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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 hrs of incubation. 30°C and pH 9.0 induced the highest
14 bioflocculant production. Agitation condition had a significant effect (P≤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). Inorganic
26 flocculating agents, such as aluminum sulfate, polyaluminum chloride, ferric chloride (Lee et al.
27 2014), and organic polymers such as polyacrylamide derivatives (Suopajarvi *et al*., 2013), are
28 frequently used in both wastewater treatment and the fermentation industries because they are

UNDER PEER REVIEW

29 not only cost-effective, but also have strong flocculating activity (He *et al*., 2002). However,
30 studies have shown that some of the chemically synthetic flocculating substances are not only
31 harmful to both humans and the environment, but are also non-degradable in nature (Taniguchi
32 *et al*., 2005).

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

41 In general, flocculants are classified into three groups: inorganic flocculants, such as aluminum
42 sulfate and polyaluminum chloride (Lee *et al*., 2014). Organic synthetic flocculants, such as
43 polyacrylamide derivatives and polyethylene amine and naturally occurring flocculants, such as
44 chitosan, gelatin, guar gum, sodium alginate (Okaiyeto *et al*., 2016). Microbial flocculants have
45 found widespread applications in several industrial and waste water treatment processes such as
46 pharmaceutical, fermentation, food industries dredging and downstream processing (Seo, 1993;
47 Zhang *et al*., 1999; Ozcan and Oner 2015). Most of high molecular weight flocculants are
48 recalcitrant. It is evident that the acrylamide monomer is not only neurotoxic and carcinogenic
49 but also non-biodegradable in nature. They have detrimental effect both on flora and fauna
50 (Vanhorick *et al*., 1983; Dearfield *et al*., 1988). Aluminium is one of the component of
51 Aluminium sulphate mostly used in the treatment of raw water for household use, has been
52 shown to cause Alzheimer's disease (Green *et al*., 2002; Kivipelto *et al*., 2005; Agunbiade *et al*.,
53 2017).

54 Many microorganisms, including bacteria, fungi and actinomycetes, have been reported to
55 produce extracellular polymeric substances, such as polysaccharides, functional proteins and
56 glycoproteins, which function as bioflocculant (Gao *et al*., 2006; Okaiyeto *et al*., 2016). Studies
57 carried out on the chemical composition of bioflocculant produced by *Bacillus subtilis* IFO3335
58 (Yokoi *et al*., 1996), *Bacillus* sp. I-471 (Kumar, *et al*., 2004), *Bacillus subtilis* DYU1 (Wu and

UNDER PEER REVIEW

59 Ye, 2007), *Halomonas* sp. V3a (He *et al*., 2010), *Paenibacillus elgii* B69 (Li *et al*., 2013) and
60 *Paenibacillus mucilaginosus* (Tang *et al*., 2014), have shown them to all be polysaccharides.
61 *Rhodoccocus erythropolis* (Takeda *et al.,* 1991) and *Nocardia amarae* YK-1 (Koizumi *et al*.,
62 1991) produce protein flocculants while *Arcuadendron* sp. TS-4 (Lee *et al*., 1995), *Klebsiella*
63 *pneumonia* (Luo *et al*., 2014), *Bacillus clausii* NB2 (Adebayo-tayo and Adebami, 2014) and
64 *Pseudomonas aeruginosa* IASST201 (Pathak *et al*., 2017) have been reported to produce
65 glycoprotein bioflocculants.

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

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

78 **MATERIAL AND METHOD**

79 **Culture preparation**

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

84 The seed culture was grown in a 250ml flask containing: Nutrient broth- 10.0g; Potato dextrose
85 broth, - 5.0g; Glycerol - 3ml; Yeast Extract - 6.5g; Sodium chloride - 1.0g in 1litre of distilled

UNDER PEER REVIEW

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

88

89 **16SrDNA Sequence determination and Phylogenetic Analysis of the Bioflocculant-**
90 **Producing *A. aquatilis* AP4**

91 *A. aquatilis* AP4 isolated from Palm oil mill effluent was identified using molecular technique
92 based on the 16SrRNA gene amplification by polymerase chain reaction (PCR) followed by
93 sequencing of the amplified gene as designed according to Gupta *et al*. (1987) and Xiong *et al*.
94 (2010).

