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Assessment of Bioflocculant Production by *Proteus vulgaris* Isolated from Ikpoba River

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ABSTRACT

Bioflocculant production potential of freshwater bacteria isolated from Ikpoba River where industrial effluents from brewery are been disposed was investigated. The 16S rDNA sequence analysis revealed 86 % similarity to Proteus vulgaris MF472597.1 and the nucleotide sequence was deposited in GeneBank as Proteus vulgaris BKG3 with accession number MK350331. Bioflocculant was optimally produced when peptone (80.90 % flocculating activity) and glucose (81.00 % flocculating activity) were used as nitrogen and carbon sources respectively. The flocculating activity of the bioflocculant was stimulated in the presence of Calcium Chloride ($CaCl_{2)}$. The highest flocculating activities for kaolin clay suspension were achieved at pH 7.0 at a dosage of 0.16 mg/ mL. Chemical analyses of the purified bioflocculant revealed that it was composed of 50 % carbohydrate and 4 % protein (wt/wt). The efficient flocculation capabilities of bioflocculant produced by Proteus vulgaris MK350331suggest potential use in wastewater treatment.

1. Introduction

Flocculation can be referred to as the separation of colloids or fine particles from liquid solutions to form loose aggregations or soft flakes, either by addition of clarifying agent or spontaneously. Flocculants are clarifying agent used to promote flocculation possessing properties that informs their use in flocculation technologies which enable them floc out suspended solutes from solvents [1]. They are applied in water and sewage treatment procedures, industries that produce cheese, downstream processing and brewery industry.

Bioflocculants (BFs) are metabolites secreted by micro-organisms during growth to reduce colloidal particles, bacteria, solid particles and cells in liquid medium [2]. The use of bioflocculant in industrial applications is due to its biodegradability, non-toxicity and harmless nature. Bioflocculants have high biopolymers such as extracellular saccharides, glycoprotein, proteins and cellulose which are used by microorganisms for attachment to solid surfaces, forming bio-films sometimes [3, 4].

The potential of various microorganisms (algae, bacteria, actinomycetes, archaea, cyanobacteria, protozoa, fungi and even crustaceans and marine worms) as producers of bioflocculants have been explored in the past few decades [5], however there has been no report on large scale commercial production of these bioflocculants due to low yields of the final product and the high costs involved in fermentation processes. This has resulted in slow progress not only in their industrial application but also in scientific research.

Bacteria that produce bioflocculants can be isolated from domestic kitchen drains, activated sludge, soils from agricultural fields, gardens and river water. For the past years, the chemical synthetic flocculants (organic flocculants; polyacrylamide, polyethyleneimine alum, inorganic flocculants; aluminium sulphate and polyaluminium chloride and natural flocculants; gelatin, starch, tannin, cellulose) have been widely used due to their effectiveness and low costs [6]. However, according to reports, synthetic flocculating agents may lead to human health problems and environment pollution. For example, acrylamide, a monomer of polyacrylamide frequently used chemical flocculant, has been shown not only to be non-degradable but also show carcinogenic and neurotoxic capabilities [7]. These negative properties of synthetic flocculants have encouraged exploration for new and safer alternatives such as bioflocculants.
In this paper, bioflocculant production potential of *Proteus vulgaris* MK350331isolated from Ikpoba River in Benin City was assessed.

2. Methodology

2.1 Source of bacteria and culture media

The bacterium was isolated from water samples collected from Ikpoba River behind Guinness Nigeria PLC where industrial effluents are disposed. The bioflocculant production medium (BPM) used was composed of 10g glucose, 0.5g Mono-potassium Phosphate (KH_2PO_4), 2g Di-potassium Phosphate (K_2HPO_4), 0.1g $CaCl_2$, 0.5g Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 1.0g peptone, dissolved in 1litre distilled water. A pH adjusted to 7.0 with Sodium Hydroxide and Hydrogen chloride was used. The preparation was sterilized by autoclaving at 121^0C for 15mins.

