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Isolation and Characterization of a High-Efficiency Bioflocculant-Producing Strain of Pseudomonas fluorescens

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ABSTRACT

This study aimed to thoroughly explore, optimize, and characterize a Bioflocculant produced by a novel Pseudomonas fluorescens PF-1 strain isolated from active sewage sludge. When examined under refined culture conditions, including an incubation temperature of 36°C, a neutral pH of 7, and the use of sucrose and yeast extract as the most effective carbon and nitrogen sources, respectively, this strain exhibited promising flocculating capabilities. An inoculum volume of 5% (v/v) further amplified Bioflocculant manufacturing. Under these conditions, the strain demonstrated remarkably high flocculating activity, reaching 87.4%. Biochemical composition analysis revealed that the extracted Bioflocculant principally comprised carbohydrates at 76.3% and proteins at 17.2%, indicating a polysaccharide-protein complex nature. FTIR spectroscopy supported the existence of functional groups, including carboxyl, hydroxyl, and amide, crucial for flocculation mechanisms. SEM imaging depicted a dense, interwoven fibril matrix perfectly suited for trapping and aggregating suspended particles. The Bioflocculant also manifested high thermal resilience up to 80°C and retained activity across a wide pH range from 4 to 8. Wastewater treatment tests employing genuine effluents showed outstanding pollutant removal proficiencies, with reductions in chemical oxygen demand (COD) and biological oxygen demand (BOD) achieving an impressive 90.2% and 88.6% respectively. These conclusions demonstrate strong potential as a Bioflocculant created by P. fluorescens PF-1 as an eco-friendly and highly effective replacement for conventional chemical flocculants in wastewater treatment applications

Keywords: Wastewater treatment, Pseudomonas fluorescens, Bioflocculant, FTIR, COD, BOD, SEM..

1. Introduction

Flocculation is a crucial physicochemical treatment unit in water and wastewater treatment, applied as an essential step to facilitate the aggregation and removal of suspended particles, colloids, and contaminants. It is based on the employment of flocculating agents, high molecular weight substances able to destabilize particles and make them aggregate into flocs that can settle or be removed through filtration. This approach is widely used in the food, mining, pulp and paper, and city wastewater treatment industries.

Conventional flocculants can be categorized as inorganic flocculants, such as aluminium sulfate (alum) and ferric chloride, synthetic organic polymers such as polyacrylamide (PAM), and natural or microbial flocculants. Although synthetically derived flocculating agents seem to be very effective, the overall long-term view raises serious health and environmental issues. For instance, acrylamide monomers that may

be found in PAM are a proven neurotoxin and possible human carcinogen [1], whereas aluminium residuals are also associated with neurodegenerative disorders such as Alzheimer's disease[2].

Consequently, bioflocculants – biopolymers synthesized by microorganisms such as bacteria, fungi, and algae have become a potential answer as eco-friendly alternatives for water treatment. These biopolymers, made mainly of polysaccharides, proteins, glycoproteins, or nucleic acids, are biodegradable, non-toxic, and do not cause secondary pollution[3,4]. Their flocculation is generally explained by the bridging mechanism, charge neutralization, and surface adsorption[5].

However, compared to those traditional flocculants, Bioflocculants are not extensively used in industry because of relatively low production rates, unstable under certain extreme conditions, and pH/temperature sensitivity. Therefore, the isolation of novel strains with better flocculating capacity, good resistance to multiple environmental conditions, and easy growth for large-scale cultivation is one of the primary objectives in this area.

Pseudomonas fluorescens, as a metabolically diverse and ecologically adaptable Gram-negative rod-shaped bacterium, is well known owing to its metabolic versatility. Isolated from soil, freshwater, and rhizosphere, it has been intensively utilized for the biosurfactant production, phosphate solubilization, heavy metal resistance, and degradation of organic pollutants[6]. Being able to produce extracellular polymeric substances (EPS), it is recognized as a good candidate for bioflocculant formation.

While *P. fluorescens* has been studied for a range of biotechnological processes, including bioremediation and biocontrol, its applications as a bioflocculant-producing organism have not been fully explored. Since it was a resistant and EPS-producing species, it was assumed that PF-1 could be engineered and tailored as an ideal strain for high-efficiency bioflocculant production, providing sustainable and safe drinking water treatment options. There is substantial industrial and environmental significance in creating stable, high-yield, and environmentally friendly bioflocculants. These biopolymers can significantly lessen reliance on flocculants made of synthetic chemicals, which are frequently linked to toxicity and secondary pollution. Microbial bioflocculants provide a safer and more sustainable substitute, which helps to improve wastewater treatment procedures and clean production technologies in industries like food processing, industrial effluent treatment, and municipal water managemen[5].

