1 **Original Research Article**
2

3 Production, Characterization and Effect of Cultural Condition on Bioflocculant Produced 4 by *Alcaligenes aquatilis* AP4

5

6 ABSTRACT

7 Bioflocculants are polymeric substance produced by microorganisms as a secondary metabolite 8 which can be used in the treatment of wastewater. A novel bioflocculant producing bacterium 9 was isolated from Palm-oil mill effluent and identified as *Alcaligenes aquatilis* AP4 using 16S 10 rRNA gene sequencing. Production, characterization and the effect of culture conditions such as 11 carbon source, cations, incubation temperature, initial pH, static/agitation incubation and speed 12 on bioflocculant produced by AP4 were investigated. Glucose supported highest production of 13 bioflocculant by the isolate at 72 hours of incubation. 30°C and pH 9.0 induced the highest 14 bioflocculant production. Agitation condition had a significant effect ($P \leq 0.05$) while shaking 15 speed of 140rpm induced maximum production of bioflocculant. Fourier Transform Infrared 16 (FT-IR) spectrophotometer analysis shows the presence of OH, NH_2, $CONH_2$ CO^- and COO^- as 17 functional groups and Chemical analyses of the purified bioflocculant revealed it to be a 18 glycoprotein.

19

20 **Keywords**: Bioflocculant, *Alcaligenes aquatilis*, flocculating activity, palm-oil effluent, 21 glycoprotein

22

23 INTRODUCTION

24 Flocculation refers to the process by which destabilized particles conglomerate into larger 25 aggregates so that they can be separated from the wastewater (Droste, 1997). In general, 26 flocculants are classified into three groups: inorganic flocculants, such as aluminum sulfate and 27 polyaluminum chloride (Lee *et al*., 2014); organic synthetic flocculants, such as polyacrylamide 28 derivatives and polyethylene amine; and naturally occurring flocculants, such as chitosan,

29 gelatin, guar gum, sodium alginate (Okaiyeto *et al*., 2016). Both organic and inorganic
30 flocculants derivatives are frequently used in both wastewater treatment and the fermentation
31 industries because they have strong flocculating activity and are also cost-effective (He *et al*.,
32 2002; Suopajarvi *et al*., 2013). However, studies have shown that some of the chemically
33 synthetic flocculating substances are not only harmful to both humans and the environment, but
34 are also non-degradable in nature (Taniguchi *et al*., 2005).

35 A bioflocculant on the other hand, is a kind of biodegradable polymeric flocculants produced by
36 many microorganisms during their growth (Gao *et al.,* 2006; Defang *et al*., 2008; Mabinya *et al*.,
37 2012). Compared with conventional synthetic organic flocculants, bioflocculant have special
38 advantages such as safety for ecosystems, potential flocculating effects, biodegradability and
39 harmlessness to humans and the environment, and as a consequence may potentially be applied
40 in drinking and wastewater treatment, downstream processing, food, pharmaceutical and
41 fermentation processing (Seo, 1993; Zhang *et al*., 1999; Salehizadeh and Shojaosadati, 2001;
42 Ntsaluba *et al*., 2013; Ozcan and Oner, 2015).

43 Moreover, most of high molecular weight flocculants are recalcitrant. It is evident that the
44 acrylamide monomer is not only neurotoxic and carcinogenic but also non-biodegradable in
45 nature. They have detrimental effect both on flora and fauna (Vanhoric *et al*., 1983; Dearfield *et*
46 *al*., 1988). Aluminium is one of the component of Aluminium sulphate mostly used in the
47 treatment of raw water for household use, has been shown to cause Alzheimer's disease (Green
48 *et al*., 2002; Kivipelto *et al*., 2005; Agunbiade *et al*., 2017).

49 Many microorganisms, including bacteria, fungi and actinomycetes, have been reported to
50 produce extracellular polymeric substances, such as polysaccharides, functional proteins and
51 glycoproteins, which function as bioflocculant (Gao *et al*., 2006; Okaiyeto *et al*., 2016). Studies
52 carried out on the chemical composition of bioflocculant produced by *Bacillus subtilis* IFO3335
53 (Yokoi *et al*., 1996), *Bacillus* sp. I-471 (Kumar, *et al*., 2004), *Bacillus subtilis* DYU1 (Wu and
54 Ye, 2007), *Halomonas* sp. V3a (He *et al*., 2010), *Paenibacillus elgii* B69 (Li *et al*., 2013) and
55 *Paenibacillus mucilaginosus* (Tang *et al*., 2014), have shown them to all be polysaccharides.
56 *Rhodoccocus erythropolis* (Takeda *et al.,* 1991) and *Nocardia amarae* YK-1 (Koizumi *et al*.,
57 1991) produce protein flocculants while *Arcuadendron* sp. TS-4 (Lee *et al*., 1995), *Klebsiella*
58 *pneumonia* (Luo *et al*., 2014), *Bacillus clausii* NB2 (Adebayo-tayo and Adebami, 2014) and

59 *Pseudomonas aeruginosa* IASST201 (Pathak *et al*., 2017) have been reported to produce
60 glycoprotein bioflocculants.

