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Screening and characterization of bioflocculant-producing bacteria isolated from domestic waste water in Bowen University, Iwo, Osun State, Nigeria.

Akinola, O.[1,2] and Adebayo, E. A.[1*]

[1]Department of Pure and Applied Biology, Ladoke Akintola University of Technology, P.M.B. 4000, Ogbomoso, Nigeria.
[2]Department of Biological Sciences, Bowen University, Iwo, Osun State.

Abstract

Bioflocculants are biodegradable polymers produced by microorganisms. Bioflocculant has some advantages over synthetic flocculant. They are safe, harmlessness to humans and environment with strong effect. However, its production and application is still at minimal level. This study aimed at screening for bioflocculant-producing bacteria isolated from eatery waste water in Bowen University, Iwo, Osun State.The flocculating activity of the isolated organism was investigated using 5g/L kaolin suspension to measure the flocculating activity at optical density (OD) of 550nm. A total of twenty-eight (28) bacteria strains were isolated from four (4) different domestic waste water samples and their bioflocculant activities were determined. Five isolates with high percentage flocculating activity were obtained as follow; Y4 (67%), P3 (61%), B8 (60%), K8 (55%)and K4 (52%). The isolates were characterized using morphological and molecular methods and they were identified as *Bacillus licheniformis* (B8, K4 and K8), *Bacillus thuringensis* (Y4) and *Bacillus cereus* (P3). The 16S rRNA sequence analysis revealed the isolates as related to the genus *Bacillus* and the nucleotide sequence were deposited in GenBank as *Bacillus* species with the accession numbers KY352342 (B8), KY352343 (P3), and KY352344 (Y4). Accession numbers for isolates K4 and K8 were not determined.

Keywords: Waste water, Bioflocculant production, Bioflocculating activity, Bacteria, *Bacillus* species.

*Corresponding author:eaadebayo@lautechedu.ng

Introduction

Flocculation is an effective and convenient method of removing suspended solids, colloids and cell debris while flocculants are the useful agent in the aggregation of colloids, cells, suspended solids. Theseare commonly used for drinking water production, waste water treatment, fermentation process and food production(Shih et al.,2001).Flocculants are substances having a synthetic or natural origin and used assedimentation aids to bring about the solid-liquid separations by the processof flocculation in industrial plants. Flocculants comprises of inorganic (Polyaluminium chloride, Aluminum sulfate), organic (polyacrylamide, polyethylene amine) and natural bioflocculants (gelatin, chitosan, sodium alginate and microbial flocculants) (Zhi-qianget al., 2007). Among the different types of flocculants in use, until recently, the chemical or synthetic flocculants has had a higher popularity due to its effective flocculating activity, low cost and versatile tailor ability (Saliehizadeh et al., 2002). However, chemical flocculants can cause health and environmental problems because they are carcinogenic, contain neurotoxic monomers and are non-degradable in nature (Suhet al., 1997; Yokoi et al., 1997).This has led to the search for alternatives. Studies on bioflocculant usage in

waste water treatment have been widely reported for their safety, eco-friendly and biodegradability nature.

Bioflocculants are natural product metabolites producedby a wide variety of microorganisms including bacteria, fungi, yeast and algae (Kuraneet al.,1986; Salehizadeh&Shojaosadati,2000)which produce extracellular polymeric substances such as polysaccharides, proteins, lipids, glycolipids and glycoproteins that function as bioflocculant (Kurane & Matsuyama, 1994).Polymeric nature and types of flocculants determine its activity. Bioflocculants are always producing during the growth of microorganisms (Lu et al., 2005; Gaoet al., 2009). Bioflocculants have widely been used to treat starch wastewaters (Deng et al., 2003), river water, brewery wastes, soy sauce brewingand meat processing wastewaters. Other uses includes treating effluents frompulp and paper mills (Gong et al., 2008), and purifyingdrinking waters at low temperature (Li et al., 2009). Bioflocculant has been reported as effective agent inremoving suspended solids, heavy metals, bacteria and in reducing the turbidity of different types of industrial wastewater effluents (Kurane et al., 1994). Bioflocculants can be produced relatively inexpensively from a variety of microorganisms such as bacteria, fungal, alga and actinomycetes (Suhet al., 1997).

There has been much research on isolation of bioflocculant-producing bacteria from different sources such as activated sludge at a wastewater treatment plant(Buthelezi et al., 2010), bottom sediment of Algoa Bay (Anthony et al., 2011), activated sludge from local hoggery (Zhang et al.,2012). However, no record is available on isolation of bioflocculant-producing bacteria from domestic waste water from cafeteria. This study therefore aimed at isolation and screening of flocculating-producing bacteria speciesfrom domestic waste water effluents from fourdifferent cafeterias in Bowen University, Iwo,Osun State, using phenotypic, morphological and molecular characterization.