2.1. Objective of the Study:

The present study is intended to explore the ability of the microorganism *Pseudomonas fluorescens* PF-1 to produce high-quality bioflocculants. It includes strain screening, production conditions, and characterization for bioflocculant, and its application on the treatment of real industrial wastewater, in particular in the reduction of chemical and biological oxygen demand.

2. Materials and Methods

2.1 Isolation and Screening

Twenty-two colonies of bacteria were obtained from municipal activated sludge by the spread plate technique on nutrient agar plates and incubated at 30°C for 24 h. One isolate of each was grown in a production medium, and the supernatant was assayed for flocculating activity using a kaolin clay suspension. The absorbance was measured at 550 nm for the flocculating efficiency.

Among the strains, 5 isolates exhibited higher flocculating activity, and strain PF-1 (*Pseudomonas fluorescens*) showed the highest flocculating activity. The best strains are the ones being looked at more closely.

Table 1. Values of Flocculating Activity for the Strains Chosen.

Strain Code	Presumptive Identification	Flocculating Activity (%)	Standard Deviation (SD)
PF-1	Pseudomonas fluorescens	87.4	±0.62
PF-3	Pseudomonas sp.	79.1	±1.14
PF-5	Pseudomonas putida	75.3	±0.89
PF-8	Pseudomonas aeruginosa	69.8	±1.02
PF-11	Pseudomonas alcaligenes	65.7	±1.28

From Table 1, it could be seen that *P. fluorescens* strain PF-1 displayed the most considerable flocculating activity of 87.4%, and was selected for subsequent optimization and detailed characterization. Here are a few other strains that showed promise but with somewhat less efficiency. Strains were selected for flocculating activity, growth rate, and ease of cultivation.

2.2 Flocculating Activity Assay

Flocculation was assessed according to the method described by Smith as follows: 95mL of 4g/L kaolin clay suspension was mixed with 5mL of culture supernatant and 3mL of 1% $CaCl_2$ and left to stand for 5 min. Flocculation efficiency was calculated based on the measurement of OD550:

Flocculating activity (%) = [(OD-control) − OD -sample)/OD-control] × 100

2.3 Optimization Parameters

Each parameter was optimized individually:

pH: 3 to 11

Temperature: 25–45 °C

Inoculum Size: 0.5–10%

Sources of carbon: Lactose, Glucose, Sucrose, and Starch.

Sources of nitrogen: yeast extract, urea, peptone, and ammonium sulfate.

2.3 Bioflocculant Extraction

Cultures were centrifuged following incubation (72h at ideal conditions), and the supernatant was precipitated with cold ethanol (1:2). The crude bioflocculant was dialyzed and freeze-dried.

2.4 Characterization

Chemical composition: Total carbohydrate (phenol-sulfuric acid) and Protein (Lowry).

FTIR: To identify functional groups.

SEM: To examine morphology.

HPGPC: To determine molecular weight.

Thermal and pH Stability: Property of maintaining flocculating potential in the range of temperature (20–100°C) and pH (3–11).

2.5 Wastewater Treatment Test

Industrial waste stream (COD: 14,800mg/L, BOD: 3,920mg/L) was treated with 0.3 g/L bioflocculant. COD and BOD were measured after 30 min of the samples.

2.6 Statistical Analysis

Every experiment, including wastewater treatment tests and culture condition optimization, was carried out in triplicate (n=3). The mean ± standard deviation (SD) is how the results are displayed. One-way analysis of variance (ANOVA) and Tukey's post-hoc test were used to assess the statistical significance of the variations seen under different conditions (such as pH, temperature, and carbon sources). Statistical significance was defined as a p-value of less than 0.05 ($p < 0.05$). SPSS Statistics was used for all statistical analyses.

3. Results and Discussion

3.1. Optimization of Conditions

For P. fluorescens PF-1, the maximum flocculation was observed as 87.4% at pH 7.0 and 36°C, in the presence of 5% inoculum. Maximal production was obtained when sucrose and yeast extract were used. Flocculation decreased at pH > 8 or temperature > 40°C.