61 The genus Alcaligenes used for this study was first classified in 1919 and since then, it has
62 undergone several changes; (Castellani and Chalmers, 1919; Van-Trappen *et al.,* 2005). From the
63 literatures, Van-Trappen *et al.* (2005) was regarded as the first to isolate and characterize
64 *Alcaligenes aquatilis* from the sediments of the Weser Estuary, Germany, and also from a salt
65 marsh on Shem Creek in Charleston Harbor, USA (Van-Trappen *et al*., 2005). *A. aquatilis* is
66 basically gram negative rod, catalase positive, oxidase positive, non-nitrate reducing, alpha
67 hemolytic, citrate positive, obligate aerobe, motile with peritrichous flagella as its peculiar
68 biochemical characteristics and it can be found in our environments such soil and water bodies
69 (Castellani and Chalmers, 1919; Bergey *et al*., 1934; Van-Trappen *et al*., 2005).

70 This research aimed at production and characterization of bioflocculant produced by *A. aquatilis*
71 AP4 which is isolated from Palm-oil mill effluent in the south-western part of Nigeria and to also
72 determine the effect of cultural conditions on the bioflocculant production.

73 **MATERIAL AND METHOD**

74 **Culture preparation**

75 Bioflocculant producing *A. aquatilis* AP4 culture isolated from palm oil mill effluent was
76 collected from the culture collection of our previous work in the Department of Microbiology
77 University of Ibadan, Ibadan Nigeria. The stock cultures were maintained on Nutrient agar,
78 incubated at 30°C for 72hrs and stored at 4°C.

79 The seed culture was grown in a 250ml flask containing: Nutrient broth- 10.0g; Potato dextrose
80 broth, - 5.0g; Glycerol - 3ml; Yeast Extract - 6.5g; Sodium chloride - 1.0g in 1litre of distilled
81 water. The pH of the medium was adjusted to 7.0 and the medium was autoclaved and inoculated
82 with pure culture from the stock culture and incubated for 24hrs.

83

84 **16SrDNA Sequence determination and Phylogenetic Analysis of the Bioflocculant-**
85 **Producing *A. aquatilis* AP4**

86 *A. aquatilis* AP4 isolated from Palm oil mill effluent was identified using molecular technique
87 based on the 16SrRNA gene amplification by polymerase chain reaction (PCR) followed by
88 sequencing of the amplified gene as designed according to Gupta *et al*. (1987) and Xiong *et al*.
89 (2010).

90 The isolate was incubated in 250-ml flasks containing 50 µl fresh LB medium for 16 h at 37°C
91 with shaking at 120 rpm. The genomic DNA of the strain was then extracted using CTAB
92 method of DNA extraction from microbes. PCR amplification was carried out to determine the
93 partial 16S rRNA gene. The PCR program was 30 cycles of 94°C (1 min), 55°C (30 s), and 72°C
94 (1.5 min). The PCR universal primers were 5-CCAGCAGCCGCGGTAATACG-3 (forward) and
95 5-TACCAGGGTATCTAATCC-3 (reverse). Purification of the PCR products and the
96 determination of sequences were performed by Macrogen USA (9700 Great Seneca Highway,
97 Rockville, MD 20850, USA). The 16S rRNA gene sequence of strain AP4 obtained was
98 compared with the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

99

100 **Bioflocculant production by *A. aquatilis* AP4**
101 The isolates were used for bioflocculant production using Bioflocculant Production Broth
102 medium (BPB). The BPB composition include: 10g glucose, 2g KH_2PO_4, 0.2g $MgSO_4.7H_2O$,
103 0.1NaCl, 0.5g $CaCO_3$, and 0.5g yeast extract. The mixture was dissolved in 1 liter deionized
104 water with the initial pH adjusted to 7.0. The medium was sterilized, inoculated with pure
105 culture of the isolate and incubated on a rotary shaker at 120 rpm and 37°C for 3 days. Kaolin
106 suspensions at a concentration of 5,000mg/l were used to evaluate the flocculating capability of a
107 series of the culture broths (Zheng *et al*., 2008).
108

109 **Determination of Flocculating Activity**

110 The flocculating activity was determined according to the method of Kurane *et al*. (1986) as
111 modified by Gao *et al*. (2006). A suspension of kaolin clay was used as test material for
112 flocculating activity determination. The kaolin clay was suspended in distilled water at a
113 concentration of 5 g/L at pH 7.0 and used as a stock solution for the subsequent assays. The