Materials and Methods

Sample collection

Samples were collected aseptically from wastewater effluents atfour different eateriessituated in Bowen University, Iwo, Osun State. Each sample was collected in a sterile conical flask, capped, coded and transported to the laboratory in dark polythene bag for further analysis.

Isolation of organism

Isolation of the micro-organism was carried out using serial dilution technique, as described by (Arora &Arora, 2007). With a sterile pipette, 9ml of water was measured into test tube and the mouth was plugged with cotton wool after which it was sterilized in an autoclave at 121°C for 15 minutes. Serial dilution was carried out in an aseptic condition and 1ml of the diluents was pipetted into a sterile petri plate from dilution 10^{-4} and 10^{-6} respectively. Having done that, moltenTryptone soya agar and nutrient agar were aseptically poured,gently swirled, allowed to setand incubated at 37°C for 24 h.Distinct colonies were selected randomly according to their different morphological characteristics and further purified by repeated streaking on solid nutrient agar and incubated at 37°C in an inverted position for 24 h.

Screening of isolated bacteria for bioflocculant production

The isolates were screened for bioflocculant production, using Bioflocculant Production Broth (BPB). The BPB composition included 10g Glucose, 2 g KH_2PO_4, and 0.2g $MgSO_4$. $7H_2O$,0.1NaCl, 0.5 $CaCO_3$and 0.5g yeast extract (Kurane & Matsuyama, 1994). The mixture was dissolved in 1litter de-ionised water with initial pH adjusted to 7.0, using 1N NaOH and the medium sterilized in an autoclave.The isolates used were firstly inoculated into peptone water after sterilization and incubated at 37°C for 24 h. From the broth culture 1ml was introduced into 25 ml of the BPB and incubated on a rotary shaker at 120rpm at 37°C for 72 h. The fermentation broth was centrifuged at 4000rpm for 30 minutes, and the supernatants were collected and examined for flocculating activity.

Measurement of flocculating activity

The flocculating activity was measured, using Kaolin Clay suspension according to the method of Kuraneand Matsuyama (1994) as modified by Gaoet al.(2006).

The stock solution used contained 5g/L of Kaolin clay suspension at pH 7 in distilled water. A volume of 9ml of kaolin clay suspension was measured into test tube 0.1ml culture supernatant and 0.25ml of 1% $CaCl_2$were mixed together in the test tube.A control tube was set up by replacing culture supernatantwith water and carried out following the same condition. Final volume was made up to 10ml for all mixtures. The mixture was vigorously shaken and allowed to settle for 5 s at 37°C, all experiments wereperformed in triplicates. The optical density (OD) of the clarifying upper phase solution was measured at 550nm with a UV Spectrophotometer and the flocculating activity was determined as follows;

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

A is the optical density of the sample experiment at 550 nm, while B is the optical density of the control experiment at 550 nm. After the determination of flocculating activity of the isolates, five isolates which produced the highest flocculating activity were selected and used for further studies.

Molecular Characterization of the isolates

Isolation of Genomic DNA, PCR amplification and 16S rRNA Sequencing

DNA was extracted from 1 ml of bacterial culture. The culture was pelleted by centrifuging at 12,000 rpm for 5 minute. Pellets were then treated with lysis buffer and protease enzyme and incubated at 65υ for 1 hr. Nucleic acids were precipitated with isopropanol by centrifuging at 10,000 rpmfor 10 minute, washed with 1 ml of 70% ethanol solution and dissolved in 0.1 ml of TE buffer. The purity and quantity of DNA were examined by recording its UV absorption spectrum and running 1.5% agarose gel electrophoresis. The DNA isolated was amplified using 16S rRNA universal ribose primers and sequence for the identification.The universal primers AGAGTTTGATCMTGGCTCAG (Forward primer 27F)and AAGGAGGTGWTCCARCCGCA (Reverse primer 1525R) were used for the amplification and sequence reaction was purified. The product from the purification was loaded on the 3131xl gene analyzer from Applied Bio-systems to give the sequences.Five isolates that produced higher percentage of bioflocculant were characterized using 16S rRNA gene sequencing in BigDye cycle Terminating sequencing kit method and the product was analyzed with BioEdit sequence Alignment editor. The genes obtained in this study were compared with known 16S rRNA gene sequences in the National Centre for Biotechnology Information (NCBI) GenBank database.