95 The isolate was incubated in 250-ml flasks containing 50 µl fresh LB medium for 16 h at 37°C
96 with shaking at 120 rpm. The genomic DNA of the strain was then extracted using CTAB
97 method of DNA extraction from microbes. PCR amplification was carried out to determine the
98 partial 16S rRNA gene. The PCR program was 30 cycles of 94°C (1 min), 55°C (30 s), and 72°C
99 (1.5 min). The PCR universal primers were 5-CCAGCAGCCGCGGTAATACG-3 (forward) and
100 5-TACCAGGGTATCTAATCC-3 (reverse). Purification of the PCR products and the
101 determination of sequences were performed by Macrogen USA (9700 Great Seneca Highway,
102 Rockville, MD 20850, USA). The 16S rRNA gene sequence of strain AP4 obtained was
103 compared with the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

104

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

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UNDER PEER REVIEW

114 **Determination of Flocculating Activity**

115 The flocculating activity was determined according to the method of Kurane *et al*. (1986) as
116 modified by Gao *et al*. (2006). A suspension of kaolin clay was used as test material for
117 flocculating activity determination. The kaolin clay was suspended in distilled water at a
118 concentration of 5 g/L at pH 7.0 and used as a stock solution for the subsequent assays. The
119 following solutions were mixed in a test tube: kaolin clay suspension (9 mL), culture supernatant
120 (0.1 mL) and 1% $CaCl_2$ (0.25 mL). A reference tube in which the culture supernatant was
121 replaced with distilled water was also included and measured under the same conditions. The
122 final volume of all mixtures was made up to 10mL with distilled water. After mixing gently, the
123 solutions were allowed to settle for 5 min. at room temperature. The optical density (OD) of the
124 clarifying upper phase solution was measured at 550nm with a UV spectrophotometer and the
125 flocculating activity determined as follows:
126 Flocculating rate (%) = $[(B - A) / B] \times 100\%$

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

128

129 **Effects of carbon source on Bioflocculant Production by *A. aquatilis* AP4**
130 Effect of carbon sources such as economic wastewaters: Palm-oil effluent, Abbatoir effluent,
131 Brewery effluent and Sewage and glucose on bioflocculant production by the isolate was
132 investigated. Kaolin assay was carried out to check the maximum flocculating activity of the
133 isolate after which the absorbance was then read using a spectrophotometer at wavelength of
134 550nm.

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

UNDER PEER REVIEW

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

146 Effect of incubation under static and agitation condition was investigated. The medium was
147 sterilized, incubated and some were inoculated under static condition while some were incubated
148 under agitation condition by varied the incubation speeds between 80 – 160 rpm. Kaolin assay
149 was carried out to check the maximum flocculating activity of the isolate after which the
150 absorbance was then read using a spectrophotometer at wavelength of 550nm.

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

152 **Extraction and Purification of the Bioflocculant**

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

165 **Chemical Analyses of the purified Bioflocculants**

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

UNDER PEER REVIEW

170 using a Fourier transform infrared spectrophotometer (Perkin Elmer System 2000, FT-IR,
171 England). The bioflocculant was ground with KBr salt at 25°C and pressed into a pellet for FT-
172 IR spectroscopy over a wave number range of 4 000-370cm^{-1}.
173

