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Bioflocculant Producing *Bacillus megaterium* from Poultry Slaughterhouse Wastewater: Elucidation of Flocculation Efficacy and Mechanism

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Abstract: The study focused on isolating bioflocculant-producing microorganisms from poultry slaughterhouse wastewater (PSW). Microorganisms (n = 20) were isolated, and the D2 isolate, identified as *Bacillus megaterium* using 16S rDNA and RpoD (sigma 70), had maximum flocculation activity. Furthermore, characteristics of the bioflocculant produced by *B. megaterium* were determined, and the optimum storage conditions, including the flocculation mechanism, were identified. The bioflocculant was composed mainly of polysaccharides and proteins and was better stored frozen in a crude form. Furthermore, the flocculation efficacy was assessed using response surface methodology at pH 4 (min) and 9 (max), bioflocculant dosage of 1% (min) and 3% (max, v/v), indicating pH 6.5 and dosage of 2% (v/v) as optimum flocculation conditions for floc formation under ambient temperature. These results were further confirmed with microscopy assessments with zeta potential measurements confirming that the bioflocculant was ionic, albeit charge neutralization was not the primary mechanism for floc agglomeration. Hydrogen bonding was predominant, indicative of a neutralization-bridging mechanism, an assertion also based on the functional groups prevalent in the isolate-*B. megaterium*. The results obtained indicate that bioflocculants can be used to treat isolates that are sourced from wastewater.

Keywords: *Bacillus megaterium*; bioflocculant; bridging; charge neutralization; flocculation activity

Citation: Mukandi, M.R.; Basitere, M.; Ntwampe, S.K.O.; Chidi, B.S. Bioflocculant Producing *Bacillus megaterium* from Poultry Slaughterhouse Wastewater: Elucidation of Flocculation Efficacy and Mechanism. *Appl. Sci.* **2024**, *14*, 3031. https://doi.org/10.3390/app14073031

Academic Editor: Juan García Rodríguez

Received: 11 January 2024
Revised: 27 March 2024
Accepted: 29 March 2024
Published: 4 April 2024

1. Introduction

Flocculation is a method that promotes solid-liquid separation. It is a cheap and efficient way of aggregating colloidal particles to form bigger flocs that can be separated from wastewater [1,2]. Flocculation is widely used as a separation technique in various floc removal facilities. Flocculation is achieved by using flocculants to extract insoluble substances from a suspension [3], as they enable dispersed particles/colloids to aggregate. They aggregate fine and subtle pollutants and form bigger flocs. Such pollutants are usually challenging to get rid of as they tend to be suspended in the liquid medium instead of settling or floating at the top; hence, the application of flocculants makes them heavier and more prominent, thus easier to separate [4].

Flocculants are characterized based on their chemical composition and are grouped into three categories, namely (i) inorganic, (ii) organic, and (iii) natural occurring [5]. Inorganic flocculants are mainly salts such as alum. They are cheap but highly dependent on operating conditions. Additionally, chemical flocculants produce by-products with high metal concentrations that are hazardous to both the environment and humans, leading to the divergence of attention to flocculants of microbial origin [6], referred to as bioflocculants.

On the other hand, organic synthetics are suitable in low dosages but have degradability problems [1]. Thus, organic and inorganic flocculants are harmful. In contrast, plant-based flocculants in the natural flocculant group are inconvenient to produce due to climate and ecological changes, leaving bioflocculants as a promising alternative [5]. Bioflocculants have excellent properties, which include low to non-toxicity, low concentration usage, and adaptability to a wide range of pH levels [1].

Bioflocculants are extracellular polymeric substances (EPS) naturally synthesized by microorganisms [7]. These metabolites are secreted during the growth of microorganisms and cell lysis. Many microorganisms such as fungi, algae and bacteria have shown the ability to produce bioflocculants [3] that are produced through microbial fermentation. This process is affected by various factors, including nutrient availability and composition, physicochemical parameters, and culture conditions [8]. The polymeric characterization of bioflocculants varies in terms of their composition of proteins, polysaccharides, polyamino acids, and sugars, with the polysaccharides and proteins receiving the most attention, as they constitute the most significant proportion of the flocculants and consequently influence their flocculation activity [4,9]. Various bioflocculation mechanisms are currently accepted, including sweeping, adsorption bridging, chemical reactions, and change neutralization [10]. The flocculation mechanisms of chemical flocculants are well known, but the mechanism related to bioflocculants needs to be fully explored [11].

According to the green chemistry analogy, chemical flocculants are unfavorable because they are environmentally hazardous; therefore, the need for safer eco-friendly alternatives does not need to be emphasized but is a necessity; consequently, bioflocculants are gaining popularity. Despite this, bioflocculants are deemed not ideal for use on an industrial scale because of their high production costs, low yields, and poor flocculation efficiency [12]. Therefore, it is of the utmost importance to screen for novel bioflocculant-producing microorganisms that produce bioflocculants with high flocculating capabilities and higher yields and understand how they function, including their optimal conditions, so that they can be applied on an industrial scale.

The main aim of this study was to isolate a bioflocculant-producing strain from poultry slaughterhouse wastewater (PSW), focusing on selecting the strain with the highest flocculation activity level. PSW is characterized by a complex composition of pollutants such as blood, feces, fats, oil and grease from the slaughtering of birds and also detergents from the sanitation of equipment and surfaces, resulting in high COD, suspended solids and other parameters being high in the effluent [13,14] including the presence of microorganisms. The inquiry included an analysis of many characteristics associated with the bioflocculant produced, including its storage conditions and the ideal parameters for flocculation. This study was conducted with the following specific objectives: (1) To isolate and identify microorganisms that produce bioflocculants that can be utilized for particle flocculation (2) To assess and characterize the flocculants, including storage conditions of cell-free bioflocculants, free and bound bioflocculant, and (3) To elucidate the flocculation mechanism of the bioflocculants. Furthermore, a comprehensive understanding of the flocculation mechanism has been achieved, shedding light on the operational principles of bioflocculants obtained from microorganisms in large-scale industrial applications. This knowledge is expected to provide significant benefits in terms of cost reduction in downstream bioflocculant processing.

