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Screening bacterial isolates: Psychrobacter sp. metabolite for wastewater treatment

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Abstract

Dewatering is a critical step in sewage sludge management, often hampered by high water retention within extracellular polymeric substances. Although chemical additives are widely used to enhance dewatering through colloidal destabilization and particle aggregation, these substances can cause secondary pollution and generate sludge residues with high metal content. In this scenario, bioflocculants represent a sustainable, non-toxic, and biodegradable alternative. This study evaluated the bioflocculant production by solar panel-isolated bacteria – *Sphingomonas paucimobilis*, *Serratia nematodiphila*, *Kocuria* sp., and *Psychrobacter* sp. While all strains exhibited flocculating activity in a model kaolin suspension, *Psychrobacter* sp. was selected for sewage sludge dewatering due to its superior efficiency. Biochemical characterization revealed that the metabolite comprised carbohydrates (24.64%), lipids (18.10%), and proteins (10.23%). Fourier-transform infrared spectroscopy (FTIR) confirmed the presence of functional groups essential for flocculation, including hydroxyl, amine, and carboxyl groups. When applied to sludge conditioning, the bioflocculant achieved flocculation efficiencies of 93.96% ± 1.13% for aerobic sludge (aeration tank) and 71.53% ± 1.37% for anaerobic sludge (Upflow Anaerobic Sludge Blanket, UASB). Remarkably, its performance was comparable to that of a conventional commercial cationic polymer. These findings demonstrate that bioflocculants derived from these bacteria are promising eco-friendly substitutes for synthetic conditioners in wastewater treatment, though future research should explore cultivation trends to identify the most favorable conditions for enhancing yields and fully comprehend the factors that influence their synthesis and efficiency.

INTRODUCTION

Wastewater treatment generates large volumes of sewage sludge, a complex matrix of water, organic matter, microorganisms, and persistent contaminants (Gherghel et al. 2019). Sludge management represents a major operational challenge, accounting for over 50% of the operating costs of Wastewater Treatment Plants (WWTPs) (Zhen et al. 2017). Mechanical dewatering is commonly applied to reduce sludge volume and, consequently, disposal costs, but high water retention within extracellular polymeric substances (EPS) limits its efficiency (Wu et al. 2020). To enhance water removal, chemical conditioning using inorganic salts or synthetic polymers is a possible strategy to promote colloidal destabilization and particle aggregation (Wei et al. 2018; Cao et al. 2021). However, these commercial additives pose significant environmental and health risks, including secondary pollution and toxic metal accumulation in sludge residues (Izydorczyk et al. 2021).

In this scenario, microbial bioflocculants have emerged as sustainable, non-toxic, and biodegradable alternatives. Synthesized by microorganisms as adaptive mechanisms for survival, which involve functions including cellular communication, nutrient acquisition, and protection against environmental stress (Fouillaud and Dufossé, 2022), these biopolymers have shown high potential for application in wastewater treatment. Nevertheless, their widespread industrial application remains hindered by high production costs compared to synthetic conditioners (Ang and Mohammad, 2020). This bottleneck

drives the search for novel approaches, such as screening highly efficient microbial strains capable of producing stable bioactive compounds, as well as strategies to reduce operational costs and enhance bioflocculant yields (Pu et al. 2020).

One promising approach to discover such strains is exploring environments subjected to harsh conditions, such as water scarcity, severe radiation, and wide temperature fluctuations, which are recognized as valuable sources of microorganisms with unique metabolic properties (Schultz and Rosado, 2019; Sayed et al. 2020). In the search for novel bioproducts of biotechnological interest, Moura et al. (2021) isolated bacterial strains from photovoltaic panels in Sorocaba and Itatiba, Brazil.

Within this perspective, the present study evaluated the potential of four of these strains (*Kocuria* sp., *Psychrobacter* sp., *Sphingomonas paucimobilis*, and *Serratia nematodiphila*) to produce bioflocculants. Furthermore, *Psychrobacter* sp., whose metabolities were identified as the most efficient, was selected for detailed biochemical and functional characterization, as well as for application in the conditioning and dewatering of aerobic and anaerobic sewage sludges.

MATERIALS AND METHODS

Microorganisms, culture media and bioflocculant production

Bioflocculant production was evaluated using four bacterial strains (*Kocuria* sp., *Psychrobacter* sp., *Sphingomonas paucimobilis*, and *Serratia nematodiphila*) isolated from photovoltaic panels (Moura et al. 2021). Inocula were grown in screening medium (Agunbiade et al. 2018) at 30°C and 150 rpm for 48 h and standardized in 0.9% NaCl (OD_{600} = 1.0) Standardized inocula (10% v/v) were transferred to production medium (g/L: glucose 10.0, peptone 1.0, $MgSO_4 \cdot 7H_2O$ 0.3, K_2HPO_4 5.0, KH_2PO_4 2.0; pH 7.0) and cultured at 30°C and 150 rpm for 120 h. Cell-free supernatants were collected every 24 h (10,000 rpm, 15 min, 4°C) to evaluate flocculating activity.

Flocculating Activity assay and process optimization

Flocculating activity (FA) was determined using a Kaolin clay suspension (4 g/L) according to Mu et al. (2018). Briefly, 1.0 mL of cell-free supernatant, 2.5 mL of $\mathrm{CaCl_2}$ solution (10 g/L), and 46.5 mL of kaolin suspension were rapidly mixed at 180 rpm for 1 min, followed by slow mixing at 60 rpm for 2 min, and allowed to settle for 10 min. The absorbance of the clarified supernatant (OD_{550}) was measured, and FA was calculated as $\mathrm{FA}\,:(\%) = [(A - B)\,/A] \times\ 100$, where A and B are control and sample absorbances, respectively. The top-performing strain was selected for bioprocess evaluation using a 2^3 Central Composite Rotatable Design (CCRD; Minitab 20.3), totalizing 17 runs. The design investigated the effects of initial pH (1.95–12.05), temperature (4.77–55.23 ºC), and inoculum size (1.59–18.41% v/v) on FA after $48\,:\mathrm{h}$ of incubation.