174 **Statistical Analysis**

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

178 **RESULTS AND DISCUSSION**

179 Bioflocculant producing *Alcaligenes aquatilis* AP4 was isolated from palm oil mill effluent. The
180 isolate was characterized genotypically and the bioflocculant produced by the isolate was also
181 characterized. A BLAST (Basic Local Alignment Search Tool) analyses of the 16S rRNA gene
182 nucleotide sequence of strain AP4 PCR amplified product showed a 97% similarity to *A.*
183 *aquatilis* (accession number KT748636). The evolutionary history was inferred by using the
184 Maximum Likelihood method based on the Jukes-Cantor model (Jukes and Cantor, 1969). The
185 tree with the highest log likelihood (-3893.6749) is shown. The percentage of trees in which the
186 associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic
187 search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix
188 of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and
189 then selecting the topology with superior log likelihood value. The rate variation model allowed
190 for some sites to be evolutionarily invariable ([+*I*], 0.0000% sites). The analysis involved 15
191 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions
192 with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps,
193 missing data, and ambiguous bases were allowed at any position. There were a total of 702
194 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.,*
195 2013).

UNDER PEER REVIEW

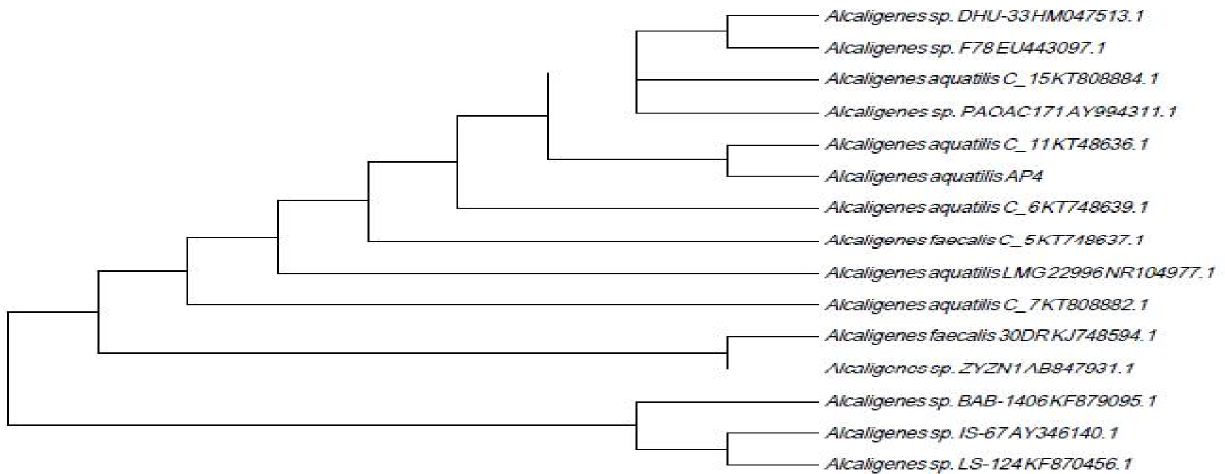
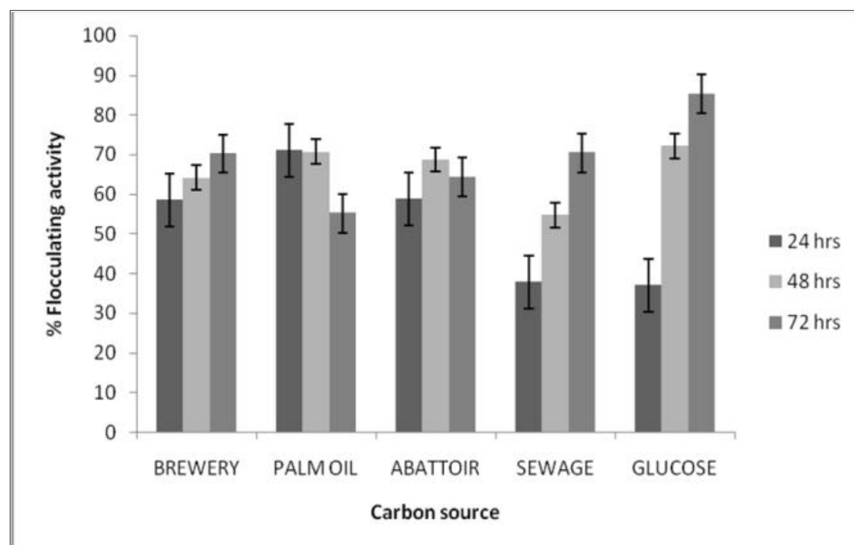