2. Materials and Methods

2.1. Microbial Isolation

Twenty ($n = 20$) morphologically different bacterial microorganisms were isolated from swabs and the poultry slaughterhouse wastewater (PSW) collected from the drainage port of a poultry slaughterhouse in Cape Town, Western Cape, South Africa, which slaughters about 1 million birds per week, and then stored at 4 °C before use. The samples were subjected to repeated dilution, resulting in a dilution factor of $\times 10^8$. This approach was used to enhance the prospect of obtaining distinct colonies. A volume of 100 µL obtained

from the preceding four dilutions was used for spread plating onto nutrient agar (31 g/L, Biolab, Diagnostics Laboratory Inc., Budapest, Hungary). The plates were inverted and incubated at 36 °C for 24 h. After the incubation period, colonies were distinguished based on their physical appearance, which included color, appearance, texture, and form. Different and single colonies were picked and subcultured onto fresh nutrient agar plates using a streak plating technique. Continued sub-culturing occurred until pure cultures were obtained [15]. The pure culture plates were coded and numbered in alphabetical order. Bacterial isolates were screened for bioflocculant production using a standardized method by determining flocculation activity [16]. The isolate with high flocculation activity was characterized using morphological features (structure, color, Gram reaction) with an Olympus light microscope (Olympus CX21 FS1, Tudortech Pty Ltd., Bryanston, South Africa) at a ×100 magnification. Isolate D2 was the selected strain and was used for further experiments.

DNA was isolated from the bacteria (isolate D2) using Zymo's Bacterial DNA extraction kit (Zymo Research LLC, Califonia, United States of America) and a Thermo Fisher Scientific (Johannesburg, South Africa) DreamTaq™ DNA polymerase and universal primers 27F (5′ AGAGTTTGATCMTGGCTCAG 3′) and 1492R (5′ CGGTTACCTTGTTACGACTT 3′) for forward and reverse reactions, respectively, were then used for amplification of the target 16S region. The amplicons were gel extracted using Zymo's Zymoclean™ Gel DNA recovery kit (Zymo Research LLC, Tustin, CA, USA) and sequenced by the ABI PRISM™ 3500xl Genetic Analyzer (Thermo Fisher Scientific, Johannesburg, South Africa). The DNA was cleaned to remove post-cycle sequencing reaction contaminants using Zymo's ZR-96 DNA Sequencing clean-up kit (Zymo Research LLC, Tustin, CA, USA), followed by sequence analysis using a QIAGEN CLC Main Workbench v.7 (QIAGEN Sciences LCL, Germantown, MD, United States of America). The resultant 16S rDNA was used to determine the most similar sequences using the Basic Local Alignment Search Tool (BLAST) via the National Centre for Biotechnology Information (NCBI) databases (https://www.ncbi.nlm.nih.gov/) [Accessed on 22 April 2022]. For RpoD, the primer sequence for the targeted region was as follows: RpoD-F 5′ATCGCAAAACGGTATGTCG3′ and RpoD-R 5′TCTTGTACGRCCRTCATCAAG3′ (Zymo's Bacterial DNA extraction kit, Zymo Research LLC, Tustin, CA, USA), and the same procedure was followed.

2.2. Bioflocculant Production

The bioflocculant production medium was similar to the one used by [16] (2014). It consisted of 3.5 g $CaCl_2$, 0.5 g glucose, 0.5 g K_2HPO_4, 0.1 g $MgSO_4.7H_2O$, 0.05 g NaCl, 1 g $(NH_4)_2SO_4$, 2.5 g peptone powder, 0.5 g yeast extract in 500 mL distilled sterile water. The medium was filter sterilized using 0.20 µm sterile membrane syringe filters (Merck Millipore, Burlington, MA, USA) as it contained some heat-sensitive compounds. A loopful of bacteria was inoculated into 50 mL of bioflocculant production medium and incubated in a shaking incubator (Labwit ZWYR-240 shaking incubator, Labwit Scientific, Melbourne, Australia) at 36.5 °C under 121 rpm for 24 h. These cultural conditions were based on the previous work by [17] (2017). After the incubation period, 5 mL of the bacterial suspension was used as an inoculum to inoculate 45 mL of bioflocculant production medium, which was further incubated in a shaker incubator under the same conditions as the inoculum. The resultant fermentation culture broth was used for further experiments.

2.3. Flocculation Activity

Flocculation activity was quantified according to [18] (2019) using a kaolin clay suspension of 4 g/L. Kaolin suspension (50 mL) was aliquoted into 250 mL Erlenmeyer flasks and mixed with 1.5 mL of $CaCl_2$ (1% w/v) and 1 mL of crude bioflocculant sample. A control was prepared the same way but with the bioflocculant being replaced by a sterile bioflocculant production medium. The mixture was swirled and transferred into a 50 mL glass measuring cylinder. This mixture was left standing for 5 min at ambient temperature to settle. A sample of the top layer was withdrawn, and its optical density was read at 550 nm using a

spectrophotometer (Jenway 7305 Spectrophotometer, Bibby Scientific Ltd., Staffordshire, UK). The flocculation activity was calculated using Equation (1), with the quantification being conducted in duplicates, and the average was used for reporting results.

$$\%\textbf{\textit{Flocculation Activity}} = \frac{\boldsymbol{A} - \boldsymbol{B}}{\boldsymbol{A}} * 100 \quad (1)$$

where:

- A = absorbance of control, and
- B = absorbance of sample.