114 following solutions were mixed in a test tube: kaolin clay suspension (9 mL), culture supernatant
115 (0.1 mL) and 1% $CaCl_2$ (0.25 mL). A reference tube in which the culture supernatant was
116 replaced with distilled water was also included and measured under the same conditions. The
117 final volume of all mixtures was made up to 10mL with distilled water. After mixing gently, the
118 solutions were allowed to settle for 5 min. at room temperature. The optical density (OD) of the
119 clarifying upper phase solution was measured at 550nm with a UV spectrophotometer and the
120 flocculating activity determined as follows:

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

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

123

124 **Effects of carbon source on Bioflocculant Production by *A. aquatilis* AP4**

125 Effect of carbon sources such as economic wastewaters: Palm-oil effluent, Abbatoir effluent,
126 Brewery effluent and Sewage and glucose on bioflocculant production by the isolate was
127 investigated. Kaolin assay was carried out to check the maximum flocculating activity of the
128 isolate after which the absorbance was then read using a spectrophotometer at wavelength of
129 550nm.

130 **Effects of pH, incubation temperature and static/ agitation on Bioflocculant Production by**
131 ***A. aquatilis* AP4**

132 Effect of pH on flocculating activity of bioflocculant produced by *A. aquatilis* AP4 was
133 determined. The initial pH of the culture media was varied between the pH range of 3–12 by
134 adjusting with either 0.1N HCl or 0.1N NaOH (Yim *et al.,* 2006). The medium was sterilized,
135 inoculated and incubated on a rotary shaker at 120 rpm and 37°C for 3 days. Kaolin assay was
136 carried out to check the maximum flocculating activity of the isolate.

137 The effect of incubation temperature on bioflocculant production by *A. aquatilis* AP4 was carried
138 out by varying the incubation temperature between 25 – 45°C. The medium was sterilized,
139 inoculated and incubated on a rotary shaker at 120 rpm and 37°C for 3 days. Kaolin assay was
140 carried out to check the maximum flocculating activity of the isolate.

141 Effect of incubation under static and agitation conditions were investigated. After sterilization
142 and inoculation of the culture medium, some were incubated under static condition while others
143 were incubated under agitation. The speed of agitation was also varied between 80 – 160 rpm.
144 Kaolin assay was carried out to check the maximum flocculating activity of the isolate after
145 which the absorbance was then read using a spectrophotometer at wavelength of 550nm.

146 **Characterization of the bioflocculant produced by *A. aquatilis* AP4**

147 **Extraction and Purification of the Bioflocculant**

148 The purification and characterization of the bioflocculant was performed using the method
149 described by Chang *et al*. (1998) and Chen *et al*. (2002). Fermentation culture was prepared
150 based on the optimal culture conditions determined earlier. After three days of cultivation, the
151 culture was centrifuged at 4,600 rpm for 30 min and at 4^{0}C to remove cells. One volume of
152 distilled water was added to the supernatant and centrifuged again for 15 min to remove
153 insoluble solutes. Two volumes of cold ethanol were added to the supernatant, and the solution
154 was mixed and left standing at 4ºC for 12 hr. The resultant precipitate was vacuum dried to
155 obtain the crude bioflocculant. The crude product was weighed and dissolved in a small volume
156 of distilled water and one volume of mixture of chloroform and *n*-butyl alcohol (5:2 v/v) was
157 added. After mixing, the mixture was left at room temperature for 12 hr. The upper phase was
158 centrifuged at 3,000 x g for 15 min and the supernatant was dialyzed against distilled water.
159 Thereafter, the dialyzate was vacuum dried to obtain a pure bioflocculant.

160 **Chemical Analyses of the purified Bioflocculants**

161 Total sugar content of the purified bioflocculant was determined by the phenol-sulphuric acid
162 method using glucose as the standard solution as described by Chaplin and Kennedy (1986).
163 Total protein content was measured by the Lowry *et al.* (1951) method using bovine serum
164 albumin as the standard solution. The functional groups of the bioflocculant were characterized
165 using a Fourier transform infrared spectrophotometer (Perkin Elmer System 2000, FT-IR,
166 England). The bioflocculant was ground with KBr salt at 25°C and pressed into a pellet for FT-
167 IR spectroscopy over a wave number range of 4 000-370cm^{-1}.