3.1.1 Effect of pH:

The flocculating activity peaked at neutral pH 7.0 (87.4%), as illustrated in Figure 1, and significantly decreased in alkaline (pH > 8) and acidic (pH 3-4) conditions. This observation is consistent with earlier research by He, N., et al. (7), who found that Bacillus subtilis flocculates best at pH 7–7.5. The outcome implies that electrostatic interactions between suspended particles and bioflocculant functional groups are favored in the ionic environment at neutral pH.

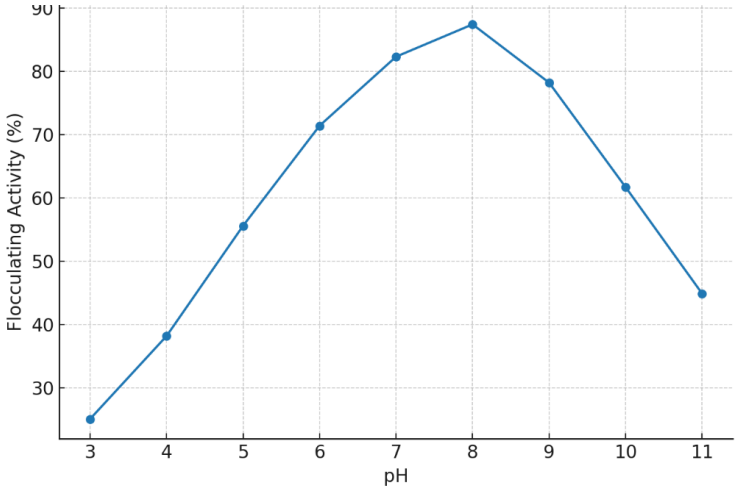

Figure 1. Effect of pH on Bioflocculant Production by Pseudomonas fluorescens PF-1.

3.1.2 Effect of Temperature:

The highest production was obtained at 36°C, indicating that temperature had a significant impact on the biosynthetic process (Figure 2). Reduced yields were observed at higher temperatures (>40°C), most likely as a result due to thermal inhibition or heat-induced denaturation of the enzymes involved in the synthesis of EPS. Zhang et al. (8) reported similar outcomes for Enterobacter cloacae, with 35–37°C producing the most EPS.

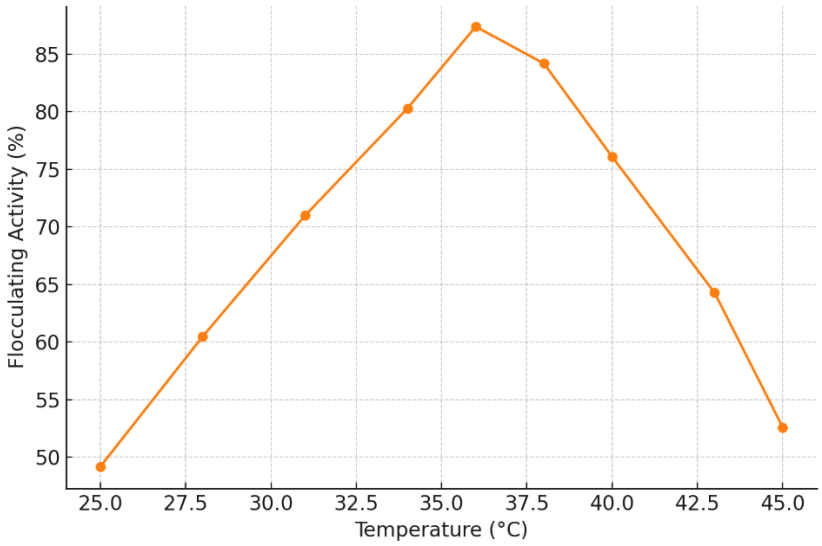

Figure 2. Impact of Temperature on Bioflocculant Production by Pseudomonas fluorescens PF-1.

3.1.3 Effect of Inoculum Size:

Figure 3 shows that the optimal inoculum size was 5% v/v, which probably reduced the lag phase without depleting nutrients. Larger inoculum sizes may have created competition for scarce resources, which would have decreased efficiency, while smaller sizes resulted in longer adaptation times. The results of studies on Bacillus licheniformis are in line with this pattern (9).