2.4. Bioflocculant Extraction and Purification

The fermentation broth from above was centrifuged at 4000 rpm using a megafuge (Heraeus megafuge 1.0, Gemini bv, Apeldoorn, The Netherlands) for 30 min to separate the cells from the supernatant. After that, the supernatant was mixed with chilled (4 °C) ethanol in a ratio of 1:2. The supernatant-ethanol mixture was swirled and then centrifuged further at 4000 rpm for 30 min to precipitate the crude bioflocculant. The supernatant was discarded, and the pellet was dialyzed against distilled sterile water overnight. After dialysis, water was discarded, and the pellet was vacuum-dried using a desiccator. The dried crude bioflocculant was used for functional group analysis using a Fourier transform infrared (FTIR) spectrometer.

2.5. Characterization of Bioflocculant Produced by FTIR

The main functional groups of the bioflocculant produced by Isolate D2 were identified using FTIR (Spectrum Two FTIR spectrometer, PerkinElmer Inc., Waltham, MA, USA). The absorption spectrum was recorded in the range of 4000–500 cm^{-1}. An IR spectrum database was used to analyze the organic compound.

2.6. Bioflocculants Stability Analysis

The effect of bioflocculant stability under different storage conditions over time was studied on a kaolin flocculation system by evaluating the flocculation activity of samples stored at room temperature, chilled samples, and frozen samples every 5 days for one month. Crude bioflocculant samples were aliquoted into Eppendorf and stored in their respective storage conditions (bench for room temperature, fridge at 4 °C for the chilled sample and freezer at −18 °C for the frozen one). The samples from the freezer were thawed at room temperature prior to use, after which the flocculation activity method was implemented.

2.7. Response Surface Methodology (RSM) Experimental Design

Charge neutralization and floc formation were studied to elucidate the flocculation mechanism of the bioflocculant produced by D2. Design-Expert (v. 11) was used to generate the statistical design of experiments. Central Composite Design (CCD), a widely adopted experimental design technique that allows researchers to efficiently explore the response surface and identify the optimal combination of factors, was used to optimize parameters (pH and bioflocculant concentration, i.e., dosage) being evaluated. Two numerical factors [pH (4–9) and bioflocculant dosage (1–3% (v/v))] were optimized. Experimental runs (n = 13; see Table 1 in the results section) were generated, and the generated conditions were used for zeta potential experiments. Zeta potential (mV), the response variable (Y), was fitted into a second-order model in the form of a quadratic polynomial equation to correlate it to the independent variables. The 3D graphs were generated directly from the software.

2.8. Zeta Potential

The surface charge/zeta potential of the particles was measured at pH, and bioflocculant dosage values were determined by RSM during the experimental design. The

procedure for flocculation activity was followed with minor changes. Kaolin clay suspension was prepared, and 50 mL was aliquoted into 250 mL Erlenmeyer flasks. The pH of each suspension was adjusted using 1 M of NaOH or 1 M of HCl to the values predetermined by CCD. 1.5 mL of 1% (*w*/*v*) of $CaCl_2$ was added to the suspensions, excluding those tested without $CaCl_2$. Predetermined dosages of bioflocculants were added to their respective flasks with a mixture of kaolin suspension and $CaCl_2$. After that, the flasks were gently swirled, and the contents were poured into 50 mL measuring cylinders and left to settle for 8 min. After settling, the top layer of the supernatant was carefully withdrawn for zeta potential measurements using a Zetasizer Nano ZS (Malvern Panalytical Ltd., Malvern, UK). The measurements made were for:

1. Kaolin clay before pH adjustments at various pHs,
2. Kaolin clay suspension with $CaCl_2$ at various pHs, and
3. Kaolin clay suspension with $CaCl_2$ and bioflocculants at various pHs and dosages.

Additionally, a loopful of the suspension was quickly recovered after addition into measuring cylinders and fixed onto slides for visual observations of floc sizes and formation under an electron Olympus CX21 FS1 microscope.

2.9. Bonding Type Determination

The bonding type of bioflocculant produced by isolate D2 was carried out using three types of chemical treatments, i.e., urea, HCl, and EDTA-Na_2. The bacterial flocculation activity described previously was carried out. However, the absorbance was measured after the settling period, and the supernatant was carefully removed. Chemical solutions of 5 M urea, 0.5 M HCl, and 10 mM EDTA-Na_2 were each added to a measuring cylinder with flocs in a manner that disturbs the flocs that had settled. The measuring cylinders were then slightly swirled and left to settle for a further 5 min. The absorbance (A_{550nm}) was measured, and qualitative observations were made [19].

3. Results and Discussion

3.1. Identification and Characterization of the Microbes

Twenty microorganisms were isolated from PSW and were screened for bioflocculant production using a kaolin clay suspension for flocculation activity assessments. Isolate D2 produced bioflocculants with the highest activity compared to the flocculants produced by other isolates. Its morphology was circular with cream-white colonies. A gram test showed a rod-shaped, arranged in linear sequences, and tested Gram-positive. The 16S rDNA results predicted that it was a Bacillus subspecies, *Bacillus arrybatai*, or *Bacillus megaterium* with 100% identity. For a group of Bacillus subspecies, sequencing the universal 16S rRNA region, which is usually expected of all bacteria, does not discriminate between them, hence making it difficult to identify them [20]. However, since the 16S gene did not sufficiently distinguish between the different species of *Bacillus*, for better resolution, RpoD was employed, which was able to distinguish well between the different *Bacillus* species. The organism was identified as *Bacillus megaterium* (98.71%), an identity with an accession number CP001983.1 which was deposited into the GenBank. Therefore, the strain and the bioflocculants were named *Bacillus* sp. D2 and D2, respectively.