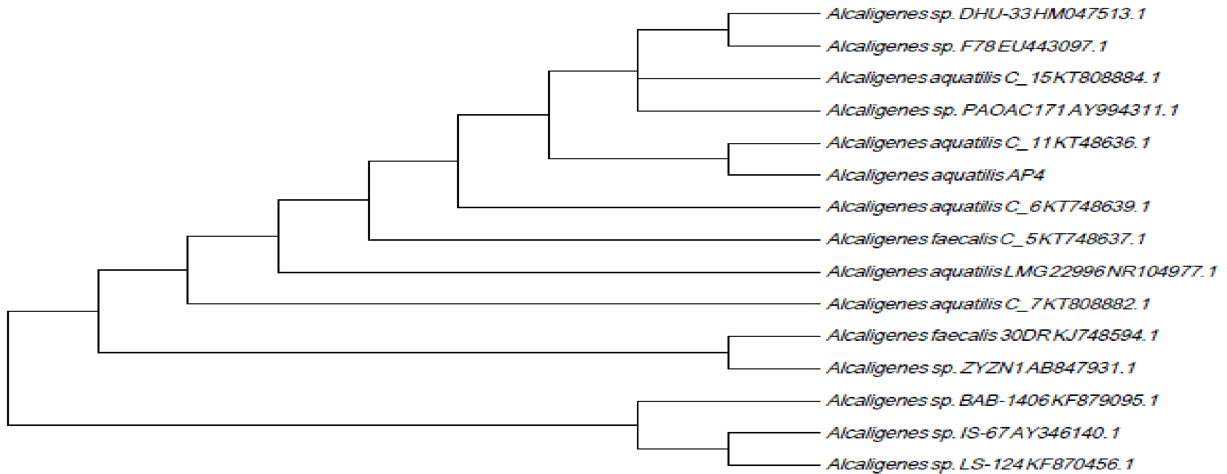
168

169 **Statistical Analysis**

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

173 **RESULTS AND DISCUSSION**

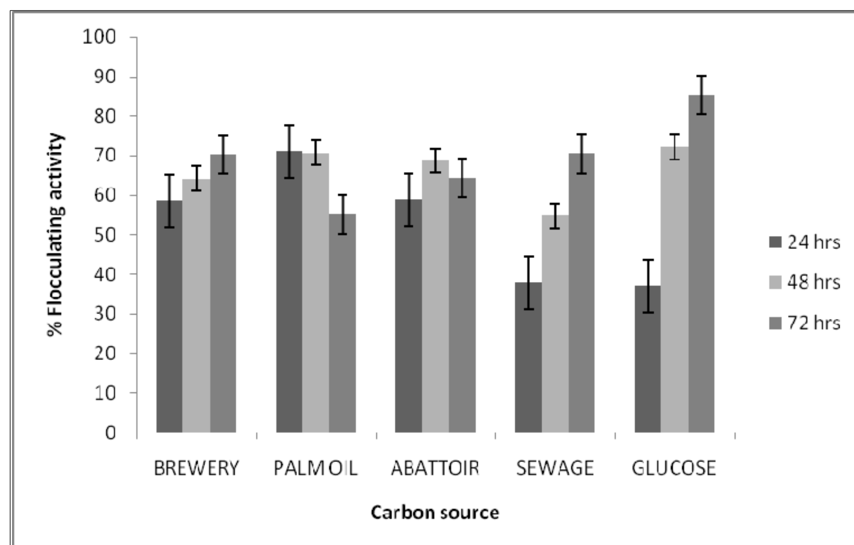
174 Bioflocculant producing *Alcaligenes aquatilis* AP4 was isolated from palm oil mill effluent. The
175 isolate was characterized genotypically and the bioflocculant produced by the isolate was also
176 characterized. A BLAST (Basic Local Alignment Search Tool) analyses of the 16S rRNA gene
177 nucleotide sequence of strain AP4 PCR amplified product showed a 97% similarity to *A.*
178 *aquatilis* (accession number KT748636). The evolutionary history was inferred by using the
179 Maximum Likelihood method based on the Jukes-Cantor model (Jukes and Cantor, 1969). The
180 tree with the highest log likelihood (-3893.6749) is shown. The percentage of trees in which the
181 associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic
182 search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix
183 of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and
184 then selecting the topology with superior log likelihood value. The rate variation model allowed
185 for some sites to be evolutionarily invariable ([+*I*], 0.0000% sites). The analysis involved 15
186 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions
187 with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps,
188 missing data, and ambiguous bases were allowed at any position. There were a total of 702
189 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.,*
190 2013).



191 *Figure 1. Molecular Phylogenetic analysis by Maximum Likelihood method of the isolate*

192

193 The effect of different carbon source on bioflocculant production by *A. aquatilis AP4* is shown in
194 Figure 2. There was a significant difference (P≤0.05) in the flocculating activity of *A. aquatilis*
195 *AP4* at different carbon sources. At 24, 48 and 72 hrs of incubation the flocculating activity
196 ranged from 37.05[e] - 77.55[a] %, 54.80[e] - 76.61[a] % and 64.29[e] - 89.58[a] % respectively in which the
197 highest flocculating activity was recorded in Abattoir effluent at 24 hrs and glucose at 48 and 72
198 hrs of incubation respectively.