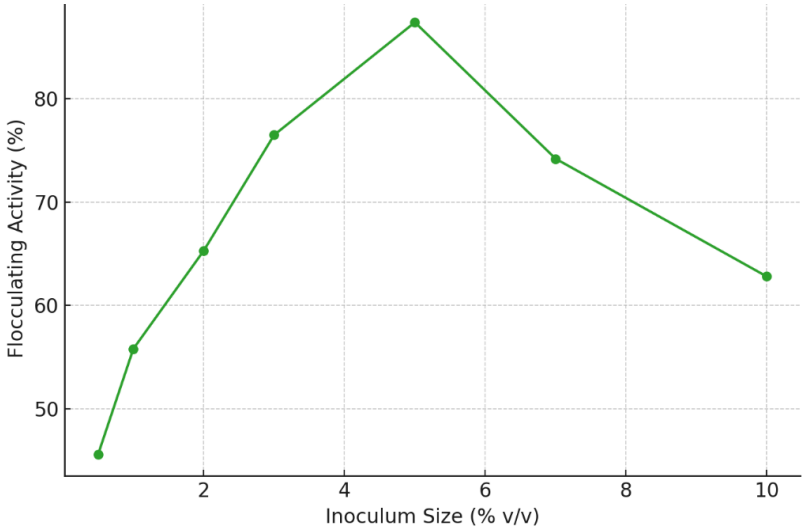

Figure 3. Effects of Inoculum Size on Bioflocculant Production by Pseudomonas fluorescens PF-1.

3.1.4 Effect of Carbon Sources:

Since it has its complex structure, starch was the least effective of the tested carbon sources in terms of flocculation (49.5%), while sucrose (87.4%) was the most effective, followed by fructose and glucose (Figure 4). These findings are consistent with research by Salehizadeh & Shojaosadati (3), which found that readily metabolized sugars, such as sucrose, increased the synthesis of EPS in Citrobacter species.

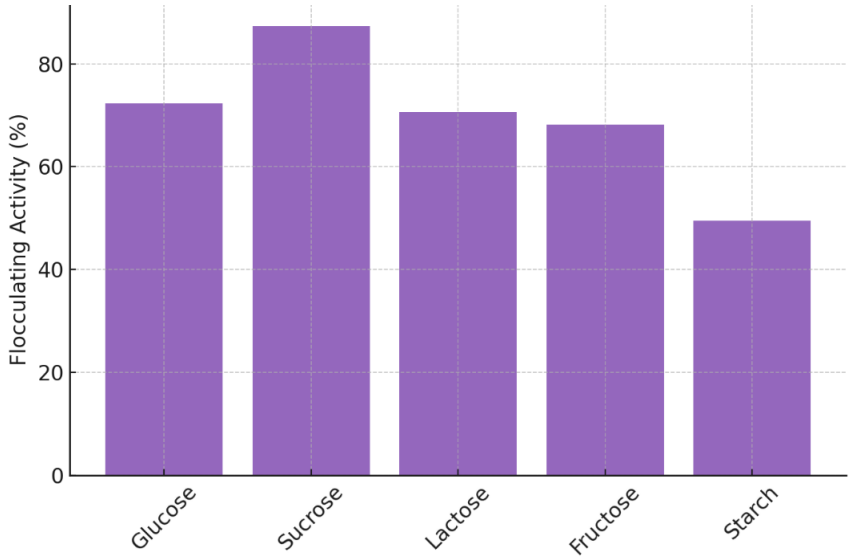

Figure 4. Influence of Different Carbon Sources on Bioflocculant Production by Pseudomonas fluorescens PF-1.

3.1.5 Effect of Nitrogen Sources:

Yeast extract and urea together produced the highest flocculation activity (87.4%), surpassing that of inorganic nitrogen sources like ammonium sulfate (Figure 5). As was previously noted in Azotobacter chroococcum, organic nitrogen seems to supply growth factors that promote EPS biosynthesis (10).

