PRINT ISSN 1119-8362
Electronic ISSN 2659-1502

Full-text Available Online at
https://www.ajol.info/index.php/jasem
http://www.bioline.org.br/ja

J. Appl. Sci. Environ. Manage.
Vol. 27 (1) 169-175 January 2023

Production of Bioflocculant through Fermentation of Spoilt Orange Juice with *Bacillus* spp Isolated from Sediment of Local Clay Pot

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ABSTRACT: The biodegradable and non-toxic Bioflocculants have attracted considerable interest as alternative to non-biodegradable chemical flocculants. However, the cost of fermentation media for bioflocculant production and low flocculation efficiency is a major challenge. In this study, we grow *Bacillus spp* isolated from sediment of local clay pot on spoilt orange juice to produce bioflocculant. The culture supernatant of the bacilli grown on spoilt orange juice were screen for bioflocculant production using Jar test method and Kaolin clay suspension as model wastewater. The effect of pH and temperature on the bioflocculant production were also studied. The effect of cations on bioflocculation rate of the bioflocculant produced were tested via hybridization of the bioflocculant with the cations. The bioflocculation efficiency of the bioflocculant on sample wastewaters were also determined. The bacilli isolated initially had flocculation rate between 31.1±1.6 % to 76.4±1.2% when cultured on screening media. Bioflocculation rate peaked to 77.9% at 40 ºC while the lowest flocculation rate (55.41%) was obtained at 25 ºC. The optimum bioflocculant production (about 78%) was recorded at pH 6 – 7 while the highest bioflocculant production (84.2%) was achieved at 96th hour of incubation. Na^+ and Fe^{3+} had serious inhibitory effect on the bioflocculant while K^+, Ca^{2+}, Mg^{2+} and Al^{3+} had less or no effect on the bioflocculant. The bioflocculant had up to 77.7% and 64.5 % on Kitchen wastewater and aquaculture wastewater. This study reveals the potential for utilization of spoilt orange juice as fermentable substrate for bioflocculant production especially if the fermentation conditions and flocculation parameters are well optimized.

DOI: **https://dx.doi.org/10.4314/jasem.v27i1.24**

Cite this paper as: MOHAMMED, J. N; MAJIYA, H; ADISA, J. M; KASSIM, Z. J. (2023). Production of Bioflocculant through Fermentation of Spoilt Orange Juice with *Bacillus* spp Isolated from Sediment of Local Clay Pot. *J. Appl. Sci. Environ. Manage.* 27 (1) 169-175

Dates: Received: 01 January 2023; Revised: 25 January 2023; Accepted: 26 January 2023;
Published: 31st January 2023

Keyword: Bioflocculant; *Bacillus;* Cationization; spoilt orange; clay pot

Bioflocculants, also known as microbial flocculants, are defined as extracellular polymers produced by microorganisms during cellular growth (Deng *et al.*, 2003). They are metabolites of microbial cells that have capacity to flocculate many suspended solids including colloidal solids and cells. Several microorganisms have been reported in literature to produce bioflocculants, including among others: *Rhodococcus erythropolis, Paecilomyces sp., Klebsiella pneumonia, Citrobacter sp*. TKF04, *Brachybacterium sp., Cellulomonas sp*. Okoh and *Bacillus subtilis* F9 (Mohammed and Dagang, 2019a). Majority of these microorganism produced extracellular polymers as polysaccharides, proteins, and Glycoproteins as common components (Mohammed and Dagang, 2019b). Bioflocculants have been applied for treatment of starch wastewater (Deng *et al.*, 2003), purification of drinking water (Li *et al.*, 2009), microalgal biomass harvest (Liu *et al.*, 2017), removal of metal ions from polluted effluents and for various applications in downstream processes. The bioflocculant have attracted substantial attention