199 *Figure 2: Effect of different carbon source (wastewater) on bioflocculant production by A.*
200 *aquatilis* AP4
201

202 The isolate was able to produce reasonable level of bioflocculant in all the carbon sources used.
203 Ability of glucose to support the highest bioflocculant production by *A. aquatilis AP4* is in
204 agreement with the report of Cosa *et al*. (2011) on *Virgibacillus* sp. Rob. Glucose has been
205 reported as a preferred carbon source in previous studies on bioflocculant production by various
206 microorganisms. Patil *et al*. (2009) reported that the bioflocculant produced by *Bacillus subtilis*
207 is enhanced by glucose and sucrose as carbon sources. In the case of *Rhodococcus erythropolis*,
208 glucose and fructose enhance elongation of the cells and the production of the bioflocculant
209 (Kurane *et al*., 1991). Cosa *et al*. (2013) found that *Virgibacillus* sp. preferred glucose as carbon
210 source and his finding was synonymous to the work of Liu and Chen (2010) on *Penicillium* sp.
211 HHE-P7 who recorded an increase in bioflocculant production when glucose was used as carbon
212 source. The findings of Gong *et al*. (2003) on bioflocculant production by *Paenibacillus*
213 *polymyxa* BY-28 showed that apart from glucose, sucrose, maltose, lactose and xylitol are also
214 suitable carbon sources. However, Shih *et al*. (2001), reported that glucose, fructose, and lactose
215 were not suitable for bioflocculant production by *Bacillus licheniformis*, instead, simultaneous
216 used of glutamic acid, citric acid and glycerol gave a better yield. Rasulov *et al*. (2017) also
217 observed that D-Mannose gave the best biomass yield and highest flocculating activity of
218 3.46g/L and 97% respectively compared to glucose with 3.46g/L and 89%.

The effect of pH on bioflocculant production by *A. aquatilis* AP4 is shown in Figure 3. There was a significant difference ($P \leq 0.05$) in the flocculating activity of *A. aquatilis* AP4 at different pH. At 24, 48 and 72 hrs of incubation the flocculating activity ranged from 36.11^{e} - 71.28^{a} %, 30.31^{e} - 78.83^{a} % and 44.58^{e} - 82.94^{a} % in which the highest flocculating activity was recorded at pH 9.0.