Figure 1. Molecular Phylogenetic analysis by Maximum Likelihood method of the isolate

The effect of different carbon source on bioflocculant production by *A. aquatilis AP4* is shown in Figure 2. There was a significant difference ($P \leq 0.05$) in the flocculating activity of *A. aquatilis AP4* at different carbon sources. At 24, 48 and 72 hrs of incubation the flocculating activity 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 highest flocculating activity was recorded in Abattoir effluent at 24 hrs and glucose at 48 and 72 hrs of incubation respectively.

UNDER PEER REVIEW



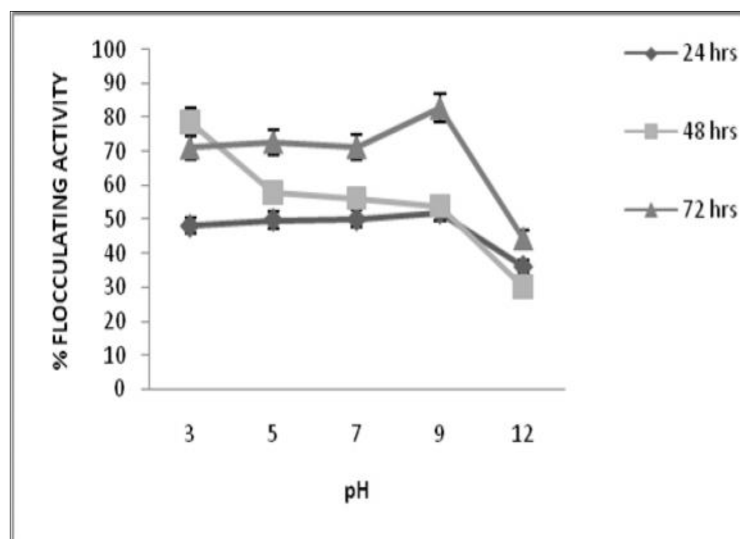
204 *Figure 2: Effect of different carbon source (wastewater) on bioflocculant production by A.*
205 *aquatilis* AP4
206

207 The isolate was able to produce reasonable level of bioflocculant in all the carbon sources used.
208 Ability of glucose to support the highest bioflocculant production by *A. aquatilis AP4* is in
209 agreement with the report of Cosa *et al*. (2011) on *Virgibacillus* sp. Rob. Glucose has been
210 reported as a preferred carbon source in previous studies on bioflocculant production by various
211 microorganisms. Patil *et al*. (2009) reported that the bioflocculant produced by *Bacillus subtilis*
212 is enhanced by glucose and sucrose as carbon sources. In the case of *Rhodococcus erythropolis*,
213 glucose and fructose enhance elongation of the cells and the production of the bioflocculant
214 (Kurane *et al*., 1991). Cosa *et al*. (2013) found that *Virgibacillus* sp. preferred glucose as carbon
215 source and his finding was synonymous to the work of Liu and Chen (2010) on *Penicillium* sp.
216 HHE-P7 who recorded an increase in bioflocculant production when glucose was used as carbon
217 source. The findings of Gong *et al*. (2003) on bioflocculant production by *Paenibacillus*
218 *polymyxa* BY-28 showed that apart from glucose, sucrose, maltose, lactose and xylitol are also
219 suitable carbon sources. However, Shih *et al*. (2001), reported that glucose, fructose, and lactose
220 were not suitable for bioflocculant production by *Bacillus licheniformis*, instead, simultaneous
221 used of glutamic acid, citric acid and glycerol gave a better yield. Rasulov *et al*. (2017) also
222 observed that D-Mannose gave the best biomass yield and highest flocculating activity of
223 3.46g/L and 97% respectively compared to glucose with 3.46g/L and 89%.