2.2 Bioflocculant Production Evaluation

A loopful of the bacteria isolates was introduced (inoculated) into 250ml flask containing 50ml of bioflocculant prepared medium and maintained (incubated) on a shaking incubator at 120 rpm for 100 hours at 28^0C. After the period of incubation, 2 ml of the fermented culture broth was centrifuged at 4500 g for 30 minutes at 4^0C, so as to separate the suspended cells. The flocculating activities of the produced bioflocculant were investigated using supernatant free of cells [8].

2.3 Assay for bioflocculant Production Potential

The assay of flocculant production potential was carried out using Kaolin clay suspension. 1.5 ml of the culture supernatant and 2 ml of 1% $CaCl_2$ were put into 75 ml of kaolin clay suspension in 100ml conical flask. The mixture was carefully stirred for 60secs and left to stand still on the

work bench for 7 min. The blank serving as control was prepared also; however, a fresh broth was used to replace the bioflocculant [8]. The turbidity in the upper phase supernatant was determined at 500 nm with a Thermoscientific GENESYS 10S UV-Vis Spectrophotometer.

The flocculating potential was estimated as

$$\text{Flocculating potential (\%)} = \{(B-A)/B\} \times 100 \quad (1)$$

Where: A is the turbidity (optical density) of the sample at 500nm; B is the (turbidity) optical density of the blank (control) experiment at 500nm. All investigations were done in triplicates for calculation of mean values.

2.4 Factors affecting Bioflocculant Production and flocculating activity

The experiment to investigate the effect of carbon source on bioflocculant production was done according to the description of [9], where glucose in the screening medium was replaced with lactose, sodium carbonate, sucrose and fructose, while the nitrogen sources was replaced with Ammonia sulphate, urea, peptone, Ammonia chloride and tryptone in order to examine the effect of nitrogen source on bioflocculant production [10].
To assess the effect of various cations on flocculating activity, the $CaCl_2$ in the flocculation assay was replaced with the metals of the following salts Potassium Chloride (KCL), Sodium Chloride (NaCl), Aluminium Chloride ($Alcl_3$) and Iron (III) Chloride ($FeCl_3$) and the flocculating potential was measured [11]. The effect of pH was assessed by adjusting the pH of the kaolin clay suspension between the range of pH 4–10 with 0.1M Hydrogen chloride (HCl) and Sodium hydroxide (NaOH) [12].

2.5 Time Course Assays

The test bacterium was first cultured in 45 mL bioflocculant production media that was in a 250 mL flask on rotary shaker (160 rpm) at 28 ^{0}C for inoculation preparation. After 24 h of cultivation, 1% of the culture broth of bacteria isolate was used as seed culture to inoculate 45 ml of production media in 250 mL conical flasks. Medium samples were taken at 24 h intervals for 168 hours and the values for pH, optical density and flocculating potential were taken note and recorded carefully [13]. All experiments were carried out in triplicates.

2.6 Bioflocculant Extraction and Purification

After fermentation for about 72 hours, the culture broth was stimulated in a centrifuge at 4000 g at 4 ^{0}C for 30 mins to remove insoluble bacteria cells. About one volume of distilled water was added to the supernatant phase and stimulated in a centrifuge at 4000g at 4 ^{0}C for 15 mins to remove other insoluble substances. Two volumes of cold aliphatic alcohol (ethanol) were later added to the supernatant, vortexed and left standing for at 4 °C for at 24 hours. The resultant precipitate was dried in a vacuum dryer to get the crude bioflocculant. The crude biopolymer was then re-dissolved in water to get a solution, thereafter one volume of chloroform and *n*-butyl alcohol mixture was added. The mixture was mixed, poured into a separating funnel and allowed to stand for another 24 hours at room temperature. The supernatant was discarded and two

volumes of cold ethanol were added to recover the precipitate and then freeze dried to obtain a purified bioflocculants carefully [14].

2.7 Jar Testing Method for Bioflocculant Dosage

Jar testing was used in investigating the maximum dosage at which purified bioflocculant will flocculate kaolin solution. Dosage concentration of bioflocculants (0.02, 0.04, 0.08, 0.16, 0.32, 0.64mg/ mL) were prepared and used to evaluate their flocculating potentials. Three grams of kaolin clay was weighed and dissolved in 1 L of distilled water. 2 mL of $CaCl_2$ and 1.5 mL of bioflocculant solution were both added to 75 mL kaolin suspension in 250 mL beakers. The solution was mixed at 200rpm for 3mins and the speed reduced to 45rpm for 10mins of agitation. The solution was thereafter poured into 100 ml measuring cylinder and sediment for 10 min and 2 ml of clear supernatant withdrawn and the flocculating activity was read at 500nm with a spectrophotometer (Thermoscientific GENESYS 10S UV-Vis Spectrophotometer) [1].