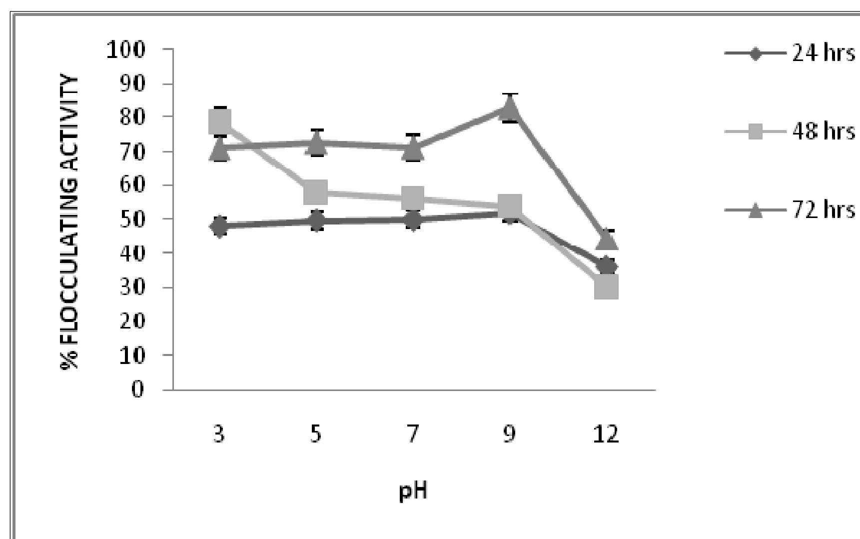


Figure 3: Effect of pH on bioflocculant production by A. aquatilis AP4

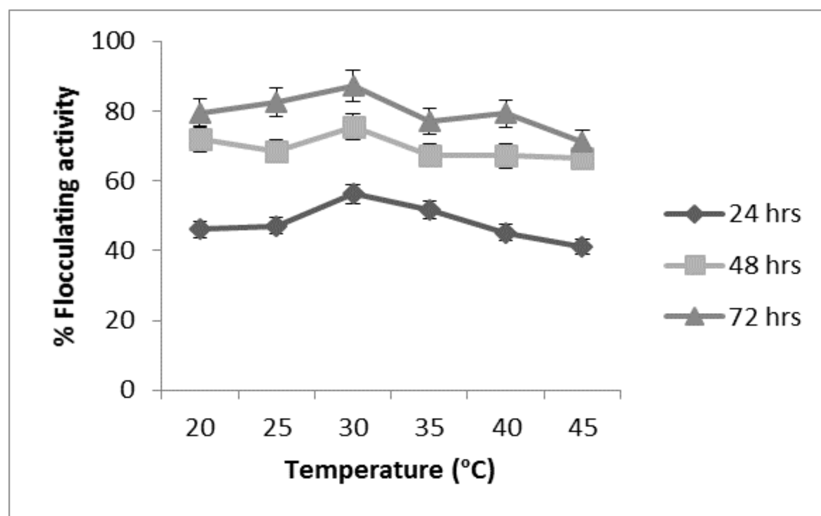
This result is in agreement with the report of Salehizadeh and Shojaosadati (2001) who reported that the initial pH of the production medium is one of the factors affecting the production and flocculating activity of the bioflocculant in that it determined the oxidation–reduction potential which could influence the absorption of nutrients in the production medium. The flocculating activity of bioflocculant produced by *A. aquatilis* AP4 was stable within pH 3.0 – 9.0 at 72 hrs of incubation. *Gyrodium impudicum* KG03 bioflocculant was reported to have maximum activity at acidic pH (4.0) (Yim *et al.,* 2007). Acidic pH was also preferred by *Aspergillus parasiticus* as reported by Deng *et al*. (2003). Liu *et al*. (2013) however reported that alkaline pH 8.0 stimulated bioflocculant production by isolate *Klebsiellus* sp. TG-1. Moreover, Haas *et al*., (1999), Patil *et al*. (2009) and Leonard *et al*. (2012) reported that *Corynebacterium xerosis, Bacillus subtilis* and *Arthrobacter* sp. 5J12A respectively had optimum bioflocculant production activity at neutral pH (7.0). After this pH range, a sharp decrease occurred at pH 12.0. This

239 contradicted the report of Cosa *et al*. (2011) on *Virgibacillus* sp. Rob who reported peak activity
240 at alkaline pH (12.0).

241 The effect of incubation temperature on bioflocculant production by *A. aquatilis* AP4 is shown in
242 Figure 4. There was a significant difference (P≤0.05) in the flocculating activity of *A. aquatilis*
243 AP4 at different incubation temperature. At 24, 48 and 72 hrs of incubation, the flocculating
244 activity ranged from 41.11^{e} - 56.22^{a} %, 66.41^{e} - 75.55^{a} % and 71.11^{e} - 87.23^{a} % respectively in
245 which the highest flocculating activity was recorded at 30°C of incubation time.

246 According to Zhang *et al*. (2007), cultivation temperature have a significant impact on the
247 enzymes responsible for bioflocculant production. Optimum temperature for maximum
248 bioflocculant production must be known which usually varies between 25°C and 37°C (Okaiyeto
249 *et al*., 2016). Nakata and Kurane (1999) reported 30°C as the optimum temperature for
250 bioflocculant production by *Citrobacter* sp. TKF04.

251



252 *Figure 4: Effect of incubation temperature on bioflocculant production by A. aquatilis* AP4

253

254 The effect of static/agitation on bioflocculant production by *A. aquatilis* AP4 is shown in Figure
255 5. There was a significant difference (P≤0.05) in the flocculating activity of *A. aquatilis* AP4
256 when the incubation was carried out in static/agitation method. In static incubation, the
257 flocculating activity ranged from 26.60^{c} - 57.25^{a} % while in agitation condition, the activity