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due to their advantages of being biodegradable, non-toxic and lack of secondary pollutant accumulation that are associated with the chemical flocculant (Cosa *et al.,* 2011). Though the bioflocculant has the advantage of being biodegradable and lack secondary pollutant accumulation, the main downside has been low flocculation efficiency that has led to large dosage requirement with obvious treatment costs implications and cost of fermentation media that is a primary need for bioflocculant production. The cost of fermentation media can be eradicated or reduced through utilization of fermentable agricultural or industrial waste while the efficiency of the bioflocculant can be promoted by increasing the surface area of the bioflocculant through hybridization with cations (Mohammed and Dagang 2019a). The Bioflocculants are made up of negatively charged functional groups mainly uronic acids which is made of carboxylic functional groups and proteins whose amino acids are mostly negatively charged glutamic and aspartic acid (Seviour *et al.*, 2010). The polysaccharides composition of Bioflocculants can get deprotonated at some pH peculiar to most wastewater and activated sludge systems (Mohammed and Dagang 2019a). Majority of the colloids in the wastewater and other liquid phases are also normally negatively charged. And could limits the efficiency of the bioflocculant (Lin *et al.*, 2013). To overcome these challenges cations can play a vital role of stimulating the bioflocculant by neutralizing and stabilizing the negative charges on the bioflocculant and bridge between suspended particles and the bioflocculant to achieve the needed flocculation. The cations reduce the distance between the colloidal particles and the bioflocculants by increasing electrostatic pull between them (Okaiyeto *et al.*, 2016). The cations can also increase the size of the floc to facilitate sedimentation. The present study focuses on the production of bioflocculant through fermentation of spoilt orange juice with bacillus species isolated from sediment of local clay pot.

MATERIALS AND METHOD

Collection and preparation of spoilt orange juice: Spoilt oranges were collected from fruit vendors in Minna under aseptic condition to avoid further introduction of contaminants. The samples were stored in a sterile plastic container and immediately transported to microbiology laboratory of Ibrahim Badamasi Babangida University, Lapai. The Oranges were thoroughly washed under tap water, peeled and cut into smaller smithereens using a sterile knife. The juice in the sliced fruits were extracted with the aid of a juice extractor. The fruit juice sample were then stored under refrigeration at 4 ºC until required for further analysis.

Microorganism and culture conditions: The bioflocculant producing bacteria was isolated from the water sediment of local clay pot. One millilitre of sediment sample was mixed in physiological saline solution and serially diluted. The serially diluted samples were streaked on Petri dishes containing nutrient agar to allow formation of discrete bacterial colonies. The plates were incubated at 37°C for 24 – 48 hours until discrete colonies were observed. Different colonies were transferred into a freshly prepared nutrient agar and incubated at 37°C for 24 – 48 hours (Abbah *et al*., 2018). The isolated colonies were preserved by preparing glycerol stock cultures and agar slant for further use.

Screening for bioflocculant producing Bacilli: The isolated bacteria with bacilli shape were screened for bioflocculant production. Two loopfuls of bacterial colonies were grown in 50 mL of screening medium (g/L of glucose, 20; casein, 0.7; yeast extract, 0.5; KH_2PO_4, 2; K_2HPO_4, 5; NaCl, 0.1 and $MgSO_4 \cdot 7H_2O$, 0.2. (Zhang *et al.*, 2007; Ugbenyen *et al.*, 2012), with slight modifications and intermittent shaking at 30°C for 72 h. Adequate portion of the resulting fermentation broth was centrifuged to separate the cells and the cell-free supernatant collected and analyzed for flocculation activity on the model wastewater (Kaolin clay). The cell-free supernatant of the bacilli that showed highest flocculation activity for kaolin clay was selected for further study.

Bioflocculant production: The bioflocculant was produced by growing the Bacilli that showed highest flocculation activity during screening in the production medium (spoilt fruit juice). The culture conditions for fermentation included temperature 37°C, intermittent shaking, incubation time 72 h, inoculum 5% and pH 7. Subsequently, the 72h culture broth was dispensed in to 50mL centrifuge tubes and centrifuged to remove the biomass and cell pellets. The bioflocculant rich culture supernatant was collected into sterile glass beaker and used as the crude bioflocculant in the subsequent analysis as described by Mohammed and Dagang, 2019**.**