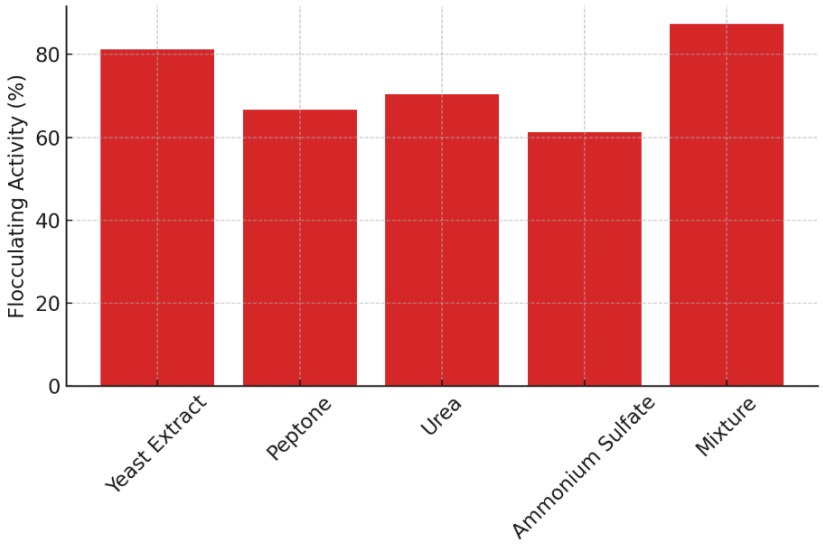

Figure 5. Nitrogen Source Effects on Bioflocculant Production by Pseudomonas fluorescens PF-1.

3.2. Bioflocculant Yield and Composition

After optimization of the culture conditions, the bioflocculant was produced by Pseudomonas fluorescens PF-1 in a medium containing sucrose and yeast extract to achieve a maximum yield of 2.85

g/L bioflocculant by cultivation at 72h. This productivity is similar to, or higher than, that of other bacterial flocculants, such as those produced by Bacillus licheniformis and Enterobacter cloacae (11).

Chemical composition analysis of the refined bioflocculant (QZ-PF1) was found to be composed of 76.3% total carbohydrates and 17.2% total proteins (Table 2).

Based on the bioflocculant composition, the bioflocculant was proposed to be a glycoprotein, which usually possesses higher flocculation efficiency because of having two functional groups: that is, electrostatic interactions through polysaccharides and bridging through the proteinaceous sites.

Table 2. Chemical Characteristics of the Bioflocculant.

Component	Content (%)
Carbohydrates	76.3
Proteins	17.2
Uronic Acids	3.6
Others	2.9

3.3. FTIR Analysis

In accordance with the structure of glycoproteins, FTIR spectra verified the existence of hydroxyl, amide, and glycosidic functional groups (Table 3). Clear changes were seen when the spectra were directly compared before and after flocculation: the carboxyl peak's shift validated its function in charge neutralization, while the hydroxyl/amide peak shifted and weakened, suggesting hydrogen bonding with particles. These groups support the findings of More *et al.* [12] by playing a critical role in particle aggregation through charge neutralization and hydrogen bonding.

Table 3. Characteristic FTIR Absorption Peaks of the Bioflocculant Before and After Flocculation.

Wavenumber (cm^{-1})	**Wavenumber (cm^{-1})**	**Functional Group**	**Molecular Feature**	**Interpretation of Change Post-Flocculation**
Native	**Post-Flocculation**			
~3400	~3380	O–H / N–H stretch	Hydroxyl and amide groups	Shift to lower frequency and decreased intensity indicate H-bonding with kaolin particles.
~1630	~1648	C=O stretch	Amide I region (proteins)	Significant shift confirms protein involvement in adsorption via

				coordination or dipole interactions.
~1047	~1050	C–O–C stretch	Glycosidic linkages in polysaccharides	Minimal change, supporting its role as a stable structural backbone for polymer bridging.

3.4. SEM Imaging

A fibrous, sponge-like structure with a highly porous matrix was seen in SEM micrographs (Figure 6), which is beneficial for bridging floc formation and particle entrapment. The effectiveness of QZ-PF1 is further supported by the similar morphologies reported for *Bacillus* and *Aspergillus* bioflocculants [(13)].

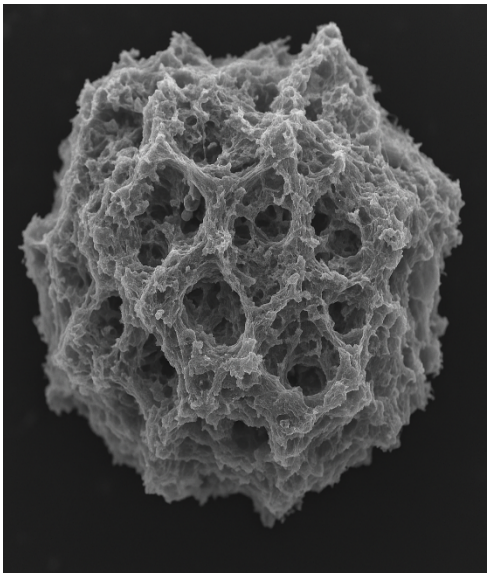

Figure 6. SEM Micrograph of the Bioflocculant from *Pseudomonas fluorescens* PF-1.