258 ranged from 50.15^{c} - 81.55^{a} % in which the highest flocculating activity was recorded in
259 agitation at 72 hrs of incubation.
260
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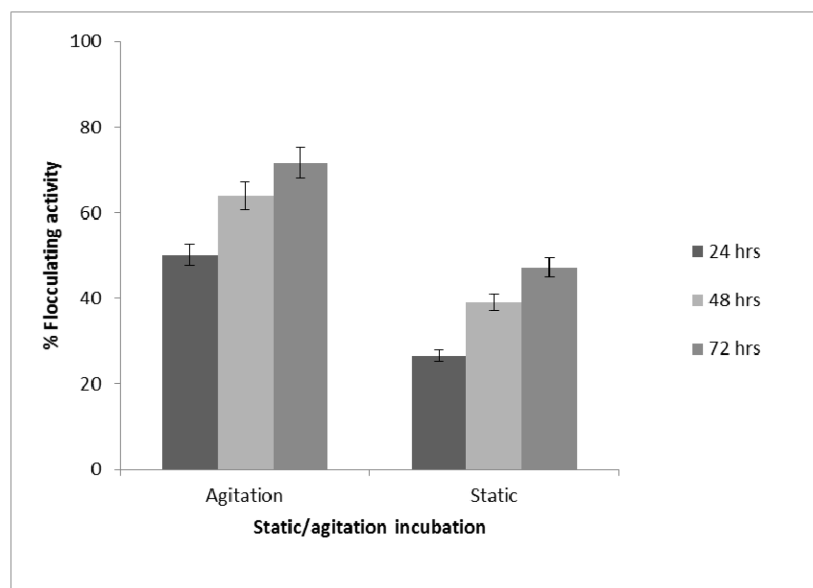
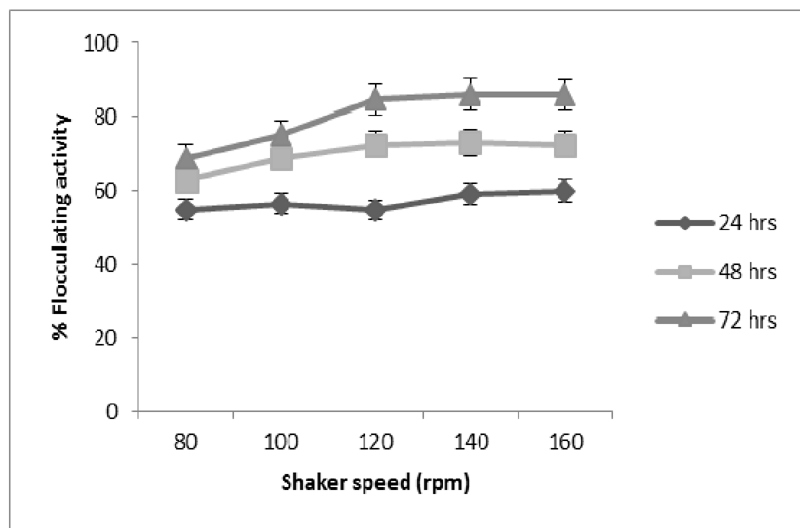


262 *Figure 5: Effect of static/agitation on bioflocculant production by A. aquatilis* AP4
263

264 Maximum flocculating activity was recorded during shaking while non-shaking condition did not
265 support bioflocculant production by the strains. This report was in contrast to what Salehizadeh
266 and Shojaosadati (2001) reported that *s*ometimes due to agitation of the culture medium, yield of
267 polymeric flocculant production by bacterial cells might be greatly reduced and it is therefore
268 important to verify if there is need to agitate the culture medium. Lopez *et al.* (2003) reported
269 that to have increased nutrient absorption and optimum enzymatic reaction, increase in dissolved
270 oxygen is very important and it can only be realized through the use of shaker for incubation.
271

272 The effect of speed (rpm) on bioflocculant production by *A. aquatilis* AP4 is shown in Figure 6.
273 There was a significant difference ($P \leq 0.05$) in the flocculating activity of *A. aquatilis* AP4 at
274 different incubation speed. At 24, 48 and 72 hrs of incubation the flocculating activity ranged
275 from 54.72^{e} - 59.80^{a} %, 62.77^{d} - 73.04^{a} % and 68.92^{d} - 86.12^{a} % respectively in which the
276 highest flocculating activity was recorded at 140rpm speed at 72 hrs of incubation. The effect of

277 shaking speed on the bioflocculant production showed that the shaking speed of 140 rpm was the
278 most preferred with *A. aquatilis* AP4 having flocculating activity of 86.12%. Decrease in
279 flocculating activity was observed when shaking below or above 140 rpm.



280

281 *Figure 6: Effect of speed (rpm) on bioflocculant production*

282

283 This may be as a result of the fact that shaking speed determines the concentration of the
284 dissolved oxygen, which can affect the absorption of nutrients and enzymatic reaction of the
285 strain (Salehizadeh and Shojaosadati, 2001). This study contradicted the work of Zhang *et al.*
286 (2007) on consortium of *Staphylococcus* sp. and *Pseudomonas* sp. with 160 rpm being the best
287 shaking speed for highest flocculating activity. Li *et al*. (2009) also reported that agitation speed
288 of 140–160 rpm was best for the bioflocculant produced by *Bacillus licheniformis* X14.

289

290 **Biochemical and FTIR Analysis of the Bioflocculant**

291 Chemical analysis of the purified bioflocculant produced by the isolate revealed that
292 carbohydrate and protein are the two components present while carbohydrate happened to be the
293 major constituent with 91.65% compared to protein with 8.35%.

294

295 The FT-IR spectroscopy performed on purified bioflocculant produced by *A. aquatilis* AP4 is
296 shown in Fig. 7. The spectrum showed a sharp, intense absorption peak at 3421.83 cm^{-1} which is

characteristic of a hydroxyl and amino group. This could be caused by the vibration of -OH or -NH in the sugar ring.