Extraction, validation, and bioflocculant characterization

Extraction, validation, and bioflocculant characterization
Based on the individual runs evaluated in the CCRD, the trends for enhanced synthesis and efficiency of the bioflocculant were statistically analyzed. To further explore these favorable conditions, a new cultivation was performed using the parameters that promoted the highest theoretical FA (pH 12.0, 23.2°C, and 5% v/v inoculum). The bioflocculant obtained under this setup was extracted from the cell-free supernatant following a modified procedure described by Xia et al. (2022) using cold ethanol precipitation (1:2 v/v, -15°C, 16 h), followed by centrifugation, drying at 45°C for 24 h, and yield quantification. To establish the optimal dosage, the extracted bioflocculant was tested in kaolin assays at concentrations ranging from 0.2 to 0.4 mg/mL. Total proteins, total carbohydrates, reducing sugars, and lipids were quantified using the methods of Bradford (1976), phenol-sulfuric acid (Chaplin and Kennedy 1994), DNS (Miller 1959), and Bligh and Dyer (1959), respectively. Functional groups were identified by FTIR-ATR (400–4000 cm^{-1}) with spectra normalized to the [0,1] range to facilitate visualization of the characteristic peaks using Origin 2018 64-bit software. Surface morphology and elemental composition were analyzed via SEM-EDS.

Sewage sludge characterization and application assays

Aerobic and anaerobic sludges from a municipal WWTP were analyzed for pH, solids series (TS, FS, VS, settleable solids; APHA 2005), and moisture content (105°C, 24 h). Baseline dewaterability and conditioning performance were evaluated using the Vaccum Filtration Time (50 kPa vacuum; Wong et al. 2016) as a primary indicator of drainability, following a modified protocol based on Tsilo et al. (2022). Diluted sludge (1:4 v/v) was mixed with 2.5 mL of $CaCl_2$ solution (10 g/L) and 1.0 mL of bioflocculant at the optimal dose (0.2 mg/mL), stirred at 180 rpm for 1 min followed by 60 rpm for 2 min, allowed to settle for 5 min, and measured at OD550. Performance was compared against untreated sludge (control) and a commercial cationic polymer (CPAM, Praestol 851 BC).

Statistical Analysis

All experiments were performed in triplicate, and data were expressed as mean ± standard deviation. Prior to statistical analysis, data normality and homogeneity of variances were verified using the Levene test. One-way Analysis of Variance (ANOVA) was performed followed by Tukey's post-hoc test ($p < 0.05$) to evaluate significant differences among treatments. All statistical analyses were conducted using Minitab Statistical Software (version 20.3) at a 95% confidence level.

RESULTS

Screening and Selection of Bioflocculant-Producing Strain

All four bacterial species (*Sphingomonas paucimobilis*, *Serratia nematodiphila*, *Kocuria* sp., and *Psychrobacter* sp.) exhibited bioflocculant activity against Kaolin suspension over 120 h of incubation under non-optimized culture conditions. However, flocculation efficiency varied significantly among strains and cultivation times (Fig. 1).

For *S. paucimobilis*, FA increased rapidly within the first 24 h, reaching a peak of 60.32%. After this initial surge, efficiency dropped sharply to 14.06% at 48 h and remained consistently low, with no statistically significant differences ($p > 0.05$) observed until the end of the incubation period.

For S. nematodiphila, FA increased from an initial 29.51% to a maximum of 41.37% at 48 h. Statistical grouping indicated that the strain maintained a stable performance over time, as no significant differences ($p > 0.05$) were observed between 24 h and 120 h. Notably, it was the only strain to sustain efficiencies above 30% up to 120 h, contrasting with the other evaluated strains that stabilized at a range between 10% and 15%.

Kocuria sp. displayed no distinct efficiency peak, maintaining a statistically stable activity ($p > 0.05$) of approximately 33.00% during the first 48 h. Subsequently, the bioflocculation efficiency experienced a significant decline, stabilizing at a lower level between 15.96% and 19.13% from 72 h to 120 h without further statistical variation.

Conversely, *Psychrobacter* sp. exhibited high and sustained performance throughout the assay, increasing from an initial 39.62% to a maximum of 54.24% at 48 h. The strain maintained a statistically stable efficiency plateau ($p > 0.05$) from 24 h up to 96 h, averaging approximately 51.00% during this broad window, before declining sharply to 13.97% at 120 h. Because of this high peak activity and remarkable stability over time, *Psychrobacter* sp. was selected for the subsequent process optimization and characterization steps.

Influence of Cultivation Parameters for Bioflocculant Production

Following strain selection, *Psychrobacter* sp. was cultured for 48 h under the experimental conditions established by the Central Composite Rotatable Design (CCRD). FA varied significantly across the 17 experimental runs ($p \leq 0.05$), ranging from 11.81% to 93.96% (Table 1).

Statistical evaluation of the CCRD showed that within the tested ranges, initial medium pH, incubation temperature, and inoculum size had no statistically significant main or interaction effects on flocculating activity, as no factor crossed the significance threshold (FigSI1). From a bioprocess perspective, this lack of significance highlights the physiological robustness of the strain, demonstrating that bioflocculant synthesis is highly resilient to operational fluctuations.

Nevertheless, CCRD remains an important tool for mapping system behavior and observing process trends. In this context, response surface and contour plots (fixing inoculum size at 5.0%) showed a clear

trend of increased bioflocculating performance under alkaline conditions, with predicted activities exceeding 80.0% at pH near 12.0 across different temperatures (FigSI2).

