feeding strategy for improvement of L-isoleucine production by *Escherichia coli*.
 543 Biotechnology & Biotechnological Equipment, 29(2):374-380.

544 Xia, S.Q., Zhang, Z.Q., Wang, X.J., Yang, A., Chen, L., Zhao, J.F., Leonard, D. and Jaffrezic-
 545 Renault, N. (2008). Production and characterization of a bioflocculant by *Proteus*
 546 *mirabilis* TJ-1. Bioresource Technology, 99:6520-6527.

547 Yim, J.H., Kim, S.J., Ahn, S.H. and Lee, H.K. (2007). Characterization of a novel bioflocculant,
 548 p-KG03, from a marine dinoflagellate *Gyrodinium impudicum* KG03. Bioresource
 549 Technology, 98:361-367.

550 Yokoi, H., Yoshida, T., Mori, S., Hirose, J. Hayashi, S. and Takasaki, Y. (1997). Biopolymer
 551 flocculant produced by an *Enterobacter* sp. Biotechnology Letters, 19:569-573.

552 Zaki, S., Farag, S., Abu, G.E., Elkady, M., Nosier, M. and Abd El Haleem. (2011).
553 Characterisation of bioflocculants produced by bacteria isolated from crude petroleum
554 oil. *International Journal of Environmental Science and Technology*, 8(4):831-840.

555 Zhang, F., Jiang, W.J. and Wang, X.D. (2008). Study on microbial flocculant produced by mixed
556 strains via distillery wastewater. *China Water and Wastewater*, 24(11):93–96.

557 Zhang, C., Cui, Y. and Wang, Y. (2012). Bioflocculant produced from bacteria for
558 decolorization, Cr removal and swine wastewater application. *Sustainable Environmental*
559 *Research*, 22(2):129-134.

560 Zhang, F., Jiang, W., Wang, X., Ji, X., Wang, Y., Zhang, W. and Chen, J. (2015). Culture
561 Condition Effect on Bioflocculant Production and Actual Wastewater Treatment
562 Application by Different Types of Bioflocculants. *Biodegradation and Bioremediation of*
563 *Polluted Systems - New Advances and Technologies*, 19-29.

564 Zhang, T., Lin, Z. and Zhu. H. T. (1999). Microbial flocculant and its application in environment
565 protection. *Journal of Environmental Sciences*, 11:1–2.

566 Zhang, Z., Xia, S., Zhaoa, J. and Zhang, J. (2010). Characterization and flocculation mechanism
567 of high efficiency microbial flocculant TJ-F1 from *Proteus mirabilis*. *Colloid and*
568 *Surfaces B:Biointerface*, 75,247-251.

569 Zhao, G., Ji, S., Sun, T., Ma, F. and Chen, Z. (2017). Production of bioflocculants prepared from
570 wastewater supernatant of anaerobic co-digester of corn straw and molasses wastewater
571 treatment. *BioResources*, 12(1):1991-2003

572 Zheng, Y., Ye, Z., Fang, X., Li, Y. and Cia, W. (2008). Production and characteristics of a
573 bioflocculant produced by *B. sp.* F19. *Bioresource Technology*, 99:7686–7691.

574 Zhou, X., Wang, J., Zhou, J. and Zhang, J. (2003). Studies on properties of a bioflocculant
575 produced by *Pseudomonas sp.* GX4-1 in fish meal wastewater. *Research of*
576 *Environmental Sciences*, 16:31-34.

577 Zuroff, T.R. and Curtis, W.R. (2012). Developing symbiotic consortia for lignocellulosic biofuel
578 production. *Applied Microbiology and Biotechnology*, 93(4):1423–1435.