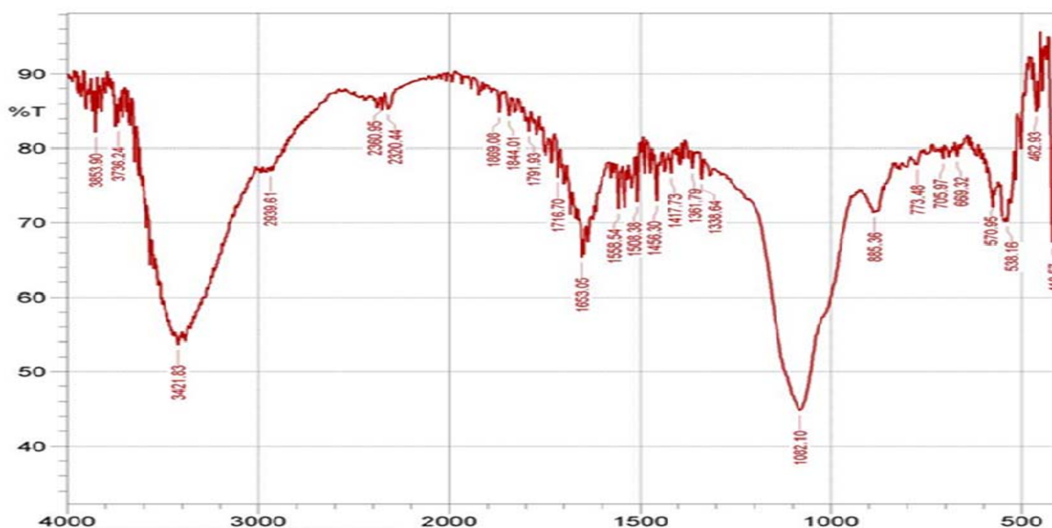


Figure 7: FT-IR spectroscopy performed on purified bioflocculant produced by the isolate

This result agreed with the report of He *et al*. (2010) and Kavita *et al*. (2013). Okaiyeto *et al.* (2014) reported that the presence of hydroxyl group was responsible for water solubility of bioflocculants. A weak peak at 2,939.61 cm^{-1} known to be typical of carbohydrates, indicated –COH asymmetrical stretching vibration. This is in accordance with the report of Yin *et al.* (2014).

A weak peak at 2360.95 and 2320.44 is typical of aliphatic band. While that of 1869.08 – 1716.70 cm^{-1} was characterized of -C=O acid chloride. Sharp peak at 1653.05 is typical of Carboxyl, -CO-NH or $-NH_2$ group. Luo *et al*. (2014) reported that carboxyl group provides adsorption sites for particle attachment which help the macromolecule of bioflocculant to adsorbed many particles.

Weak asymmetrical stretching peak observed from 1558.54 – 1338.64 cm^{-1} is characteristic of NH band vibration -CONH. A very sharp stretching peak at 1082.10 cm^{-1} indicated asymmetrical stretching vibration of a -C-O-C- ester linkage. Sharp peak at 885.36 cm^{-1} could be associated with glycosidic linkages between the sugar monomers.

314 The weak peak at 773.48 – 669.32 cm^{-1} is typical of Benzene rings. The presence of
315 characteristic peak for carbohydrate and amide shown by infrared spectral indicated that the
316 bioflocculant produced by *A. aquatilis* AP4 is a glycoprotein. Gao *et al*. (2006) reported that
317 many microorganisms such as bacteria, fungi and actinomycetes produce extracellular
318 substances which could composed of Polysaccharide, protein and glycoprotein. Zaki *et al*.
319 (2013), Luo *et al*. (2014) and Ntsangan *et al*. (2017) reported that the bioflocculants produced by
320 *Bacillus velezensis* 40B, *Klebsiella pneumonia and Bacillus* sp. AEMREG4 respectively are
321 glycoprotein. However, the bioflocculants produced by *Nocardia amarae* (Koizumi *et al*., 1991)
322 was discovered to be protein while that of *Halomonas sp.* V3a (He *et al*., 2010), *Paenibacillus*
323 *elgii* B69 and (Li *et al*., 2013) are polysaccharide.
324

325 **Conclusion**

326 In conclusion, *Alcaligenes aquatilis* AP4 could be regarded as novel bioflocculant producer since
327 there is no reported history of any bioflocculant produced by this same species in the past.
328 Cultural conditions such as carbon source, pH, incubation temperature and agitation have
329 significant effect on the bioflocculant production. Glucose, 30°C, pH 9.0, agitation and shaking
330 speed of 140 rpm were the best for maximum production of bioflocculant by the isolate. The
331 bioflocculant is a glycoprotein consisting of hydroxyl, amide and carboxyl as its functional
332 groups. Isolate AP4 is a good agent with high flocculating activity (89.58%), it therefore has the
333 potential to be used on a large scale for bioflocculant production, which could serve as a possible
334 substitute for non-biodegradable, carcinogenic and harmful chemical flocculants which is often
335 used in the treatment of water today. Further studies on the biotechnological application of the
336 bioflocculant and the genes responsible for flocculation are in progress.

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