Bioflocculation Test: A model wastewater (kaolin clay suspension) was used to determine the flocculation efficiency of the bioflocculant. Two gram of Kaolin clay was dissolved in 1 L of deionized water to form a model wastewater. Subsequently, 2 ml of culture supernatant obtained was added to 200ml of kaolin suspension in a 400-ml beaker and the pH adjusted to 7.0 using NaOH or HCl. The solution was manually stirred vigorously for 1 min, and slowly for 5 min. The mixture was allowed to stay without stirring for 5 min to allow for floc collection. The optical density (OD)

of the upper liquid (treated water) was determined with the aid of a spectrophotometer. The control experiment, in which 2ml of culture supernatant was replaced with 2 mL of fresh culture medium (spoilt orange juice), was also set up. The flocculating rate was calculated and expressed in percentage using the formular below (Khiew *et al.*, 2016).

$$FC = [(A - B/A) \times 100\%] \qquad (1)$$

Where FE = Flocculation efficiency; A= the OD of the control at 550 nm; B = OD of the sample at 550 nm.

Effect of Cations on the Bioflocculant: The effect of cations on the bioflocculant was determined in accordance with the methods demonstrated by Aljuboori et al., 2015 and Xia *et al.* 2018. Briefly, 2mL crude bioflocculant was added to 200mL of kaolin suspension in a 400-mL beaker and the pH adjusted to 7.0 using NaOH or HCl. Different doses (1 – 10mL) of 1% of the cation of interest was added as the bioflocculant stimulant. The cations considered included Na^+, K^+, Ca^{2+}, Mg^{2+}, Al^{3+} and Fe^{3+} and were all added as chloride salts. The flocculation efficiency was determine following same procedure explained above

RESULTS AND DISCUSSIONS

Flocculation Activities of the Isolated Bacilli: In all, five different pure Bacilli colonies were isolated from the Bosso Dam water sediments. The bioflocculation activity recorded from culture supernatant of each of the pure bacilli as estimated with the ability of the supernatant to flocculate Kaolin clay suspension (model wastewater) is as depicted in Table 1. The highest flocculation efficiency of 76.4 ± 1.2% was recorded from culture supernatant of isolate 3 followed by isolates 2 and 4 which had efficiencies of 70.5 ± 0.9% and 66.0 ± 1.8% respectively. Lower flocculation efficiencies of 31.1 ± 1.6% and 41.5 ± 5.9% were respectively recorded from isolate 1 and 5. Thus, isolate 3 was selected for the subsequent studies.

Effect of the culture temperature on bioflocculant synthesis: The incubation temperature can influence bioflocculant synthesis because microbial metabolism is directly related to the culture temperature. The effect of the changes in incubation temperature on bioflocculant production by the bacillus spp grown on spoilt orange juice is displayed in figure 1.

Table 1: Flocculation activities of the isolated Bacilli

Colonies	Flocculation1	Flocculation 2	Flocculation 3	Flocculation %	Stdev%
1	32.6	29.5	31.3	31.1	1.6
2	70.5	69.6	71.3	70.5	0.9
3	75.8	77.7	75.6	76.4	1.2
4	67.1	64.0	67.0	66.0	1.8
5	46.1	43.2	35.0	41.5	5.9

Although there was bioflocculant synthesis at all the temperatures tested, the bioflocculant synthesis was enhanced between 30°C to 40ºC and peaking at 40 ºC (77.9%). The bioflocculation activities at 25 ºC (55.4%) and 45 ºC (56%) were low. Researchers (Zhang *et al.*, 2007) had earlier observed that enzymes responsible for bioflocculant production are activated for different microorganisms and substrates at an optimum temperature. Although many studies (Chen *et al.*, 2016; Bukhari *et al.*, 2018; Mohammed and Dagang, 2019) indicated optimum bioflocculant production at temperatures between 25°C and 37°C, The optimum production at 40°C in this study agrees with the report of Aljuboori *et al.* (2013) who also reported highest at same temperature. Generally, polymer-specific synthesis enzymes such as hexokinase that convert glucose to glucose phosphate (phosphorylation), pyro-phosphorylases that convert sugar nucleotides and glycosyltransferases that transfer the nucleotides across the periplasmic membrane to form repeated units have been indicated to play vital role in bioflocculant synthesis (Agunbiade, 2017). The catalytic activities of these enzymes can be affected by the cultivation temperature as the maximum activity is expected to occur at optimum temperature (Mohammed and Dagang, 2019).