Table 1
– Central Composite Rotatable Design (CCRD) matrix with coded and uncoded variables and the corresponding experimental responses for Flocculating Activity (FA).

Runs	Coded variables			Uncoded variables			Response
	pH	Temperature	Inoculum size	pH	Temperature (ºC)	Inoculum size (% v/v)	FA (%)
1	-1	-1	-1	4.00	15.00	5.00	11.81 ± 1.67[i]
2	1	-1	-1	10.00	15.00	5.00	93.96 ± 1.38[a]
3	-1	1	-1	4.00	45.00	5.00	45.01 ± 0,91[d]
4	1	1	-1	10.00	45.00	5.00	78.08 ± 4.35[b]
5	-1	-1	1	4.00	15.00	15.00	23.82 ± 1.95[g,h]
6	1	-1	1	10.00	15.00	15.00	62.34 ± 6.12[c]
7	-1	1	1	4.00	45.00	15.00	18.90 ± 2.23[h,i]
8	1	1	1	4.00	45.00	15.00	51.05 ± 1.49[d]
9	-1.681	0	0	1.95	30.00	10.00	64.44 ± 1.82[c]
10	1.681	0	0	12.05	30.00	10.00	49.41 ± 3.06[d]
11	0	-1.681	0	7.0	4.77	10.00	31.36 ± 1.82[f,g]
12	0	1.681	0	7.0	55.23	10.00	29.92 ± 2.39[f,g]
13	0	0	-1.681	7.0	30.00	1.59	44.62 ± 3.78[d,e]
14	0	0	1.681	7.0	30.00	18.41	45.47 ± 4.18[d,e]

Runs	Coded variables			Uncoded variables			Response
	pH	Temperature	Inoculum size	pH	Temperature (°C)	Inoculum size (% v/v)	FA (%)
15	0	0	0	7.0	30.00	10.00	67.13 ± 1.39[c]
16	0	0	0	7.0	30.00	10.00	68.50 ± 1.97[c]
17	0	0	0	7.0	30.00	10.00	35.96 ± 2.53[e,f]

*Different lowercase letters denote significant differences ($p < 0.05$) within each run, with 'a' indicating the highest flocculating efficiency, followed alphabetically by lower values.

To further investigate these operational boundaries, response optimization analysis was performed. Based on the mapped trends, the optimization established an initial medium pH of 12.0, an incubation temperature of 23.18°C, and an inoculum size of 5.0% v/v as the configuration to maximize the response, reaching a maximum theoretical FA of 93.16% (d = 0.9902). However, the predictive tool highlighted that further increases in incubation temperature and inoculum size beyond these values resulted in lower expected flocculation efficiency.

Validation of the Mapped Operational Trend, Bioflocculant Yield, and Dosage Influence

Cultivation of *Psychrobacter* sp. under the specific conditions identified by the trend analysis (initial pH 12.0, 23.2°C, and 5.0% v/v inoculum) for 48 h yielded a mean bioflocculant dry mass of 2.9 g/L. Applying the extracted bioflocculant in kaolin clay suspension assays verified the mapped tendencies in practice, yielding a flocculation efficiency of about 82%. To establish the ideal application rate, FA was evaluated at three distinct concentrations: 0.2, 0.3, and 0.4 mg/mL. No statistically significant differences in performance were observed between the 0.2 (87.19% ± 2.61%) and 0.3 mg/mL (84.42% ± 3.14% doses. However, increasing the dosage to 0.4 mg/mL led to a slight reduction in flocculation efficiency (74.62% ± 5.85%). Therefore, the 0.2 mg/mL concentration was selected as the most effective dosage for the subsequent sludge dewatering assays.

Biochemical and Structural Characterization of the Bioflocculant

Chemical composition of the extracted bioflocculant produced by *Psychrobacter* sp. revealed a predominantly polysaccharide-lipid complex (Table SI1). Expressed as relative proportions among the

characterized organic components, polysaccharides, lipids, and proteins accounted for 46.52%, 34.17%, and 19.31%, respectively.

Qualitative elemental analysis via energy-dispersive x-ray spectroscopy (EDS) indicated a composition predominantly composed of oxygen (59.90% wt) and carbon (18.60% wt), along with phosphorus (12.54% wt) and potassium (9.01% wt). Surface morphological characterization by scanning electron microscopy (SEM) displayed an irregular, granular micro-structure with particle sizes ranging from approximately 5 µm to 100 µm (Fig. 2).

Fourier-Transform Infrared (FTIR) spectroscopy identified 12 distinct absorption bands across the 4000 – 400 cm^{-1} range (Fig. 3). A broad absorption band centered at 3262 cm^{-1} corresponds to the stretching vibrations of hydroxyl (–OH) and amine (–NH) groups. Sharp absorption peaks at 1653 cm^{-1} and 1636 cm^{-1} indicate carbonyl (C = O) stretching associated with amide or carboxylate functional groups, supported by asymmetrical/symmetrical carboxylate vibrations at 1375 cm^{-1}. Moderate bands observed between 1200 – 1000 cm^{-1}, specifically at 1015 cm^{-1} and 889 cm^{-1}, correspond to C-O-C and C-O stretching vibrations characteristic of polymeric carbohydrates.

Sewage Sludge Characterization and Dewatering Performance

Physicochemical characterization of raw aerobic and anaerobic sludge matrices from the municipal WWTP revealed distinct baseline properties, particularly in terms of solid fractions and EPS content (Table SI2). Baseline Filtration Time assays demonstrated low dewaterability for both untreated matrices, with aerobic sludge requiring 21.0 min to collect 15 mL of filtrate (50 kPa vacuum), while anaerobic sludge caused rapid filter cake blinding (> 30.0 min).