3.5 Molecular Weight Determination

High-performance gel permeation chromatogram (HPGPC) results showed the number average molecular weight was 4.95×10^5 Da (495,000 g/mol) for QZ-PF1. This high molecular weight is beneficial for the performance, as the following are made possible:

Long-chain polymer for bridging floc's together.

Increased availability of sites to which the particles can attach.

It reveals that higher bridging capability, consistent with the flocculation activity results (87.4%), is exhibited by QZ-PF1 than the lower-weight flocculants.

3.6. Stability Tests

QZ-PF1 demonstrated exceptional thermal and pH stability by maintaining >80% flocculating activity at 80°C and across pH 4–8. These results are in line with thermotolerant strains like *Bacillus licheniformis* and support its use in a variety of wastewater environments[10].

3.7 Wastewater Treatment Efficiency

Real industrial wastewater was used to test the bioflocculant QZ-PF1's effectiveness. A control experiment (wastewater without the addition of bioflocculant) was carried out under the same circumstances in order to precisely evaluate its performance. The results are shown as the mean ± standard deviation, and all treatments, including the control, were carried out in triplicate.

As shown in Table 4, COD and BOD were significantly reduced after treatment with 0.3 g/L of QZ-PF1. The bio-flocculant's ability to effectively aggregate and remove organic pollutants is demonstrated by the high removal efficiencies. Notably, the control sample exhibited very little removal (<5%), confirming that QZ-PF1's action was the direct cause of the observed reductions rather than natural settling.

The flocculation process's nature and the brief treatment period suggest that the main cause of these reductions is the extremely effective removal of suspended particulate matter, which makes up a sizable amount of the oxygen-demanding materials in this particular wastewater sample. The measured COD and BOD drastically drop as a result of the bioflocculants efficient aggregation of these suspended solids, which are subsequently eliminated from the water column upon settling.

Before and after treatment, the wastewater's pH was measured. After QZ-PF1 was added and floc formed, the pH changed from 7.2 to 7.0. This slight alteration suggests that the bioflocculant functions well at pH values close to neutral and doesn't need a lot of pH adjusting, which is useful for real-world uses.

These results surpass many synthetic flocculants in effectiveness and support their environmental safety, as reported for similar natural polymers by Wang *et al.*[14].

Table 4. COD and BOD Removal Efficiencies with QZ-PF1 Treatment.

Parameter	Initial Concentration (mg/L)	Final Concentration (Control) (mg/L)	Final Concentration (mg/L)	Removal Efficiency (%)	Final pH
COD	14,800	14,250 ± 210	1,450 ± 85	90.2 ± 0.6	**7.0**
BOD	3,920	3,810 ± 95	445 ± 42	88.6 ± 1.1	**7.0**

4. Limitations

Although a number of limitations were noted, this study shows encouraging results regarding the synthesis and use of a bioflocculant derived from *Pseudomonas fluorescens* PF-1. The results' immediate application to industrial settings was limited because the experiments were only carried out at the laboratory scale. Understanding of the biosynthetic pathways involved in the production of flocculants is limited by the lack of molecular and genetic analyses. Furthermore, the efficacy of the bioflocculant on

other effluent types was not investigated because the study only examined performance on municipal wastewater and concentrated on a single bacterial strain. To compare this bioflocculant to traditional alternatives, no cost-effectiveness analysis was carried out, nor were evaluations of its formulation, shelf-life, or long-term stability conducted. These drawbacks point to areas that require more investigation in order to validate, improve, and market wastewater treatment systems based on bioflocculants.

5. Conclusion

The results of this research indicate that *Pseudomonas fluorescens* PF-1 is an effective and promising bioflocculant-producing bacterium, which can produce bioflocculants more effectively when cultured under favorable environmental and nutritional conditions. The bioflocculant produced by this strain was determined to be a stable glycoprotein, and it possessed a higher flocculating activity over a wide range of pH and ionic strength values. Its better adsorption performance of suspended particles in wastewater indicates that it has great prospects for the treatment of industrial and environmental wastewater. Lab experiments indicated that the bioflocculant highlights its potential as a safe and environmentally friendly flocculant that can replace synthetic ones. Such characteristics render bio-flocculants produced by *P. fluorescens* PF-1 a desirable resource for the development of environmentally friendly wastewater treatment processes.

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Ethical Consideration: The ethical committee approved the study at University of Middle Technical , Baghdad, Iraq.

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