UNDER PEER REVIEW

224

225 The effect of pH on bioflocculant production by *A. aquatilis* AP4 is shown in Figure 3. There
226 was a significant difference (P≤0.05) in the flocculating activity of *A. aquatilis* AP4 at different
227 pH. At 24, 48 and 72 hrs of incubation the flocculating activity ranged from 36.11[e] - 71.28[a] %,
228 30.31[e] - 78.83[a] % and 44.58[e] - 82.94[a] % in which the highest flocculating activity was recorded at
229 pH 9.0.



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

231

232 This result is in agreement with the report of Salehizadeh and Shojaosadati (2001) who reported
233 that the initial pH of the production medium is one of the factors affecting the production and
234 flocculating activity of the bioflocculant in that it determined the oxidation–reduction potential
235 which could influence the absorption of nutrients in the production medium. The flocculating
236 activity of bioflocculant produced by *A. aquatilis* AP4 was stable within pH 3.0 – 9.0 at 72 hrs of
237 incubation. *Gyrodium impudicum* KG03 bioflocculant was reported to have maximum activity at
238 acidic pH (4.0) (Yim *et al.,* 2007). Acidic pH was also preferred by *Aspergillus parasiticus* as
239 reported by Deng *et al*. (2003). Liu *et al*. (2013) however reported that alkaline pH 8.0
240 stimulated bioflocculant production by isolate *Klebsiellus* sp. TG-1. Moreover, Hass *et al*.,
241 (1999), Patil *et al*. (2009) and Leonard *et al*. (2012) reported that *Corynebacterium xerosis,*
242 *Bacillus subtilis* and *Arthrobacter* sp. 5J12A respectively had optimum bioflocculant production
243 activity at neutral pH (7.0). After this pH range, a sharp decrease occurred at pH 12.0. This

UNDER PEER REVIEW

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

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

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

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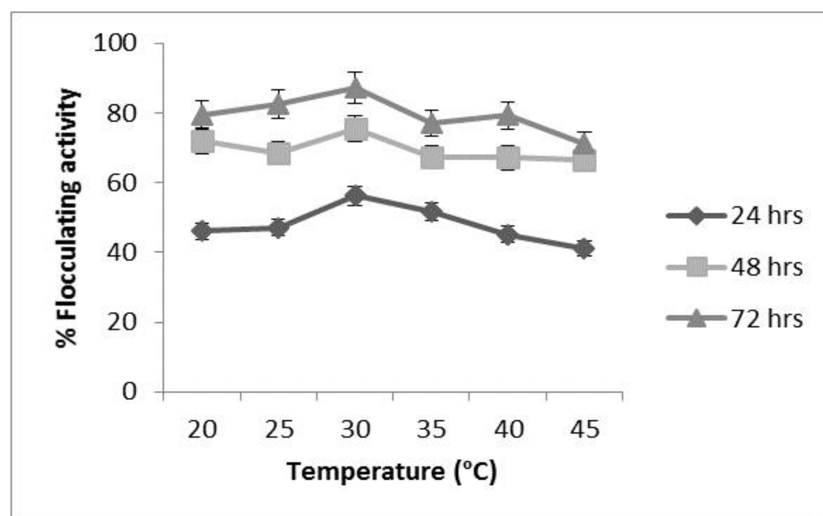

257 *Figure 4: Effect of incubation temperature on bioflocculant production by A. aquatilis* AP4
258