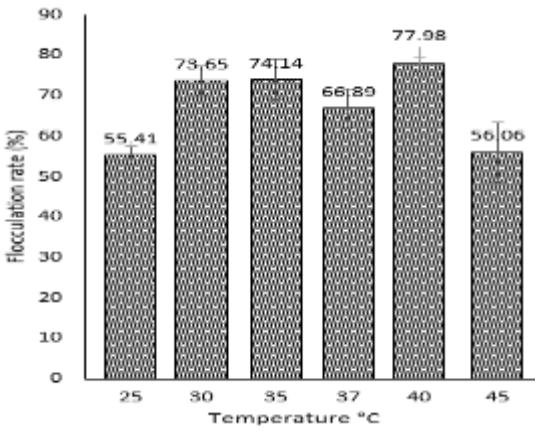

Fig 1: Flocculation rate of culture supernatant at different temperature. The error bars are standard deviation of the triplicate data.

Effect of pH on Bioflocculant Synthesis: The initial pH of the culture media can considerably affects the ionic concentrations of the production medium, electric charges of the microorganisms and the nutrient uptake and integration by the microorganisms the microbial cells (Salehizadeh and Yen, 2014). The effect of the initial pH of the spoilt fruit broth on bioflocculant synthesis was investigated over the pH range of 4 to 9. As shown in figure 2, the flocculation rate was consistently above 60% at all the pH tested but rise to about 78% at pH 6 and 7. These findings are consistent with the reports of Aljuboori *et al*. (2013) *P. aeruginosa* bioflocculant and our previous studies on *Aspergillus flavus* bioflocculant grown on chicken viscera, both studies recorded optimum flocculation rate bioflocculant production at pH 7. Although optimum bioflocculant were recorded at or close to neutral pH in most of studies as reviewed in our earlier report (Mohammed and Dagang, 2019b) few reports such as Zheng *et al.*, 2008, Liu *et al.*, 2010 and Zhao *et al.*, 2017, indicated optimum production at pH 9, pH 11 and pH 3.3 respectively.

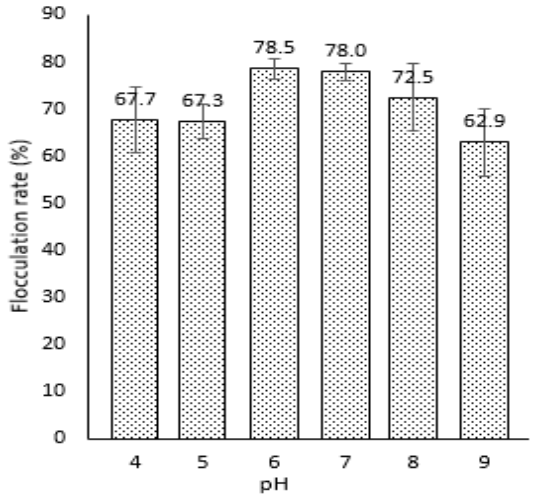

Fig 2: Flocculation rate of culture supernatant on Kaolin clay suspension at different pH. The error bars are standard deviation of the triplicate data

Time course of bioflocculant production: The time course of bioflocculant production is shown in figure 3 below. The flocculation rate increased steadily from 51.4% at 24th hour, 59.8% at 48th hour, 71.4% at 72nd hour and peaked to 84.2% at 96th hour. The flocculation rate then decreased to minimum of 47.9% at 168th hour. The flocculation rate was related to the biomass throughout except at the 120th hour at which the biomass was still at rise despite the decrease in the flocculation rate. The parallel flocculation rate with the biomass as the incubation period increases signifies the growth dependent of the bioflocculant synthesis in this study. The nature of production medium, the type of the microorganism couple with other culture conditions play vital role in growth stages (lag, exponential, stationary, and death phase) of the microorganisms. The microbial bioflocculant synthesis may be related / unrelated to the microbial growth or growth synonymous. Generally, growth of microorganisms used to be low at the lag since the microbes are still adapting to the new medium (Okaiyeto et al. 2016). The flocculation rate was initially low at the beginning due to the acclimatization of the organism after which the flocculation rate increased to peak at 96th hour at which the organism attains the stationary face. However, the flocculation rate begins to decline at 120th hour downward. The decline in bioflocculant synthesis as indicated by the flocculation rate is attributable to nutrient depletion in the production broth, depletion of the oxygen accessible to the microorganism and accumulation of the toxic wastes of the microbial metabolism within the culture broth (Mohammed and Dagang, 2019c). The potentials of the exoenzymes to bring about deflocculation through depolymerisation of the biopolymers (the bioflocculant) into degradable monomeric units after depletion of principal nutrient source in the culture broth to be used as nutrient and energy source for growth was also explained by Aljuboori *et al.* (2013). The exoenzymes have ability to breakdown the macromolecules including bioflocculant formed during microbial fermentation to bring about deflocculation. The bioflocculant produced is thus degraded at stationary phase and beyond thereby retarding the flocculation rate (More *et al.*, 2014).