Sludge conditioning with the *Psychrobacter* sp. bioflocculant (0.2 mg/mL) supplemented with $CaCl_2$ (10 g/L) markedly improved clarification efficiency, reaching 93.96% ± 1.13% of FA in aerobic sludge and 71.53% ± 1.37% in anaerobic sludge. These results were comparable to those obtained with commercial cationic polyacrylamide (CPAM), which achieved FA values of 96.32% ± 0.60% and 97.72% ± 0.12% for aerobic and anaerobic sludges, respectively.

Visual analysis of aggregate formation indicated structural differences between treatments. CPAM produced large, cohesive, high-shear-resistant flocs. Conversely, although the *Psychrobacter* sp. bioflocculant effectively reduced supernatant turbidity and accelerated particle settling, it resulted in small, dispersed aggregates deposited at the bottom of the flask. These smaller aggregates exhibited lower structural cohesion compared to those formed by the commercial polymer (Fig. 4).

DISCUSSION

Mechanisms and Temporal Dynamics of Bioflocculant Production

Different aspects may be related to the variations in flocculation efficiencies observed among the evaluated bacterial species. The nature of the molecule plays a fundamental role in flocculation efficiency, as it results in specific structural characteristics such as functional groups and surface charges (Wei et al. 2018; Kurniawan et al. 2020). Bioflocculants with high molecular weights are generally more efficient because they establish stronger bonds and possess higher adsorption capacity, allowing the formation of larger flocs (Alias et al. 2022). Thus, it is likely that the evaluated species synthesize molecules with distinct properties, conditioning process efficiency.

Furthermore, bioflocculant synthesis can occur at different stages throughout the incubation time. The higher efficiencies observed within the first 48 h, followed by a reduction in flocculation rates over time, suggest that these molecules originate from direct biosynthesis rather than cell autolysis. When produced in parallel with cell growth, bioflocculant synthesis can occur in distinct phases for each species. For instance, bioflocculant synthesis by *Bacillus subtilis* occurred in parallel with the exponential growth phase, peaking at 60 h and decreasing during the cell death phase (Giri et al. 2015). Conversely, *Klebsiella pneumoniae* bioflocculant production occurred only at the end of the exponential growth phase at 18 h, with subsequent production peaks observed after 70 h and 90 h (Nie et al. 2011).

The reduction in FA for *Sphingomonas paucimobilis*, *Kocuria* sp., and *Psychrobacter* sp. over 120 h can be associated with the consumption of these molecules by the bacteria as nutritional sources. Secondary metabolites can be degraded as carbon and energy sources under nutrient scarcity (More et al. 2014). However, this reduction was not observed for *Serratia nematodiphila*, suggesting that the system had not yet reached the cell death phase at 120 h and/or that this species does not produce bioflocculant-degrading enzymes (Makapela et al. 2016; Ding et al. 2021).

Under the adopted cultivation conditions, the bioflocculant produced by *Psychrobacter* sp., with maximum activity at 48 h, presented high efficiency with minor variations over time, justifying its selection for subsequent optimization assays. Nevertheless, bioflocculants synthesized by *S. paucimobilis*, *S. nematodiphila*, and *Kocuria* sp. also show potential if evaluated under individualized optimal growth conditions.

Influence of Culture Conditions, Process Optimization, and Bioflocculant Dosage

Although statistical analysis via the Pareto chart indicated that initial pH, incubation temperature, and inoculum size did not exert main or interaction effects above the significance threshold within the evaluated ranges, response surface and optimization plots were analyzed in terms of operational trends. FA above 80% was predominantly associated with highly alkaline conditions, particularly near pH 12.0. The wide parameter intervals adopted in the experimental design allowed for the identification of these

extreme operational boundaries, culminating in the theoretical ideal conditions of initial pH 12.0, incubation temperature of 23.18°C, and 5.0% v/v inoculum size.

Validation under these conditions yielded a dry bioflocculant concentration of 2.9 g/L. This yield is comparable to those reported for other bacterial bioflocculants, such as *Bacillus salmalaya* (2.7 g/L; Abu Tawila et al. 2018), *Pantoea* sp. (2.9 g/L; Ayangbenro et al. 2019), *Bacillus pumilus* (3.1 g/L; Maliehe et al. 2020), and *Bacillus megaterium* (3.01 g/L; Selepe et al. 2022). Although the obtained yield was satisfactory, optimizing additional cultivation factors not evaluated in this study, such as nutritional components, agitation speed, and aeration, may further enhance biopolymer productivity and efficiency.

Bioflocculant dosage is a critical factor in flocculation processes, as it directly conditions treatment efficiency and economic feasibility. Insufficient dosages fail to provide adequate polymer chains to establish interparticle bridges, whereas excessive dosages induce steric hindrance and electrostatic repulsion, hindering particle sedimentation (Gong et al. 2008; Zulkeflee et al. 2012). Restabilization phenomenon occurs when excess bioflocculant completely saturates the colloid surfaces, blocking available binding sites required for cross-linking with adjacent particles (Cosa and Okoh 2014). These mechanisms may explain the reduction in FA observed when the *Psychrobacter* sp. bioflocculant dosage was increased to 0.4 mg/mL.

Consequently, the 0.2 mg/mL dosage was selected, striking a balance between high efficiency and process economy. This dosage aligns with effective concentrations reported for other microbial bioflocculants, including *Halobacillus* and *Oceanobacillus* strains (0.2 mg/mL; Cosa and Okoh 2014), *Streptomyces platensis* (0.2 mg/mL; Agunbiade et al. 2018), and *Pichia kudriavzevii* (0.4 mg/mL; Tsilo et al. 2022).