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

UNDER PEER REVIEW

263 ranged from 50.15[c] - 81.55[a] % in which the highest flocculating activity was recorded in
264 agitation at 72 hrs of incubation.
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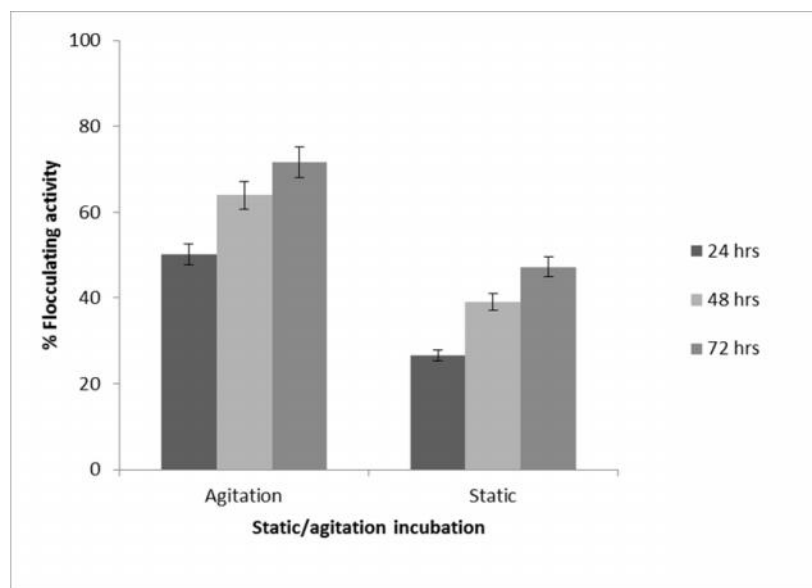


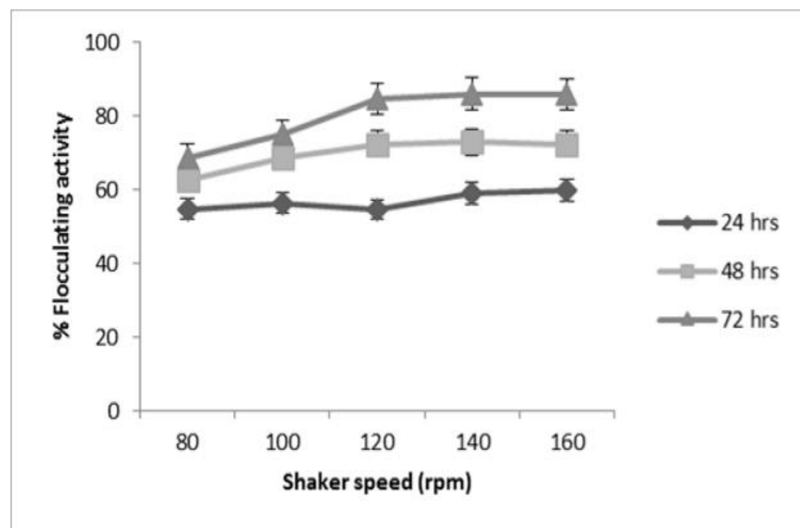
267 *Figure 5: Effect of static/agitation on bioflocculant production by A. aquatilis* AP4
268

269 Maximum flocculating activity was recorded during shaking while non-shaking condition did not
270 support bioflocculant production by the strains. This report was in contrast to what Salehizadeh
271 and Shojaosadati (2001) reported that *s*ometimes due to agitation of the culture medium, yield of
272 polymeric flocculant production by bacterial cells might be greatly reduced and it is therefore
273 important to verify if there is need to agitate the culture medium. Lopez *et al.* (2003) reported
274 that to have increased nutrient absorption and optimum enzymatic reaction, increase in dissolved
275 oxygen is very important and it can only be realized through the use of shaker for incubation.
276

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

UNDER PEER REVIEW

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



285

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

287

288 This may be as a result of the fact that shaking speed determines the concentration of the
289 dissolved oxygen, which can affect the absorption of nutrients and enzymatic reaction of the
290 strain (Salehizadeh and Shojaosadati, 2001). This study contradicted the work of Zhang *et al.*
291 (2006) on consortium of *Staphylococcus* sp. and *Pseudomonas* sp. with 160 rpm being the best
292 shaking speed for highest flocculating activity. Li *et al*. (2009) also reported that shaker speed of
293 140–160 rpm was best for the bioflocculant produced by *B. licheniformis* X14.