It is worth noting that several researchers [5,21–24] have studied or characterized bioflocculant produced by *Bacillus megaterium*, which was either isolated from various niches or taken from a culture collection. The results or characteristics of the bioflocculants in these studies vary, which can be attributed to the origin of the microorganism, the methods used for flocculants recovery, and other factors. In addition, because bioflocculants are produced through microbial fermentation, this process is influenced by several variables, such as the availability and composition of nutrients, physicochemical parameters, and culture conditions, resulting in bioflocculants with varying properties [8]. Therefore, intensive research is required to understand their properties better if microbial flocculants are to be implemented on an industrial scale.

3.2. Characterization of the Produced Bioflocculant

The flocculation characteristics of bioflocculants are contingent upon the presence of functional groups [25]. Diverse microorganisms can create bioflocculants that exhibit distinct compositions and chemical structures [26]. FTIR was used to analyze the functional groups of the bioflocculant (D2). The resultant spectrum shown in Figure 1 had a broad polymeric stretch of the hydroxyl (O-H) group at 3296.94 cm^{-1}. A peak identified at 1631.88 cm^{-1} is related to the carbonyl group from amides, which represents the presence of proteins [27]. This was followed by a weak stretch at 1406.04 cm^{-1}, indicating the presence of the carboxylate group. Hydroxyl and carboxyl groups are favorable for flocculation, as they are known to participate in hydrogen bonding with the particles or pollutants [28]. The peaks at 1083.65 and 990.76 cm^{-1} depict ethers, typically sugar derivatives. Overall, it was evident from the FTIR analysis that bioflocculant D2 is made up of polysaccharides and proteins. This agrees with the literature reviewed that proteins and polysaccharides make up the highest composition of EPS, and they indirectly affect flocculation activity [4].

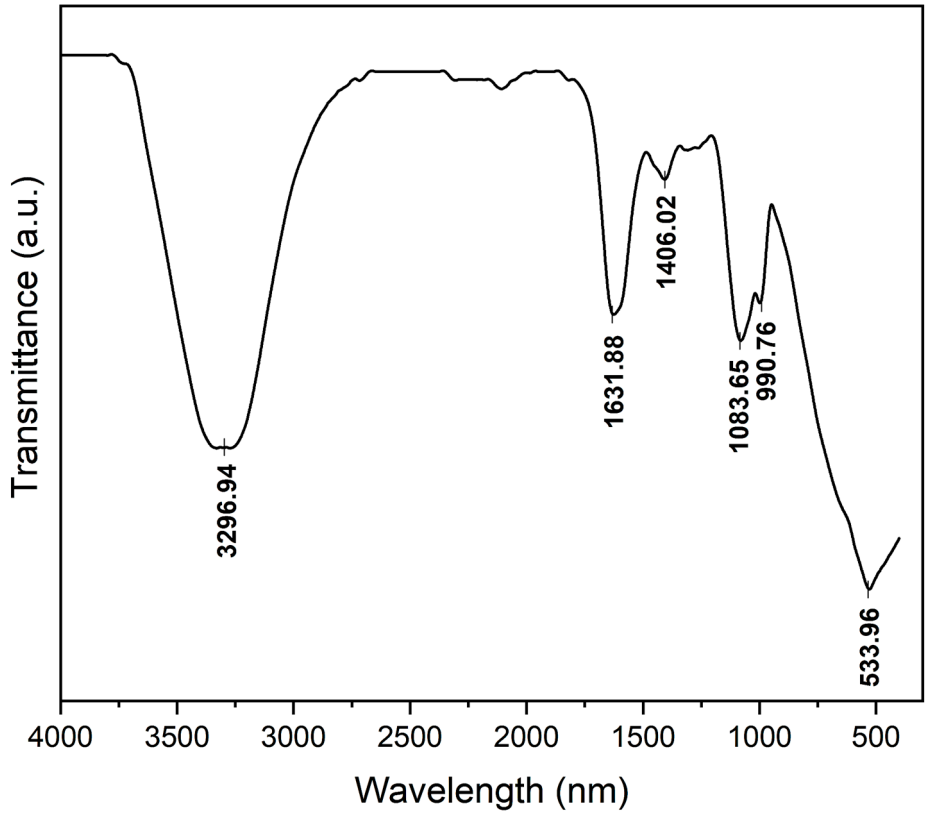

Figure 1. FTIR spectrum of bioflocculant produced by *Bacillus* sp. D2 (from *Bacillus megaterium*).

3.3. Effect of Storage Conditions

According to [12] (2023), the manufacturing cost has been identified as a significant obstacle to the widespread implementation of bioflocculants in industrial settings. Consequently, there is a pressing need to explore strategies to mitigate these costs. The current study examined crude bioflocculant as a cost-effective alternative in downstream solid-liquid separation processes. These processes often include using chemicals, resulting in additional processes and, thus, expenses that may be avoided. Numerous methods are employed for the downstream processing of bioflocculant, i.e., the purification step, which utilizes various chemicals. Chemicals such as chloroform and n-butyl-alcohol in the proportion of 5:2 (*v*/*v*) [29], 2% hexadecyltrimethylammonium bromide [30], chloroform and methanol at a ratio of 2:1 (*v*/*v*) [31] and 32% (*w*/*v*) sulfuric acid [32], have been used for bioflocculation purification processes. When manufacturing bioflocculants, it is necessary to avoid using an excessive amount of these chemicals, mainly if the production is to

be scaled up for wastewater treatment. Therefore, the storage conditions of the crude bioflocculant were evaluated to determine the bioflocculants' viability.