2.8 Chemical Composition of the Purified Bioflocculant

The total protein content of the purified bioflocculant was determined using Folin-Lowry method and bovine serum albumin (BSA) was used as the standard solution [15]. The total sugar content was determined by phenol- sulphuric acid using glucose as a standard solution [7].

2.9 Molecular Identification of Bioflocculant-Producing Bacterial Isolates

DNA extraction was conducted using boiling method. Here, a loopful of pure single colonies of the bacterium was dispensed into appendorf tube containing 200µL of nuclease free water. The appendorf tube was vortexed and inserted inside the heating block machine to heat open the cells giving rise to the exposed DNA at 100 °C for 15 minutes the appendorf tube was removed and inserted into a micro-centrifuged to spin at 11000 rpm for 2 minutes so as to separate the debris of the cells from the supernatant. The supernatant was gently transferred to a clean tube, stored at 4 °C to be used as the DNA template. The 16SrRNA gene was amplified by PCR using universal primer (27F-5GTGCCAGCAGCCGCGCTAA-3) and (1492R-5 AGACCCGGGAACGTATTC-3 [16].The PCR master piece contained 12.5 µL one taq master mix, 1.25 µL forward and backward primers, 5.0 µL nuclease free water, and 5.0 µL DNA template The PCR process was performed in Gene PCR Cycler with the recommended guideline of an initial denaturation (94 °C for 5 min), 36 cycles of denaturation (94 °C for 30 s), annealing (56 °C for 30 s) and extension (72 °C for 45 min), and a final extension (72 °C for 7 min) and hold at 4^0C. Gel electrophoresis of PCR products were conducted on 1.5 % agarose gel to confirm that a fragment of the correct size had been amplified [16].

2.10 Statistical Analysis

All experimental analysis was done in triplicates and results expressed as the mean ± standard deviations. Data was subjected to one-way analysis of variance (ANOVA) using minitab student release 12. A significance level of $P \leq 0.05$ was used. Microsoft Excel 2010 package was used to chart graphs.

3. Results and Discussion

3.1 Screening and identification of bioflocculant producing bacterium

Over 114 freshwater bacteria isolated from Ikpoba River in Ikpoba Okha Local Government Area of Edo State, Nigeria were screened for bioflocculant producing abilities. Among these was the test bacterium showing 81% flocculating activity. PCR amplification of the 16S rRNA gene of the bacterium produced a PCR product of amplicon size of 1.5 kb size. Basic Local Alignment Search Tool (BLAST) analyses of the nucleotide sequence of the amplified product showed 86% similarity to *Proteus vulgaris* MF472597.1 and the nucleotide sequence was deposited in GenBank as *Proteus vulgaris* BKG3 with accession number MK350331.

Morphologically, *Proteus vulgaris* is about 1-3 µm × 0.5 µm (micrometer), can be arranged singly, in pairs or in short chains and sometimes in clusters. It is actively motile and well known for the swarming growth on ordinary medium like nutrient agar medium.

Proteus vulgaris is rod shaped, nitrate reducing, indole + and catalase positive, hydrogen sulphide producing, Gram negative bacterium that can be found in water, soil and faecal matter. It is a non-sporing and flagellated bacterium with peritrichous flagella arrangement with no capsule. The positive aspects of the bacterium presence in water and soil are connected with exceptional features displayed by autochthonic *Proteus* spp. strains detected in these environments [17]. These rods acquire various metabolic abilities allowing their adaptation to different environmental conditions such as high concentrations of heavy metals or toxic substances, which may be exploited as sources of energy and nutrition by the bacteria. *Proteus* spp. abilities to tolerate or utilise polluting compounds as well as promote plant growth provide a possibility of employing these microorganisms in bioremediation and environmental protection. *Proteus vulgaris* is able to successfully implant itself in a complex cheese ecosystem and contribute to the organoleptic properties during ripening [18].