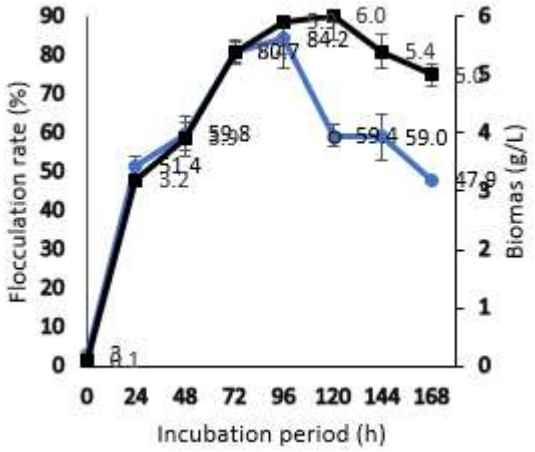

Fig 3: Time course of Bioflocculant production. The error bars are standard deviation of the triplicate data

Effect of cations on flocculation activity of the crude bioflocculant: Cations have been demonstrated to exert a vital influence on flocculation activity of negatively charged bioflocculant by neutralizing and stabilizing the anions of the active groups of the

bioflocculant thereby bridging between the suspended particles in the wastewater and the bioflocculant. The cations reduce the distance between the floating particles in wastewaters and the flocculants by increasing their electrostatic affinity (Mohammed and Dagang, 2019a). However, cations have also been found to hinder bioflocculation rate especially if the bioflocculant is chiefly made up of positively charged moieties. The effect of monovalent, divalent and trivalent cations on the present bioflocculant is displayed in figure 4. No of the cations significantly increased the flocculation rate of the bioflocculant when compared with the show results on the time course of the bioflocculant production as well as those results obtained with on the effect of pH and temperature on the bioflocculant production. The highest flocculation rate obtained with cation hybridization is 82.2% when the lowest dose (1mL) of Mg^{2+} was used. Meanwhile, Na^{+} and Fe^{3+} generally inhibited flocculation at all the doses tested. The flocculation rate falls to as low as about 26% when 10mL of Na^{+} was used and to 32.2% when 8mL Fe^{3+} was used. The inhibitory effect of monovalent metal ions such as sodium is attributable to its monovalent nature, small size and high hydration radius. The water molecules around the sodium can also prevents it approach to the surface of the colloids. while trivalent metal ions have been demonstrated in several studies and linked to the fact that trivalent ion increase the cationic density of the bioflocculant as well the cationic density over the exterior of the suspended particles with its superfluous electron. This modifies the equilibrium of the system and block flocs build up between the cationized bioflocculant and the particles (Zheng, 2008). Although K^{+} and Al^{3+} have been reported to posses' inhibitory effect on some bioflocculant, their effect on the present bioflocculant appears very low as flocculation rate of up to 77.6% and 74.4% was recorded 4mL K^{+} and 3mL Al^{3+} respectively. The divalent metal ions, Ca^{2+} and Mg^{2+} gave flocculation rate of 80.9 and 82.2% indicating slight positive effect on the bioflocculant. Both metal ions have valency of 2^{+} on their outer electron configuration, with such outer electron configuration, it is possible for them to form two ionic bonds with the flocculant and the kaolin particles. Such bonds brings the bioflocculant and the kaolin particles closer and stronger together (Khiew *et al.*, 2016). Though in this study, the divalent metal ions only show a trivial stimulation of the bioflocculant, they have generally been found to enhance bioflocculation activities of especially the bioflocculant that are made of negatively charged functional groups. Overall, it is convenient to conclude that the present bioflocculant may contain more positively charged functional groups and couldn't have been stimulated much by the cations to flocculate the kaolin particles.