Biochemical Features and Structural Characterization

Chemical characterization of the *Psychrobacter* sp. bioflocculant revealed a composition predominantly composed of polysaccharides (46.52%), lipids (34.17%), and proteins (19.31%). These results contrast with standard literature reports, where lipid content is rarely highlighted as a major proportional component in microbial bioflocculants. Most studies describe bioflocculants as predominantly polysaccharide or glycoprotein-based complexes (Okaiyeto et al. 2015; Zhong et al. 2020; Ding et al. 2021; Xia et al. 2022). Notably, the chemical analysis in the present study was performed on the crude bioflocculant. The inclusion of a downstream purification step could yield polysaccharide and protein proportions closer to those reported in the literature.

Qualitative elemental analysis indicated a major presence of oxygen (59.9%) and carbon (18.6%), along with phosphorus (12.54%) and potassium (9.01%). While phosphorus and potassium levels were likely influenced by residual salts from the culture medium, carbon and oxygen represent the essential structural backbone of bioflocculants (Ntsangani et al. 2017; Alias et al. 2022). The predominance of C and O aligns with elemental profiles reported for bioflocculants from *Pseudomonas aeruginosa* (Pathak et al. 2017), *Streptomyces platensis* (Agunbiade et al. 2018), *Bacillus salmalaya* (Tawila et al. 2019), and *Bacillus* spp. (Alias et al. 2022).

Morphological evaluation via SEM revealed granular microstructures ranging from 5 µm to 100 µm. These features differ from those observed for bioflocculants produced by *Serratia marcescens* (Kurniawan et al. 2022) and *Bacillus megaterium* (Selepe et al. 2022), but share similarities with the biomolecule from *Pichia kudriavzevii* (Tsilo et al. 2022). Morphological variations compared to other

studies may stem from differences in sample preparation, such as freeze-drying versus grinding, or the extraction method utilized, which can alter biopolymer arrangement (Kanamarlapudi and Muddada 2017).

FTIR spectroscopy identified 12 absorption bands, reflecting a complex polymeric structure and suggesting a high-molecular-weight molecule (Coates 2000; Nandiyanto and Ragadhita 2019). The broad band at 3262 cm^{-1} corresponds to hydroxyl (–OH) and amine (–NH) stretching (Muthulakshmi et al. 2023). Peaks at 1653 cm^{-1} and 1636 cm^{-1}, combined with the band at 1375 cm^{-1}, indicate amide or carboxylate groups ($–COO^{–}$) (Cosa et al. 2013; Nandiyanto and Ragadhita 2019). Furthermore, bands at 1015 cm^{-1} and 889 cm^{-1} are characteristic of sugar derivatives and carbohydrate linkages (Cosa et al. 2013; Ntsangani et al. 2017).

Carboxyl and hydroxyl groups are fundamental structural elements governing bioflocculating activity (Ntsangani et al. 2017). Hydroxyl groups enhance water solubility, while carboxyl groups facilitate binding with divalent cations (Ca^{2+}), promoting charge neutralization and interparticle bridging with colloidal particles (Agunbiade et al. 2022). The spectral profile observed in this study displays strong similarities to those reported by Maliehe et al. (2020) and Selepe et al. (2022), who characterized their respective bioflocculants as functional glycoproteins.

Sewage Sludge Dewatering Performance and Flocculation Mechanisms

The physicochemical characteristics of the sludge matrices directly influenced their baseline dewaterability and response to chemical conditioning. The rapid filter cake blinding observed for raw anaerobic sludge during filtration time assays (> 30 min) stems from its high total solids content (TS = 53.87 g/L) and elevated proportion of dispersed fine solids. High suspended solids increase sludge viscosity, reducing cake permeability and restricting water passage (Cao et al. 2020). Moreover, filtration resistance correlates directly with the dispersion degree of fine colloids (Mowla et al. 2013).

Conversely, for aerobic sludge, the formation of a gelatinous layer on the filter medium indicates that its higher EPS carbohydrate content (EPS_c = 1.55 g/L) played a decisive role in low baseline drainability. Although EPS are crucial for floc integrity, excess accumulation can increase repulsive forces and clog filter pores (Wu et al. 2020). These baseline limitations underscore the necessity of conditioning prior to mechanical dewatering.

The differences in EPS content between matrices reflect microbial community specificities and metabolic regimes. Anaerobic bacteria frequently consume extracellular polymers as energy substrates due to metabolic constraints, reducing overall EPS levels in the system (Houghton et al. 2001). Additionally, oxygen availability modulates EPS production, where aerobic environments promote carbohydrate synthesis within extracellular matrices, whereas oxygen restriction induces floc disintegration (Sheng et al. 2010).

Sludge conditioning using the *Psychrobacter* sp. bioflocculant (0.2 mg/mL) with $CaCl_2$ demonstrated high clarification efficiency in aerobic sludge (93.96% ± 1.13%), comparable to commercial CPAM (96.32% ± 0.60%). These results match or exceed performances reported for commercial coagulants/flocculants (Cainglet et al. 2020, 2023; Piaskowski et al. 2023) and microbial bioflocculants applied to wastewater treatment (Vimala 2019; Zhong et al. 2020; Gupta et al. 2023). However, bioflocculant efficiency was lower in anaerobic sludge (71.53% ± 1.37%). This discrepancy is attributable to the high solids burden in the anaerobic matrix, which requires higher bioflocculant and Ca^{2+} dosages to overcome repulsive forces and achieve complete system destabilization.

Distinct aggregate morphologies were observed between treatments. Commercial CPAM induced large, cohesive, shear-resistant macro-flocs, whereas the bioflocculant with Ca^{2+} formed smaller, rapidly settling aggregates. CPAM performance is governed by its extremely high molecular weight, enabling extensive interparticle bridging and forming compact, robust networks (Lapointe and Barbeau 2020; Li et al. 2021). Bridging mechanisms generally produce flocs with higher mechanical resistance compared to charge neutralization or sweep flocculation (Jiang 2015). For the *Psychrobacter* sp. bioflocculant, the formation of smaller, rapidly settling aggregates resembles patterns observed in sweep flocculation or ion-bridging interactions (Koul et al. 2022).