3.2 Time course of bioflocculant production

The inter relationship between Optical density; pH and flocculating activity was investigated over a growth period of 168 hours. The flocculating potential of the bioflocculant increased with increased cell growth depicted as Optical density, cell growth increase started at 48 hours with 49.5 % flocculating activity and a maximum activity of more than 88 % attained at about 144 hours (Figure 1). Similar result was reported by Liu *et al*. [4] for bioflocculant produced by *Chryseobacterium daeguense*. The pH of the culture medium decreased from an initial pH 7 at 0 hours to a pH 5.23 at 24 hours, and a steady decrease continued until pH 3.51 was attained at 168 hours. A decrease in pH observed is as a result of organic acid produced during metabolism of glucose since glucose was the major component of the production medium [19] or the production of organic acid during bacteria metabolism. The optical opacity (density) of the medium also increased with increase in culture time from an initial of 0.24 to 1.55 after 144 hours and thereafter a decrease was observed with increase in cultivation time. The cell growth curve was directly parallel to flocculating activity curve indicating that production of bioflocculant was through biosynthesis and not cell death. This result is in accordance with a report by Deng et al.

[20] where cell growth curve was parallel to flocculating activity curve for bioflocculant produced by *Aspergillus parasiticus.*

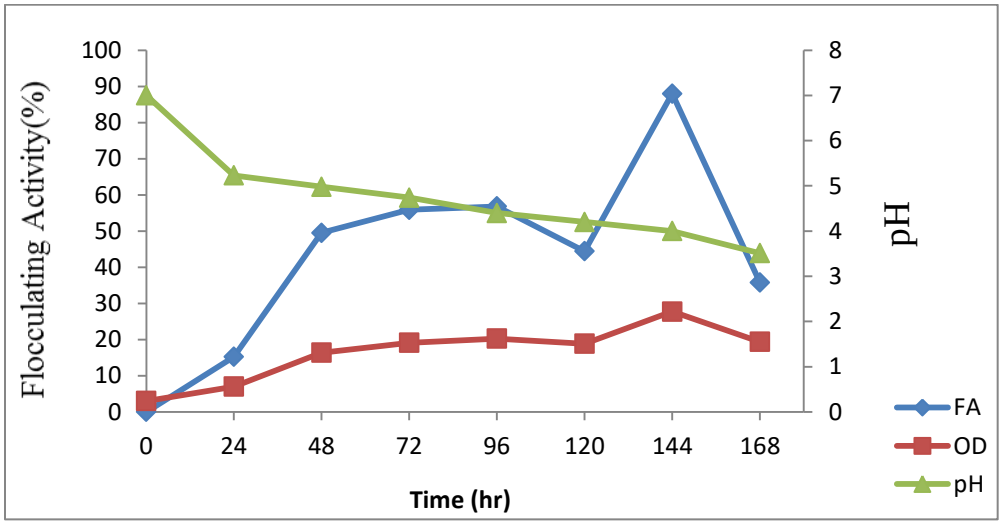

Figure 1: Time course assay for bioflocculant production by *Proteus vulgaris* MK350331

3.3 Effects of carbon and nitrogen sources on bioflocculant production

Carbon and nitrogen sources help in improving bioflocculant yield, productivity and flocculating potential carefully [14]. It has been reported that nitrogen and carbon sources significantly favour the growth of bacteria hence improving production of bioflocculants along with other constituents such as salt ions [21].

This current study investigated the effects of carbon sources such as fructose, sodium bicarbonate (Na_2CO_3), glucose, lactose and sucrose. From the results obtained, glucose supported the highest bioflocculant production with an optimum flocculating activity of 81% at $P \leq 0.05$ compared to that of fructose (39%), Na_2CO_3 (13%), lactose (32%) and sucrose (45%). Sodium bicarbonate was the least favoured carbon source by *Proteus vulgaris* MK350331 with a 13% flocculating activity (Figure 2). Glucose had the most pronounced effect because it gets readily utilized by microorganisms compared to other sugars; hence the wide report as a preferred carbon source in various studies for production of bioflocculant by various microorganisms [22]. This result finding is similar to study by Okaiyeto et al. [23] where glucose also showed significant support for bioflocculant production by *Bacillus* sp.