Results

For the screening of novel flocculant producing bacteria from this study, out of the twenty-eight bioflocculant-producing bacteria initially isolated from four different cafeterias in Bowen University, only five isolates with high flocculating activity were selected. Table 1 shows the percentage mean result of the five isolates with highest flocculating activity ranged from 52% to 67%. The isolateY_4(Y352344) has highest value of 67% followed by $P_3$with 61% (KY352343), $B_{8,}$ 60% (KY352342), while K_8 and K_4 have the least values of 55% and 52% respectively and their accession numbers were not determined.Sequence data were analyzed in the NCBI database by using BLAST (Basic Local Alignment Search) program available on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The nucleotide sequence of 16SrRNA was deposited in GenBank database as *Bacillus* species with the following accession number KY352342, KY352343, and KY352344.Amplification of the 16SrRNA gene of the bacterium resulted in polymerase chain Reaction (PCR) at 700 bp on 1 Kb DNA marker as shown in Figure 1. The biochemical characterizations of the bacteria isolates with good flocculating activity were shown in Table 2.

The strains isolated in this study are indicated by solid triangles. Statistical significance of the tree topology was tested by 1000 bootstrap replication. Bootstrap values are displayed at the nodes. The relatedness of the strains at the genetic level was revealed by 16S rRNA gene sequenced analysis. *B. licheniformis* (NGIWBAC1- KY352342) and *B. thuringensis* (NGIWBAC4-KY352344) were located at the 9thsubcluster which has 93% similarity to *B. anthracis* (GenBank accession no. KF475846)

with 18 bootstrap, while *B. cereus*(NGIWBAC2-KY352343) was located at cluster one with 20 bootstrap value to the *B. amyloliquefaciens* (GenBank accession no. KY362201) (Fig. 2). The closeness in bootstrap values and the location of the strains at cluster one and 9th subcluster on the phylogenetic tree suggested that the organisms have the same ancestral origin.

Table 1: Names and Accession numbers of high flocculating bacteria

Code	Names of organism	Mean (%) of Flocculation activity	Accession Number
B_8	B.licheniformis	60	KY352342
P_3	B. cereus	61	KY352343
K_4	B.licheniformis	52	Not Determined
K_8	B. licheniformis	55	Not Determined
Y_4	B. thuringensis	67	KY352344