Agitation speed also critically influences polymer dispersion, particle collision frequency, and shear-induced floc breakage (Kurniawan et al. 2020; Hyrycz et al. 2022). Floc disruption under excessive shear can cause irreversible residual turbidity (Marques and Filho 2016). Future investigations monitoring zeta potential variations, particle size distribution dynamics, and bioflocculant molecular weight are required to fully elucidate the predominant interaction mechanisms promoted by the *Psychrobacter* sp. bioflocculant.

CONCLUSION

This study demonstrated that *Psychrobacter* sp., *Serratia nematodiphila*, *Sphingomonas paucimobilis*, and *Kocuria* sp. are bioflocculant-producing bacteria capable of clarifying kaolin suspensions. Under unoptimized baseline conditions, *Psychrobacter* sp. exhibited superior performance and temporal stability, achieving a peak FA of 54.24% ± 4.26% after 48 h of incubation. Although initial pH, temperature, and inoculum size did not show significant main effects within the evaluated ranges, the investigation of process trends resulted in a dry bioflocculant yield of 2.9 g/L.

Biochemical characterization of the crude macromolecule synthesized by *Psychrobacter* sp. revealed an organic composition dominated by polysaccharides (46.52%), lipids (34.17%), and proteins (19.31%). Functional group analysis via FTIR confirmed the presence of amine, hydroxyl, and carboxyl groups, which act as active binding sites governing flocculation. SEM and EDS analyses further indicated granular microstructures ranging from 5 µm to 100 µm and an elemental profile predominantly composed of oxygen (59.9%), carbon (18.6%), phosphorus (12.54%), and potassium (9.01%).

Application of the *Psychrobacter* sp. bioflocculant in real municipal wastewater treatment achieved 93.96% ± 1.13% flocculation efficiency in aerobic sludge, performing comparably to a commercial cationic polyacrylamide polymer. In contrast, lower efficiency (71.53% ± 1.37%) was observed in anaerobic sludge, which can be attributed to the high concentration of suspended particles requiring elevated dosages for full system destabilization.

Future investigations should focus on molecular weight determination, zeta potential monitoring, and particle size distribution analysis to elucidate the precise interaction mechanisms. Furthermore, investigating unmapped cultivation factors, such as incubation period, agitation speed, and nutrient sources, presents a promising pathway to increase bioflocculant yields and efficiency for sustainable sludge conditioning.

Statements and Declarations

Author Contributions: LPN, MAA and ICSD conceived and designed research. LPN and MAB conducted experiments. LPN and MAB analyzed data. LPN wrote the manuscript. LPN, MAB, MAA and ICSD reviewed and edited the manuscript. LPN, MAB, MAA and ICSD read and approved the manuscript.

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Figures

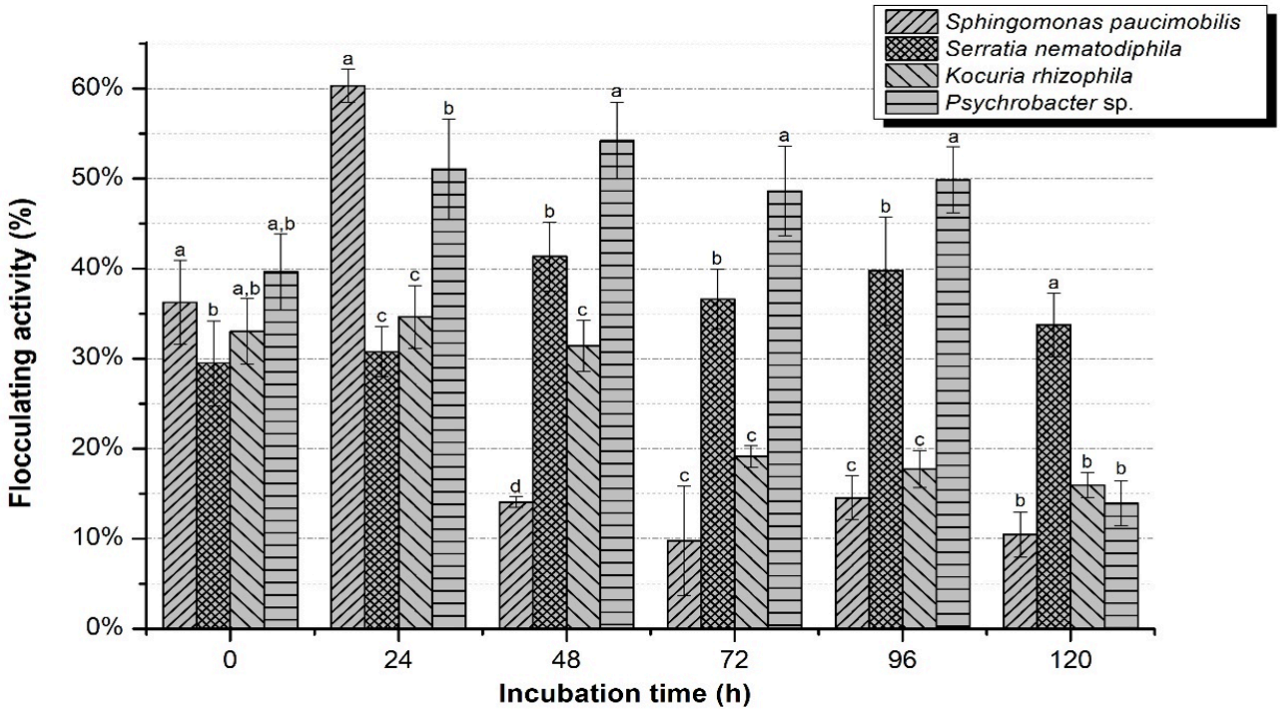


Figure 1

Comparison of the flocculating activity of *Sphingomonas paucimobilis*, *Serratia nematodiphila*, *Kocuria* sp., and *Psychrobacter* sp. as a function of incubation time

*Different lowercase letters denote significant diferences ($p < 0.05$) within each analyzed time, with ‘a’ indicating the highest flocculating efficiency, followed alphabetically by lower values.