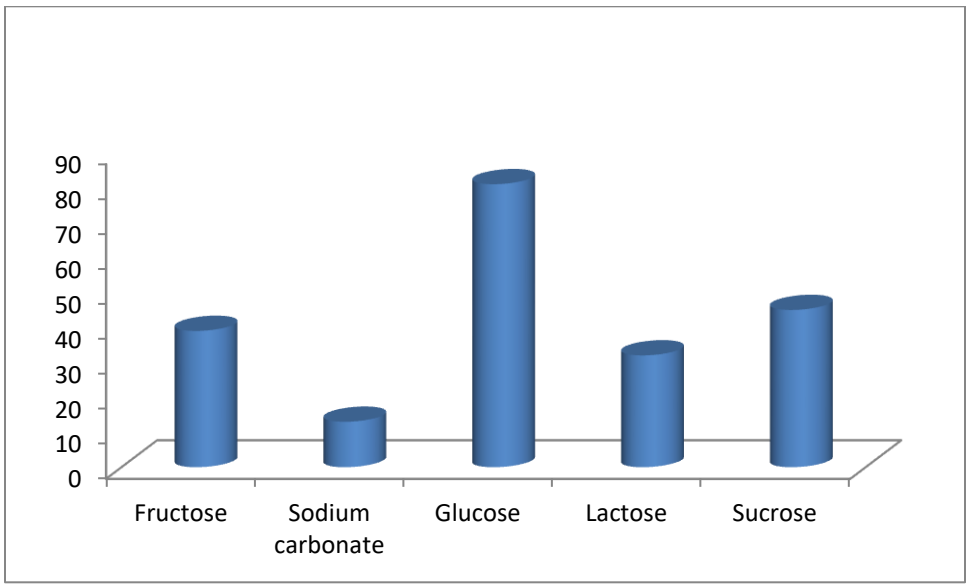

Figure 2: Effects of carbon sources on bioflocculant production by *Proteus vulgaris* MK350331

The production medium was further optimized with different nitrogen sources such as Ammonia Chloride (NH_3Cl), Ammonia sulphate ($(NH_4)_2SO4$), tryptone, peptone and urea. It was observed that peptone showed the highest flocculating activity of 80.90% while NH_3Cl showing the least flocculating activity of 23.00 % for bioflocculant production by *Proteus vulgaris* MK350331 (Figure 3). This finding is in accordance with the results obtained by Piyo et al. [24] for production of bioflocculant by *Bacillus* sp. in which peptone was found to be suitable nitrogen sources. It was observed from the study that *Proteus vulgaris* MK350331 preferred organic nitrogen sources for production of bioflocculant. The ability for microorganisms to use organic nitrogen sources easily may be due to the fact that they are readily available in nature and nitrogen is easily available from organic compounds [8]. Microorganisms may either use organic nitrogen sources, inorganic sources of nitrogen or a combination for bioflocculant production.

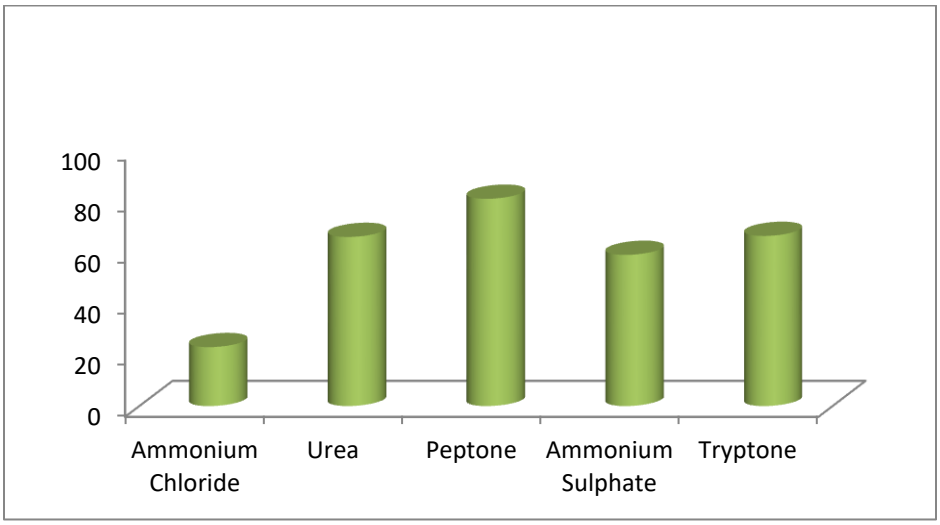

Figure 3: Effects of nitrogen sources on bioflocculant production by *Proteus vulgaris* MK350331