Figure 2 shows the differences in flocculation activity of the bioflocculant when stored under chilled, frozen, and room temperature conditions over twenty days. The results show reduced efficiency as the days progressed in terms of the various conditions. The worst form of preservation was observed at room temperature, with activity above 80% on day 0 and down to below 30% within five days of storage. The flocculants lost their efficiency as the days progressed to days 10 and 15. This may have been caused by the proliferation of microorganisms in the crude bioflocculant, as centrifugation may not have eliminated all cells. Overall, ambient temperature is conducive to the growth of various microorganisms. It is close to the optimal temperature for cultivating the microorganisms used in the experiments, thereby increasing the likelihood of survival and growth. In addition, *Bacillus* spp. are known to be spore-forming [33], so they may have resurrected and produced EPS in response to the conditions they were exposed to, rendering the bioflocculant ineffective. The chilled flocculant lost efficiency gradually over time until there was no activity. This could be because the temperature (4 °C) was unfavorable for the rapid revival of spores and the rapid growth of microorganisms if there were any remaining cells. It was noted that contamination risks exist at ambient temperature and in chilled environments. However, the frozen bioflocculants maintained a reasonable flocculation efficacy over time (n = 20 days). The minor decrease in flocculation efficiency may have been due to water accumulation under frigid conditions, as the chilling temperature used was 0 °C or during thawing. The results indicate that crude bioflocculant can be retained for future use under frozen conditions because it does not lose much of its effectiveness.

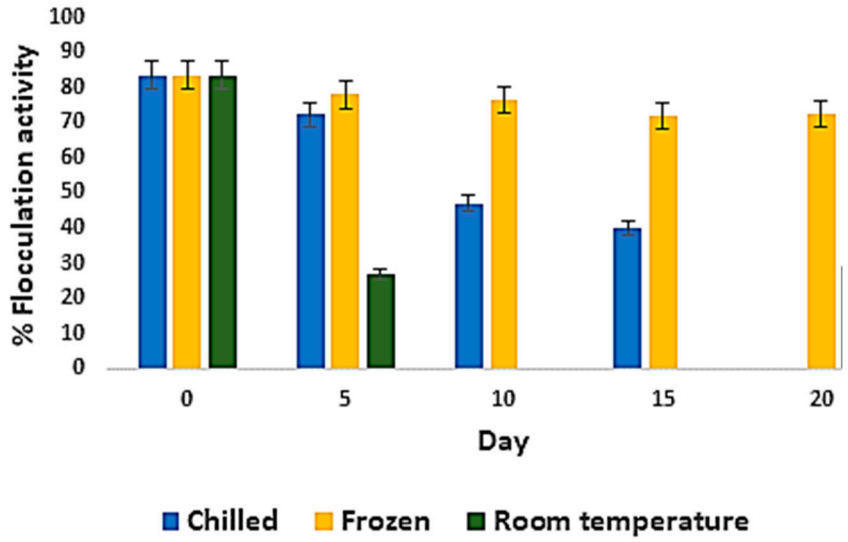

Figure 2. A graphical illustration of changes in flocculation activity of bioflocculant, *Bacillus* sp. D2 (from *Bacillus megaterium*), stored in various conditions over twenty days.

3.4. Zeta Potential

Zeta potential measures the force of repulsion between suspended particles and the distance that must be covered/overlapped for agglomeration [31]. The zeta potential measurements were used to determine if charge neutralization was the responsible mechanism and to determine the optimal conditions for flocculation using central composite design (CCD). Table 1 shows the zeta potential results under different physicochemical conditions. The zeta potential of kaolin clay decreased with an increase in pH values, i.e., it was −19.8 mV at pH 2.96 and decreased to −48.3 mV at pH 10.04. This resulted from increased electrostatic repulsion force on the negatively charged flocs due to raised charged densities from pH increases. However, the zeta potential increased after the addition of $CaCl_2$ for all pH conditions because Ca^{2+}, which is a divalent cation, enhances flocculation activity through charge neutralization and stabilization of negative charges [34]. It was further

noted that adding bioflocculant D2 to a suspension of kaolin clay and $CaCl_2$ decreased the zeta potential's negative charge. This observation confirms that the bioflocculant has a negative charge, namely an anionic charge, which, therefore, leads to the observed drop, hypothetically reducing the interfacial double layer (DL). The negative zeta potential of the bioflocculant may also be attributed to the presence of functional groups possessing negative charges, as shown by the FTIR analysis. The reduction observed may be attributed to the electrical repulsion resulting from the presence of similarly charged layers between the bioflocculants and kaolin clay [35]. This implied that the primary mechanism of flocculation is not charged neutralization since it was shown to occur only after the introduction of a cation. This indicates the involvement of a bridging mechanism, therefore necessitating further investigation by a bonding-type test. Similarly, some bioflocculants, such as poly(γ-glutamic acid) (γ-PGA) produced by *Bacillus subtilis* with a high flocculating activity stimulated by 0.325 mM of Mg^{2+}, provide an alternative source of biodegradable, eco-friendly Bioflocculant [36]

Table 1. Zeta potential results.