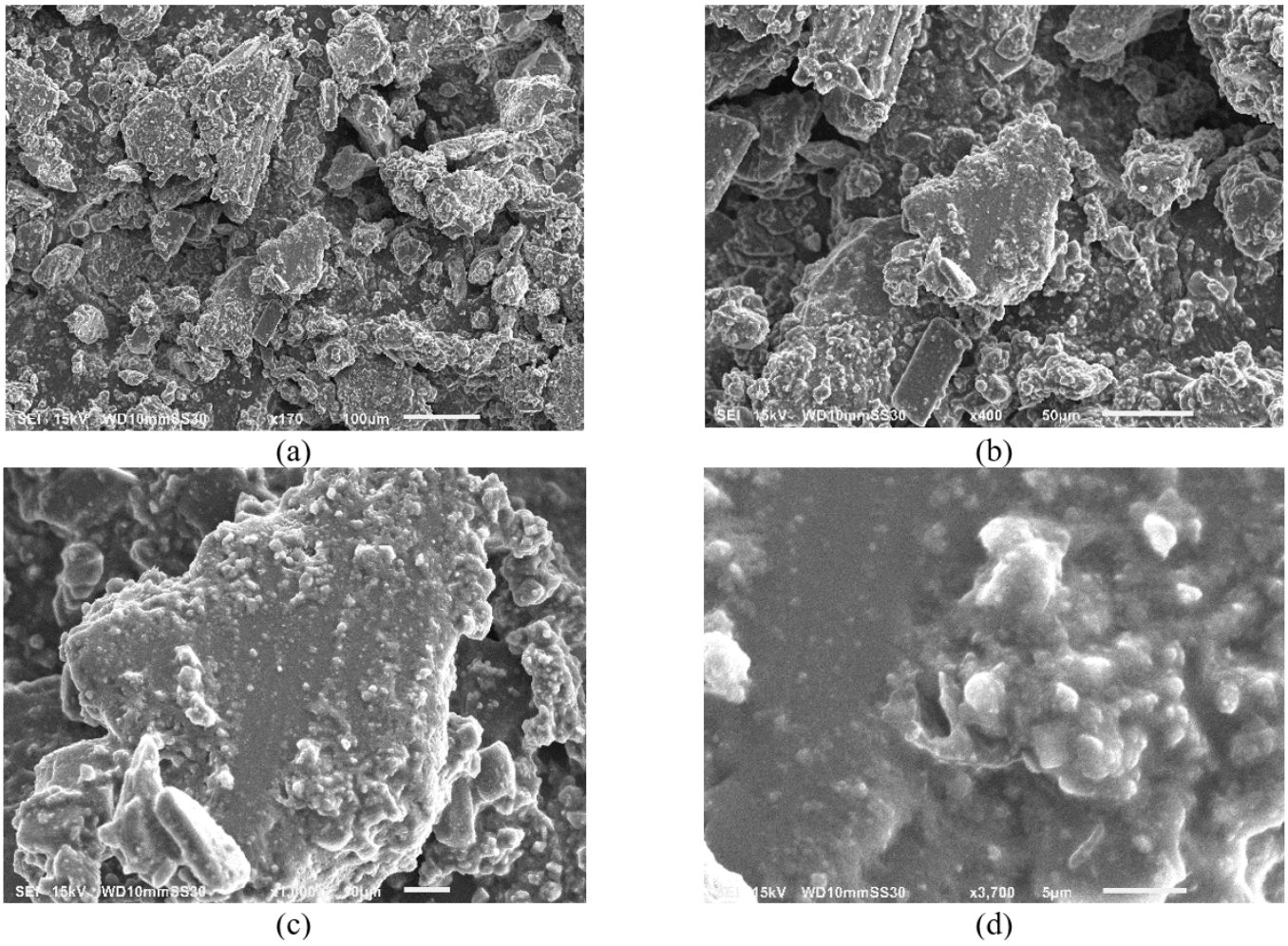


Figure 2

Scanning electron micrographs of the bioflocculant produced by *Psychrobacter* sp. at magnifications of (a) 170x, (b) 400x, (c) 1000x, and (d) 3700x