Bioflocculant are biopolymer that are negatively charged hence the limitations observed during its application in water purification since most water pollutants carry negative charge [22]. It is imperative to utilize coagulants so as to render the negative charge on both the suspended particles and bioflocculant inactive. For high flocculating activity to be achieved, cations are needed as coagulants to aid the flocculation mechanisms [25].

The effects of different cations were investigated, and observation revealed that flocculation process was highly stimulated by cations addition; with Calcium Chloride ($CaCl_2$) enhancing bioflocculant production with 80 % flocculating activity by *Proteus vulgaris* MK350331 and the least flocculating activity of 30 % was observed when the medium was optimized with Sodium Chloride (NaCl).(Figure 4).

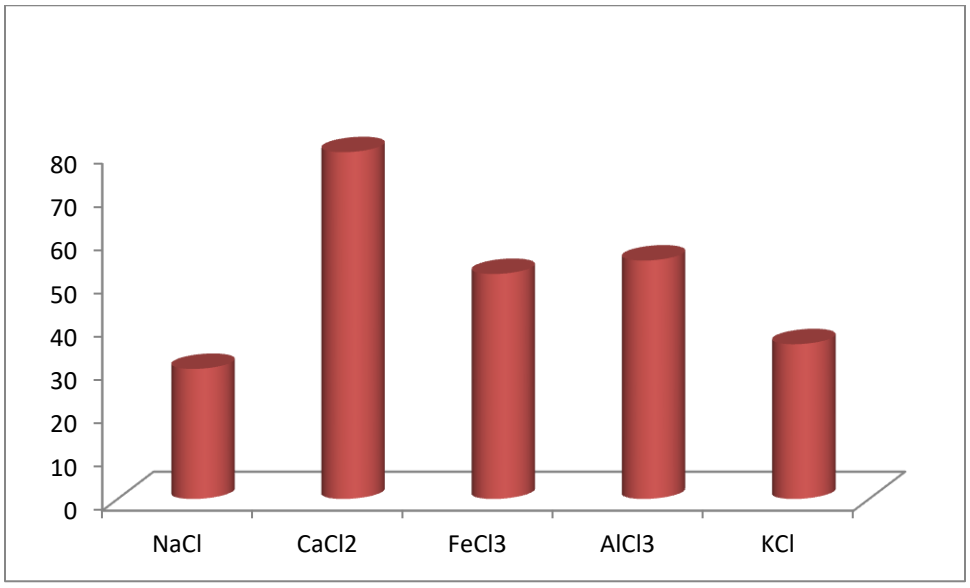

Figure 4: Effects of cations on bioflocculant production by *Proteus vulgaris* MK350331

3.4 Effect of bioflocculant concentration on flocculating activity of purified bioflocculant

The appropriate bioflocculant concentration for subsequent experiments was determined by investigating different bioflocculant concentration ranging from 0.02 – 0.64 mg/ mL as shown in Figure 5. Concentration requirement is very important when determining the performance of bioflocculant in industrial application in order to minimize cost and attain better performance. The inter-relationship that occurred between bioflocculant dosage and flocculating potential was investigated in this study; it was observed that 0.16 mg/mL was the optimal dose for bioflocculant production with flocculating activity of above 85% by *Proteus vulgaris* MK350331. From previous reports, bridging flocculation mechanism will not be improved at a low bioflocculant dosage, insufficient bioflocculant concentration might not effectively make the negative charges on kaolin particles to be inactive [22], and consequently high dosage will result in high viscosity blocking adsorption site and reducing flocculation [1]. Higher or lower dosages result in lower flocculating potential.