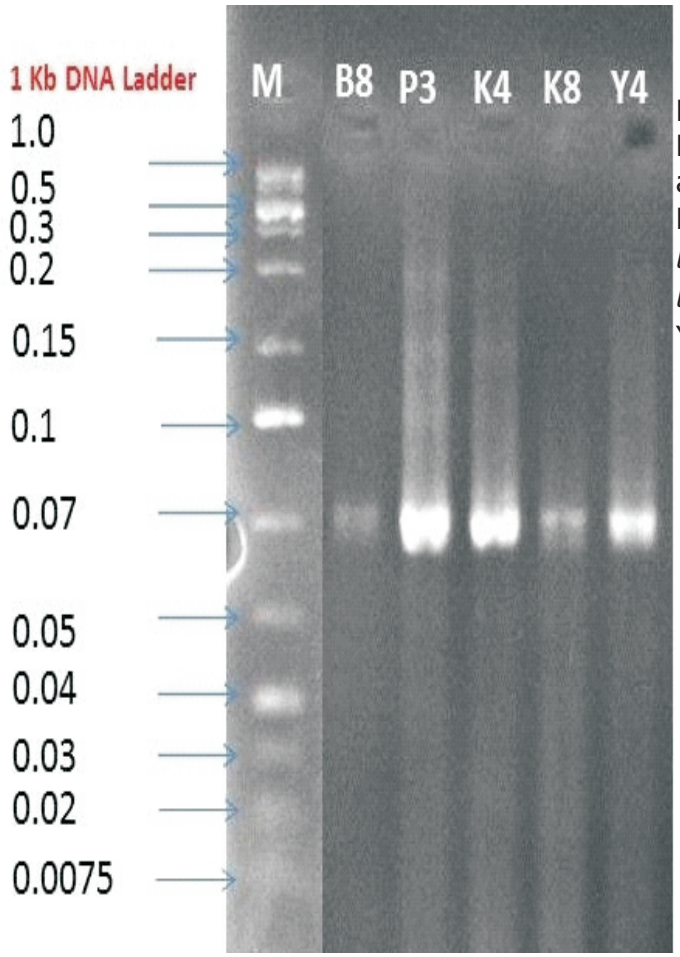
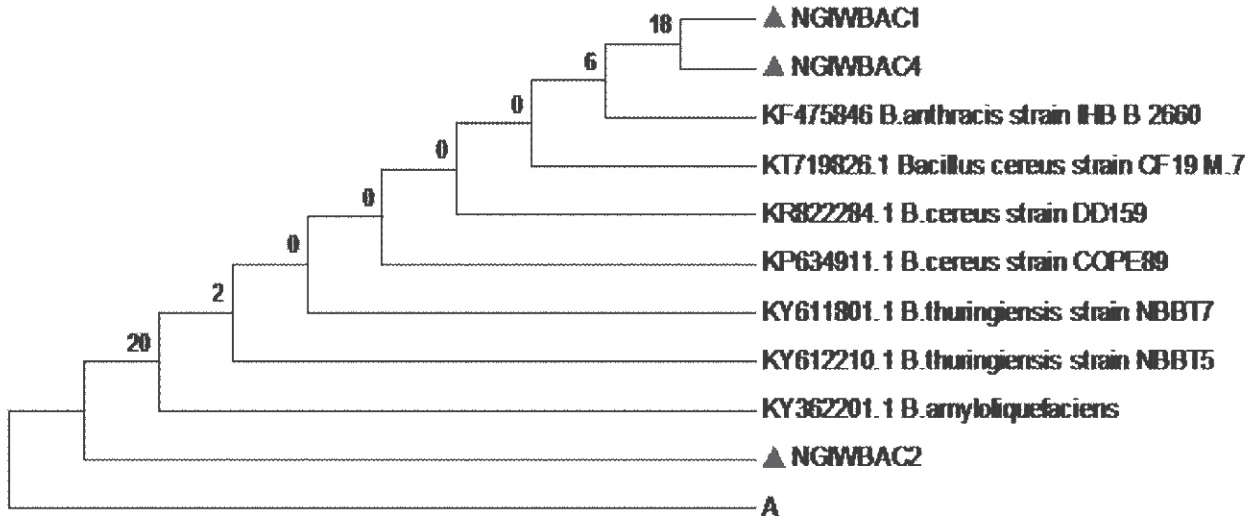


Figure 1: 16S rRNAfingerprint of Bacillus species amplified on 1.5% agarose gelLane M: Marker. (1KB DNA Marker; GeneRuler); B8: *B. licheniformis*; P3: *B. cereus*; K4: *B. licheniformis*; K8: *B. licheniformis*; Y4: *B. thuringensis*

Table 2: Biochemical characterization of the isolate with high flocculating activity

Isolate code	Gram's Reaction	Shape	Catalase	Indole	Citrate	Starch	MR	VP	Glu	Man	Lac	Suc	Probable Organism
B_8	+	Rod	+	-	+	+	+	+	+	+	+	+	*B.licheniformis*
P_3	+	Rod	+	-	+	-	+	+	+	+	+	+	*B. cereus*
K_4	+	Rod	+	-	+	+	+	+	+	+	+	+	*B.licheniformis*
K_8	+	Rod	+	-	+	+	+	+	+	+	+	+	*B.licheniformis*
Y_4	+	Rod	+	-	+	-	+	-	+	-	+	+	*B.thuringensis*

KEY: + = Positive/hydrolysed, - = Negative/unhydrolysed, Glu = Glucose, Man = Mannitol, Lac = Lactose, Suc = Sucrose, MR = Methyl Red, VP = VogesProskauer



NGIWBAC4 (KY352344) *B. thuringensis*

Discussions

The characterized organisms fall within the genus '*bacilli*' the probable organisms were *Bacillus licheniformis, Bacillus thurigenesis* and *Bacillus cereus*. Salehizadeh & Shojaosadati (2001) previously reported that *Bacillus* sp significantly secrete biopolymer flocculant during growth. The genus *Bacillusare* generally believed to be industrially important species and with more emphasis on safe use in food industry. In the recent times, cyclic peptides and bacteriocins produced from *Bacillus* sp. have been used in bio-preservation of food materials (Nath et al., 2015). This study observed the highest flocculating activity of 67% with *Bacillus* sp (KY352344) obtained from isolate Y_4 and this is in line with the work of Bajlanet et al. (2013). He reported bacterial isolates with flocculating activity of 62.7% from domestic waste water. However, the flocculating values produced by organisms obtained in this study may be rated as low when compared with the values reported by some other authors from other waste water sites. Simphiweet al. (2010) have observed flocculating activity of 89% from waste water treatment plant in South Africa, while Piyoet al. (2011) reported organisms which produced at 72% from sediment of Alga bay. The differences obtained in the level of bioflocculants production in this study from previous works may be as result of waste water sources, waste water compositions and environmental factors.