Conditions		Zeta Potential (mV)		
pH	**Bioflocculant (% *v*/*v*)**	**Kaolin**	**Kaolin/$CaCl_2$**	**Kaolin/$CaCl_2$/Bioflocculants**
2.96	2	−19.8	−4.64	−16.9
4	1	−31.7	−11.1	−20.4
4	3			−21.6
6.5	0.59			−21.0
6.5	2	−41.2	−20.3	−22.6
6.5	3.41			−21.4
9	1	−44.0	−17.9	−21.1
9	3			−20.6
10.04	2	−48.3	−19.7	−21.2

NB: In kaolin clay samples and Kaolin clay $CaCl_2$, bioflocculants were not added.

Additionally, it should be noted that the zeta potential results do not provide a direct means of determining the optimal conditions for achieving maximal flocculation activity. The results underwent Analysis of Variance (ANOVA) to statistically assess the suitability and significance of the model generated to describe the association between zeta potential, pH and bioflocculant dosage. However, it was determined that the model needed to be improved. The results from the ANOVA are shown in Table 2. According to [8] (2019), a *p*-value of less than 0.05 indicates statistical significance for the analyzed model or component. The current model had a significance level of 0.0281, indicating its significance.

Table 2. Analysis of variance (ANOVA).

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value	
Model	22.97	5	4.59	5.05	0.0281	significant
A-pH	4.18	1	4.18	4.59	0.0694	
B-Biofloculant dosage	0.2002	1	0.2002	0.2200	0.6533	
AB	0.7225	1	0.7225	0.7937	0.4026	
A^2	17.26	1	17.26	18.96	0.0033	
B^2	1.74	1	1.74	1.91	0.2094	
Residual	6.37	7	0.9103			
Lack of Fit	6.37	3	2.12			
Pure Error	0.0000	4	0.0000			
Corr. Total	29.34	12				

R^2 = 0.7828, adjusted R^2 = 0.6277, predicted R^2 = −0.5442.

Nevertheless, when evaluating the model's dependability using the coefficient of determination (R^2), it was determined that there was an insufficient correlation between the projected R^2 and the experimental response. The corrected R^2 value was determined

to be 0.6277, while the anticipated R^2 value was found to be −0.5442. This discrepancy exceeds the established threshold of 0.2, as [37] (2022) specified, indicating that the model was inadequate.

The second order model was established in terms of coded factors expressing the relationship between zeta potential, pH and bioflocculant dosage. This is shown by Equation (2), while the depiction of the response is shown in Figure 3.

$$Zeta\ potential(\mathrm{mV}) = -22.60 - 0.7226A - 0.1582B + 0.4250AB + 1.57A^2 + 0.5000B^2 \quad (2)$$

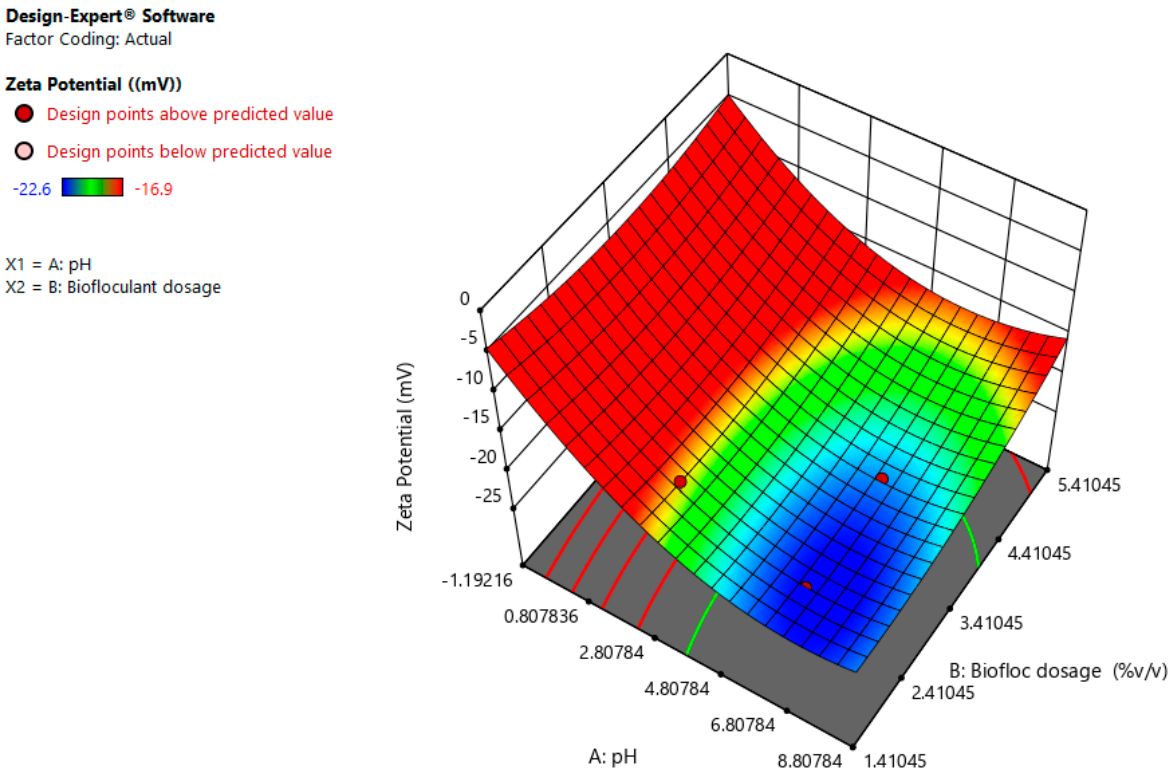

Figure 3. Graphical illustration of zeta potentials' relationship with changes in pH and bioflocculant dosage.

In addition, the three-dimensional quadratic model surface plot underscores the significance of pH as a determining factor in the modulation of zeta potential, as seen by the asymmetrical distribution observed in the graph. Given the unsatisfactory findings of the zeta potential analysis in establishing the optimal circumstances for maximal flocculation activity, an alternative approach was taken by observing formed flocs under a light microscope, as seen in Figure 4.