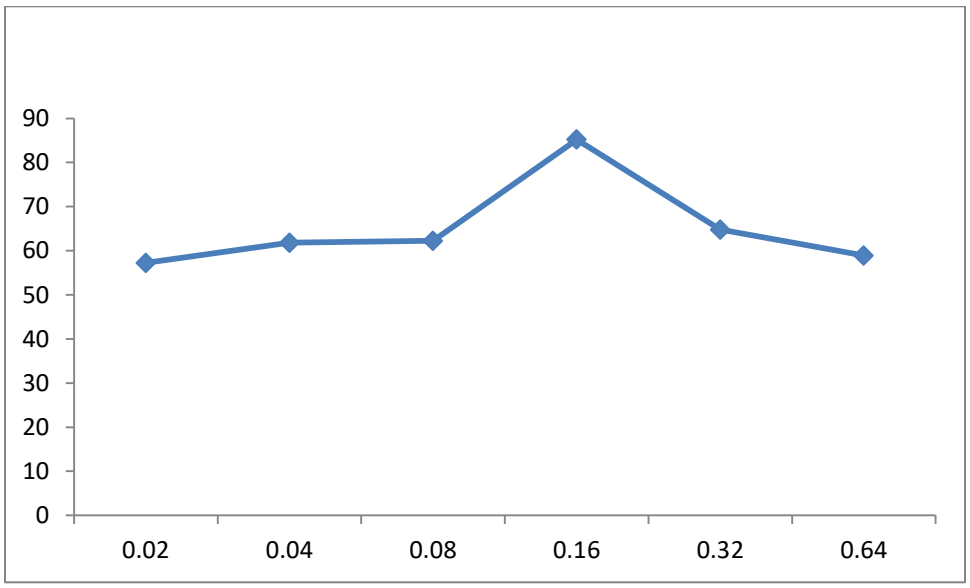

Figure5: Effect of concentration on flocculating activity of bioflocculant produced by *Proteus vulgaris* MK350331

The reduction in flocculation potential that occurred due to increase in bioflocculant concentration is as a result of a mechanism called "flocculation deterioration mechanisms" where some tiny suspended particles were entrapped by flocculants that are concentrated, resulting in lower flocculating potential [25].

3.5 Effect of pH and Temperature on flocculating activity of purified bioflocculant

The pH of culture broth is a major factor affecting bioflocculation mechanism and the optimum pH for bioflocculant production varies with different organisms [27]. When cultivation of microorganisms takes place at unfavourable pH, cell growth as well as the production of bioflocculant may be limited. As shown in Figure 6, *Proteus vulgaris* MK350331 produced bioflocculant optimally at pH 7.0 (86% flocculating activity) and flocculating activity decreased as pH migrated towards alkalinity. Kurane *et al.* [28] reported that bioflocculant produced by *Rhodococcus erythropolis* was stimulated at a neutral pH, Cosa *et al.* [29] also reported that bioflocculant produced by *Virgibacillus* sp. Rob preferred alkaline pH conditions.