Previous studies have indicated that bacteria utilized the nutrient in the culture medium to produce high molecular weight polymers within the cell catalyzed by the specific enzymes and excreted extracellularlly into the medium. The bacteria converted the simple substances found in their surrounding into complex polymers which were later used as flocculant. Glucose which was the major carbon source was used in the media preparation. The role of carbon source is significant especially in cell growth and extracellular polysaccharide production. It was observed from the study that the media used (Bioflocculant Production Broth) supported the flocculating ability (67%, 61% & 60%) of the isolates. The isolates grew and produced a reasonable flocculants with the glucose in the medium. This result agreed with the report of Cosaet al. (2011) who reported that using glucose as carbon source yielded bioflocculant with the highest flocculating activity (70%). Glucose had been vastly documented as fine substrate for bioflocculant production (Suhet al., 1997). Cosaet al. (2011) reported that *Virgibacillus* species rod produced bioflocculant optimally using glucose as the sole carbon source, the result also confirmed that bacteria prefer organic carbon sources for bioflocculant production.

The electric charge of the cells and the oxidation-reduction potential may be determined by the initial pH culture medium and eventually affect absorption of nutrients and enzymatic reaction of growing cell. Hence, the alteration in pH medium composition may affect tbioflocculant activities. As illustrated in the obtained results, it is evident that the acidic conditions supported bioflocculant production. The current study differs from Cosa et al. (2011) work, who reported alkaline pH 10 to be optimum. Although the electric charge of the cells and the oxidation-reduction potential may be determined by the initial pH culture medium. The absorption of nutrients and enzymatic reaction of growing cellin one organism may differ from another and can be affected by the pH value of the culture medium (Salehizadeh&Shojaosadati, 2001). Mabinya et al. (2011) reported that *Halomonas* sp. OKOH optimally produced bioflocculantat pH 7. Kuraneet al. (1994) documented that pH between(8.0-9.5) favours the production of bioflocculant by *Rhodococcuserythropolis*. In the case of the present study, the pH for flocculating activity observed in this study decline and ranged from 3.4 – 5.6, which might have been due to the production of organic acids from glucose. This is in agreement with the reports of Salehizadehet al., (2000) and Piyoet al. (2011), which have observed optimal production of bioflocculants at pH values of 3.7 and 3.0 respectively.All the strains analyzed using 16S rRNA were homogeneous, producing the same band pattern with amplicon lengths around 700bp on 1Kb DNA ladder. Senesi et al. (2000) identified different species of *Bacillus* that produced the same band with amplicon length, ranging from 800 to 150 bp, while Rajashekhar et al. (2017) reported an amplicon length of about 1500 bp and Singh et al. (2016) documented an amplicon length of 1271 bp of different species of *Bacillus*. It is very clear from the available literature that amplicon length obtained by using 16S rRNA for *Bacillus* species identification were varied from one species to the other. BLAST (Basic Local Alignment Search Tool) analyses of the nucleotide sequence revealed the entire organism to be *Bacillus* sp and the nucleotide sequence were deposited in GenBank with accession numberKY352344 for isolate Y_4while isolate P_3 and B_8 have accession number KY352343 and KY352342. The accession number for isolate K_4 and K_8 were not determined.

The relatedness and similarity of isolated flocculating producing bacteria *B. licheniformis* (NGIWBAC1- KY352342) and *B. thuringensis* (NGIWBAC4-KY352344)with *B. anthracis* (GenBank accession no. KF475846) and *B. cereus* (NGIWBAC2- KY352343) to *B. amyloliquefaciens* (GenBank accession no. KY362201) has established the identity of the bacteria. The 16S rRNA analysis of isolated strains showed significant homology with known *Bacillus* species in the GenBank database, hence the isolated strains are new species belonging to genus *Bacillus*, and this was also established by biochemical tests.

Conclusion

The current study revealed the ability of isolated bacteria to convert the simple substances found in their surrounding into complex polymers which were later used as flocculant. These bioflocculants producing bacterial strains have satisfactorily produced flocculants at pH of 7 and in the presence of glucose as carbon source. The bioflocculating producing bacteria were identified as *Bacillus* species, and they were characterized as *B. licheniformis* (KY352342), *B. thuringensis* (KY352344) and *B. cereus* (KY352343) with accession numbers deposited in Genbank.

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