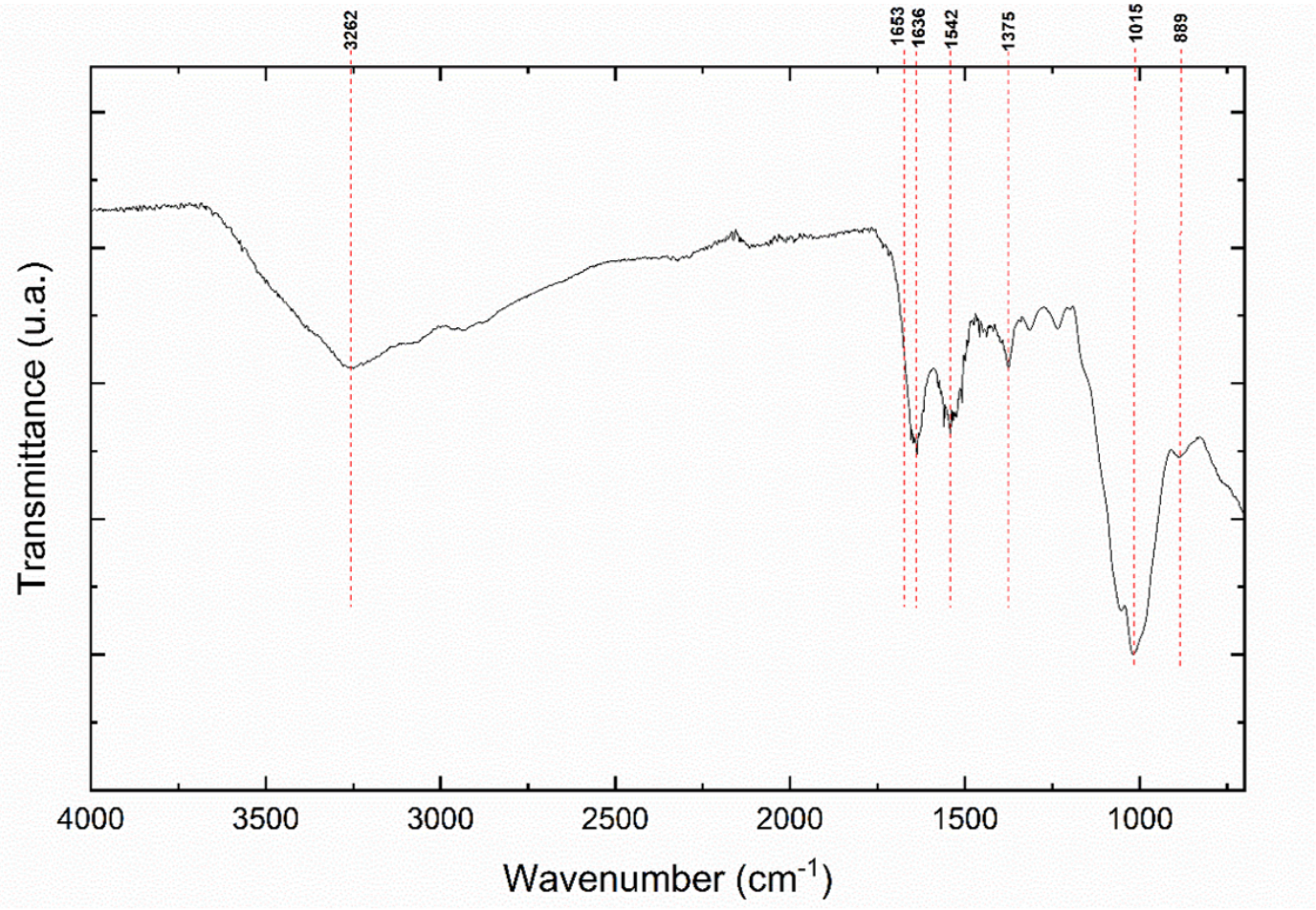
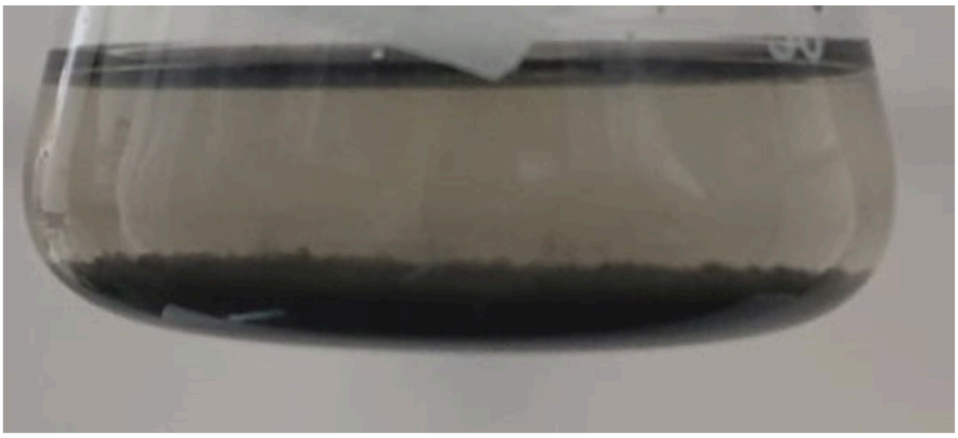


Figure 3

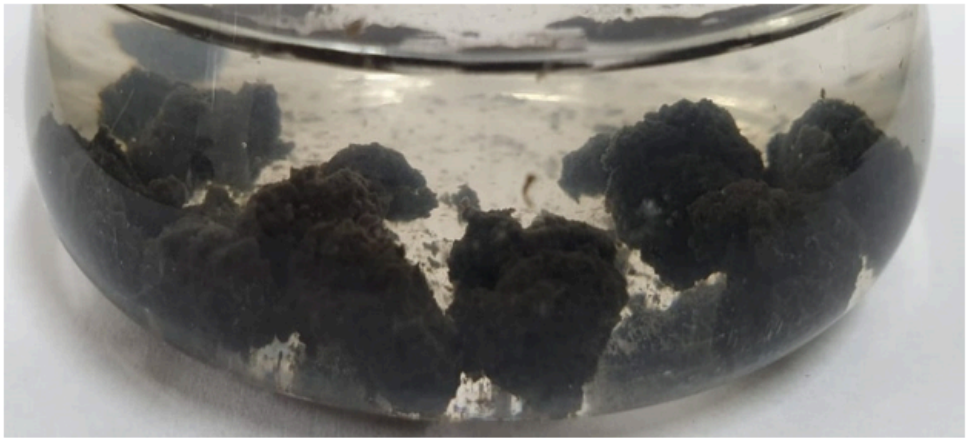
Fourier-transform infrared (FTIR) spectrum of the bioflocculant synthesized by *Psychrobacter*sp



(a) Aerobic sludge – control sample



(b) Aerobic sludge treated with bioflocculant



(c) Aerobic sludge treated with CPAM

Figure 4

Comparative flocculation performance of the *Psychrobacter*sp. bioflocculant and a commercial flocculant in aerobic sludge

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