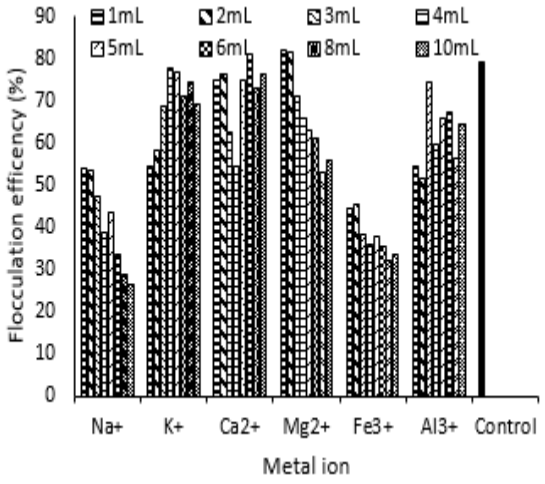

Fig 4: Effect of cations on flocculation activity of the crude bioflocculant produced from spoilt orange juice

Flocculation efficiency of the crude bioflocculant on different wastewaters: The flocculation efficiency of the crude bioflocculant produced from the spoilt orange juice on different wastewater (Activated carbon, suspended soil solids, Aquaculture wastewater, Algae solution and dye wastewater using jar test is presented in figure 5. The results indicates that the highest flocculation efficiency (77.7%) was recorded on the kitchen wastewater while the lowest efficiencies were recorded on dye wastewater (40.9) and activated carbon (42.2).

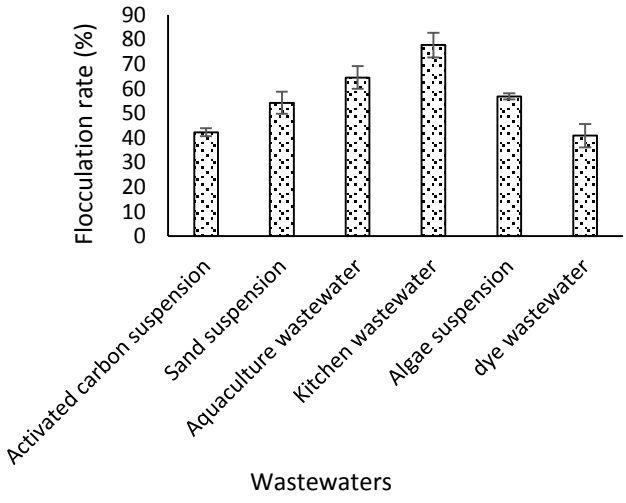

Fig 5: Flocculation efficiency of the crude bioflocculant on different wastewaters

Sand suspension, aquaculture wastewater and algal suspension had 54.2%, 64.5% and 56.8% respectively.

The flocculation efficiencies recorded on the various wastewaters tested is an indication of good flocculation properties of the crude bioflocculant that can be enhanced through characterization of the wastewaters, optimization of the flocculation conditions, optimization of the bioflocculant dose as well as cationization of the bioflocculant for the wastewaters that compose mainly of positively charged functional groups. Activated carbon for example is characterized with broad adsorption and attraction for exopolymer flocculants to form complex flocs (Aljuboori *et al.*, 2015).

Conclusion: The result of this study indicates the potential of spoilt orange juice as fermentation substrate for bioflocculant production and bioflocculant production ability of the bacilli spp isolated from water sediment of local clay pot. The optimum production was obtained at culture conditions. While monovalent cations showed inhibitory effect on the bioflocculant, divalent and trivalent had less or no effect on the bioflocculant. The bioflocculant obtain can be used to treat various wastewater especially when the culture conditions and flocculation parameters are well optimized.

Acknowledgement: The authors wish to acknowledge Tertiary Education Trust Fund, Nigeria for sponsoring this study under Institution Base Research and Ibrahim Badamasi Babangida University for providing basic facilities for the study.

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