294

295 BIOCHEMICAL AND FTIR ANALYSIS OF THE BIOFLOCCULANT
296 Chemical analysis of the purified bioflocculant produced by the isolate revealed that
297 carbohydrate and protein are the two components present while carbohydrate happened to be the
298 major constituent with 91.65% compared to protein with 8.35%.

299

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

UNDER PEER REVIEW

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

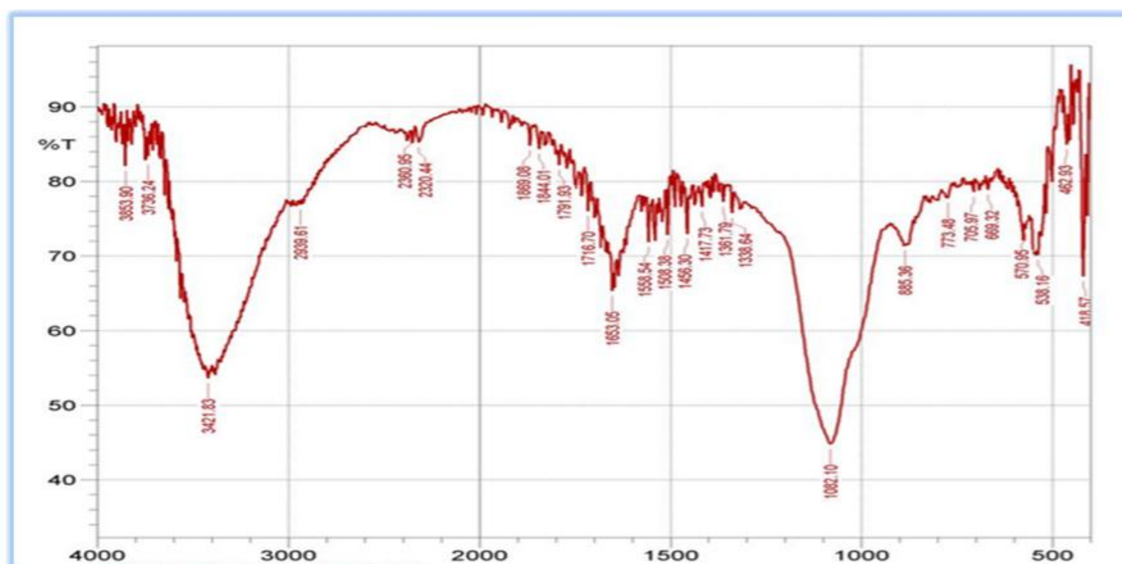


Figure 7: FT-IR spectroscopy performed on purified bioflocculant produced by the isolate

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.

UNDER PEER REVIEW

319 The weak peak at 773.48 – 669.32 cm^{-1} is typical of Benzene rings. The presence of
320 characteristic peak for carbohydrate and amide shown by infrared spectral indicated that the
321 bioflocculant produced by *A. aquatilis* AP4 is a glycoprotein. Gao *et al*. (2016) reported that
322 many microorganisms such as bacteria, fungi and actinomycetes produce extracellular
323 substances which could composed of Polysaccharide, protein and glycoprotein. Zaki *et al*.
324 (2013), Luo *et al*. (2014) and Ntsangan *et al*. (2017) reported that the bioflocculants produced by
325 *Bacillus velezensis* 40B, *Klebsiella pneumonia and Bacillus* sp. AEMREG4 respectively are
326 glycoprotein. However, the bioflocculants produced by *Nocardia amarae* (Koizumi *et al*., 1991)
327 was discovered to be protein while that of *Halomonas sp.* V3a (He *et al*., 2010), *Paenibacillus*
328 *elgii* B69 and (Li *et al*., 2013) are polysaccharide.
329

330 **Conclusion**

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

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