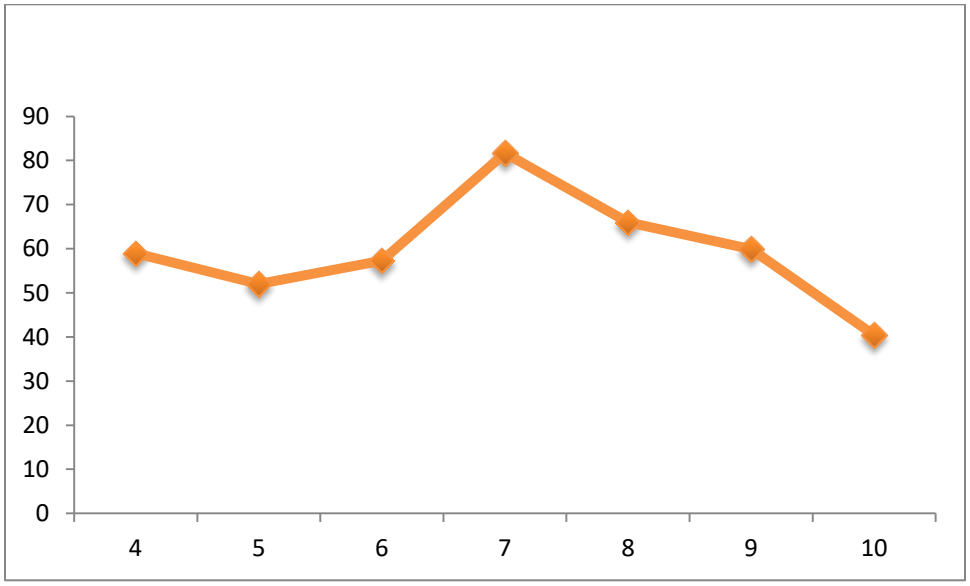

Figure 6: Effect of pH on flocculating activity of bioflocculant produced by *Proteus vulgaris* MK350331

The maximum flocculating activity of 80% was achieved at 30^{o} C. The activity decreased when the temperature level increased above 40^{o} C suggesting that cultivation temperature affects bioflocculant production (Figure 7).

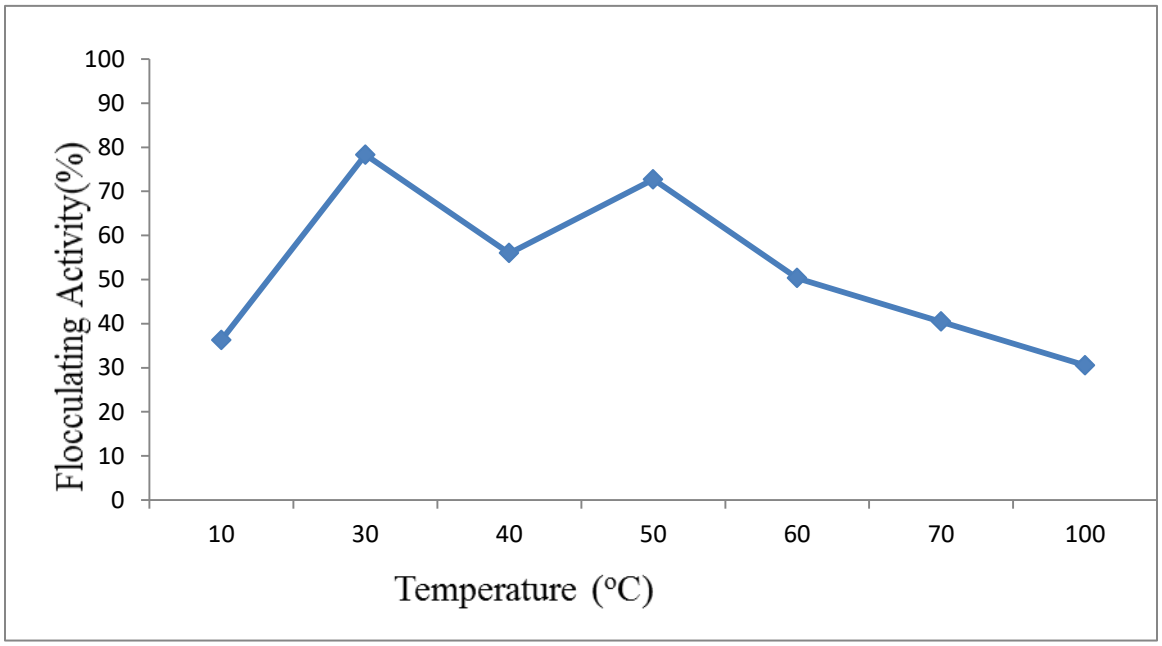

Figure 7: Effect of temperature on flocculating activity of bioflocculant produced by *Proteus vulgaris* MK350331

The bioflocculant produced by *Proteus vulgaris* MK350331 was composed of 50% (wt/wt) carbohydrates and 4% (wt/wt) protein.

4. Conclusion

A bioflocculant producing bacterium isolated from freshwater habitat, identified as *Proteus vulgaris* and named *Proteus vulgaris* BKG3 flocculated optimally at pH 7, at temperature of 30^oC with the aid of peptone and glucose and stimulated in the presence of $CaCl_2$ has demonstrated high flocculating activity of 92% for kaolin clay suspension at a dosage of 0.16mg/mL making it viable for practical application in wastewater treatment.

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