The optical microscope images revealed that the kaolin clay particles had a minimum and dispersed distribution before introducing any other substances. The suspension or dispersion of kaolin clay particles in a solution is due to their negative charge, leading to an electronic double layer [38]. The addition of $CaCl_2$ resulted in a modest increase in density, which may be attributed to the process of charge neutralization. The occurrence of flocculation is evident in the subsequent images after the introduction of the D2 bioflocculant. Larger, more compact aggregates were seen to have developed. The flocs exhibited a discernible shape in contrast to the aggregation of particles induced by $CaCl_2$, providing more evidence that the flocculation process involves charge neutralization followed by bridging [39].

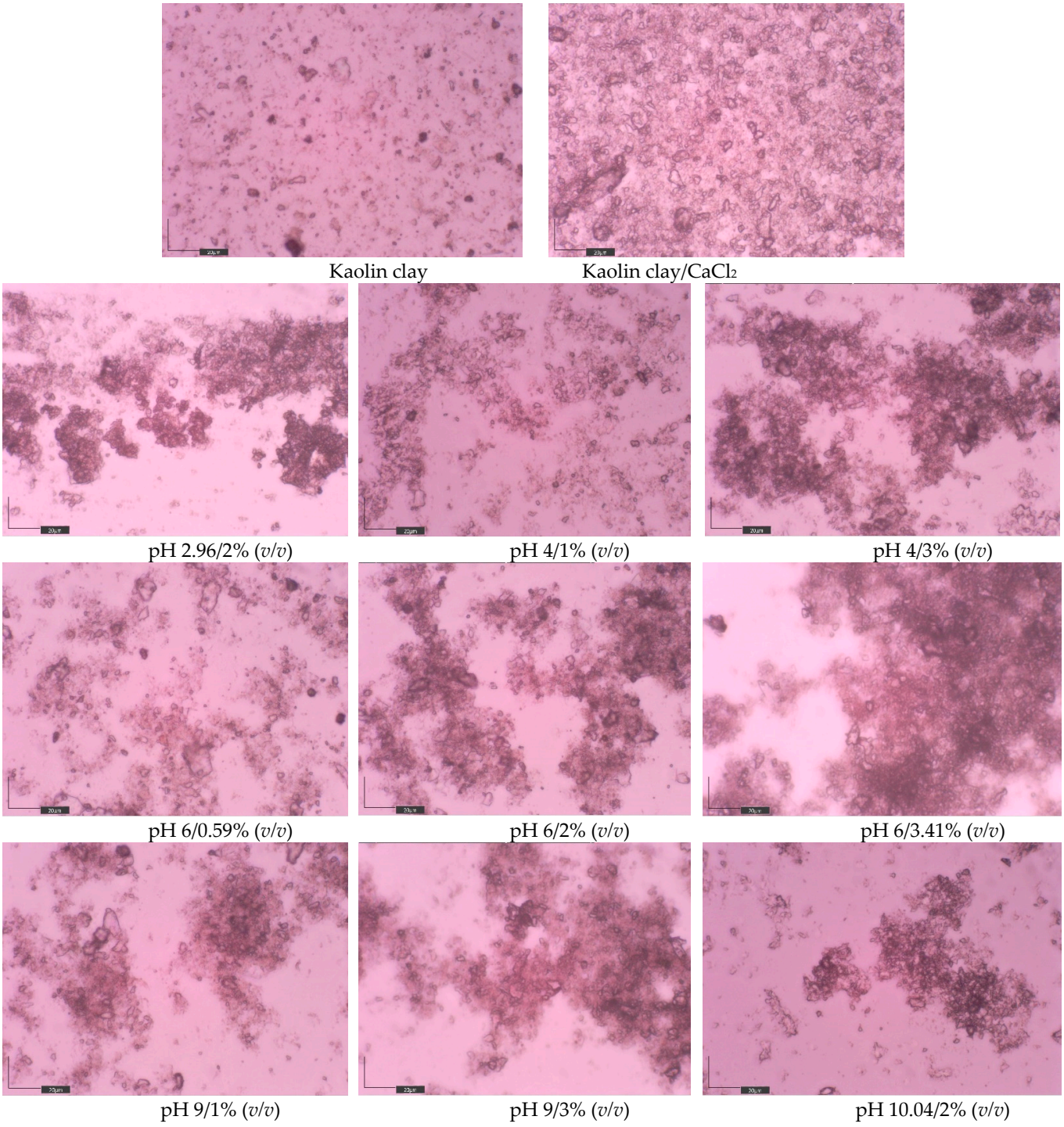

Figure 4. Microscopic images of floc formation at various pHs and bioflocculant dosage (20 µm scale bar).

Furthermore, it was apparent from the visual representations that the most favorable aggregation of flocs occurred at a pH level of 6.5, accompanied by a dose of 2% (*v*/*v*). Under such circumstances, the flocs exhibit a moderate size while the dose remains within an optimal range, avoiding excessive or insufficient amounts. Upon examination of the minimum dose, namely 0.59% (*v*/*v*) at pH 6.5, it became evident that the flocs exhibited a diminutive size and poor density. According to [23] (2020), the flocculation activity is diminished at lower doses due to reduced surface area available for adsorption. The floc exhibited a higher density than the other samples when the bioflocculant was administered

at its maximum dose of 3.41% (v/v) under pH 6.5. However, it was not chosen as the most suitable option because its dosage was about 1.5 times higher than the optimal level. This indicated that the dosage was too high. At a dosage of 2% (v/v), the bioflocculant exhibited effective flocculation activity.

Consequently, using the maximum dosage would result in wastage. Generally, the bioflocculant D2 exhibited favorable performance throughout a broad pH spectrum when used at an optimal dose. Moreover, its application for suspended solid elimination is supported by its beneficial influence on the aggregation of suspended particles into flocs.

Assessingly, a double layer does affect the stability of flocs. By using flocculation, liquid-solid perturbation occurs, leading to the thinning of the double layer and reducing the magnitude of repulsion between the particles. This is also enhanced by the roughness of the particles. This means that bubble movement increases particle movement, resulting in particle/kaolin collisions and thinning the double-layer structure. Thus, the electrical force decreases, i.e., reduced polarizability and double-layer relaxation, as the double layer of individual particles overlaps as flocs form [40]. As zeta potential evaluates the size of the magnitude of the double layer repulsive force, increases in pH from acidic to alkaline, as observed, tend to reduce the zeta potential in kaoline-water suspensions [41], even when CaCl2 is added to the suspension [42].

3.5. Confirmation of Flocculation Mechanism through Bonding Type Test

The flocs underwent three chemical treatments, namely 5 M Urea, 0.5 M HCl, and 10 mM EDTA-Na_2. According to [38] (2015), EDTA-Na_2 and HCl have been seen to disrupt ionic connections, whereas urea has been found to disrupt hydrogen bonds. The observation results indicated that the flocs treated with urea had a significant impact. This phenomenon may be attributed to the interaction between urea and kaolin clay particles, disrupting hydrogen bonds between the particles and the bioflocculant. Consequently, the flocs created experienced a collapse, resulting in the turbidity seen in the reaction system. The flocs treated with EDTA-Na_2 exhibited a minor disruption level, indicating weak ionic connections between the bioflocculant and the kaolin particles. In addition, applying hydrochloric acid (HCl) to the flocs resulted in little or insignificant impact. This implies that hydrogen bonding is mostly the primary bonding mechanism between D2 flocculants and kaolin particles. Hydrogen bonding occurs when hydroxyl and amide functional groups are present [43]. The chemical structure of bioflocculants contains functional groups that facilitate the bridging process by offering binding sites to attach contaminants or particles [44].

The results contrast those of [23] (2020), who discovered that the bioflocculant generated by B. megaterium exhibited an ionic bonding nature in the bonding type test. This observation underscores the need for a more comprehensive comprehension of the behavioral dynamics of bioflocculant-producing microorganisms to effectively leverage their economic potential, given their susceptibility to multifarious circumstances and situations. In summary, the underlying mechanism of action for bioflocculant D2 involves a two-stage process. The first phase involves the neutralization of charges via the interaction with Ca^{2+} ions, forming Ca^{2+} and kaolin complexes. This interaction serves to stabilize and neutralize the overall charge. Subsequently, a bridging process ensues, whereby this mechanism gives rise to a three-dimensional configuration, wherein the functional groups present in the bioflocculant, mostly polysaccharides, serve as the bridging component connecting the kaolin particles. The presence of these functional groups facilitates two forms of interaction, namely ionic and hydrogen bonding, resulting in the adsorption of bioflocculants onto the surface of kaolin clay particles [45].

4. Conclusions

The study identified a microorganism capable of producing a bioflocculant from PSW with strong flocculating properties. Additionally, the study focused on finding the best conditions for flocculation and understanding the mechanism behind the flocculation

process. Isolate D2, identified as Bacillus megaterium, exhibited the highest level of flocculation activity. Analysis of the bioflocculant indicated the presence of hydroxyl, amide, and carboxyl functional groups, indicating that it is mainly made of polysaccharides and proteins. The optimal storage conditions for the crude bioflocculant were found to be freezing temperatures, as opposed to room temperature or refrigeration. In addition, the RSM was used to identify the optimal flocculation conditions and investigate the underlying floc formation mechanism. This was achieved by assessing the zeta potential as the response variable while considering pH and bioflocculant dose as the independent factors. It has been determined that charged neutralization is not the fundamental mechanism for flocculation. The zeta potential findings yielded equivocal outcomes concerning the identification of optimal flocculation settings.

Consequently, the flocs were examined using a microscope. The best flocculation conditions were determined based on microscopy observations, with a proposed pH of 6.5 and a bioflocculant dose of 2% (v/v). Based on the results of a bonding type test, hydrogen bonding was the prevailing bonding mechanism, albeit the flocculation process was postulated to proceed in two distinct stages. The first stage involves neutralizing charges by introducing Ca^{2+} ions, while the second stage is facilitated by the bridging action of functional groups, providing more support for this hypothesis. It is advisable to do thorough research on bioflocculants to enhance our understanding of their suitability for industrial implementation, given their susceptibility to many influencing circumstances.

Author Contributions: Conceptualization, M.R.M., S.K.O.N. and M.B.; methodology, M.R.M.; software, M.R.M.; validation, S.K.O.N. and M.B.; formal analysis, M.R.M.; investigation, M.R.M.; resources, S.K.O.N. and M.B.; data curation, M.R.M.; writing—original draft preparation, M.R.M.; writing—review and editing, S.K.O.N., M.B. and B.S.C.; visualization, S.K.O.N.; supervision, S.K.O.N., M.B. and B.S.C.; project administration, M.R.M.; funding acquisition, M.B. and S.K.O.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Research Foundation Thuthuka Funding, cost center R017, Cape Peninsula University of Technology South Africa, grant number RK45, and the CPUT Vice Chancellor Achiever's Award. Furthermore, a scholarship for Melody Mukandi was provided for by Mwalimu Nyerere African Union scholarship scheme.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study is available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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