

Exploring Microbial Diversity for Bioflocculant Production: Isolation, Media-Specific Screening, and Evaluation of Flocculating Efficiency

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This study isolates and systematically evaluates bioflocculant-producing bacteria from Municipal Solid Waste (MSW) and Pharmaceutical Effluent Treatment Plants (ETPs) to identify sustainable, high-efficiency alternatives to synthetic chemical coagulants. Utilizing selective isolation techniques, 73 distinct bacterial isolates were recovered. Morphological screening identified 45 isolates exhibiting pronounced mucoid and ropy phenotypes, indicative of robust extracellular polymeric substance (EPS) production. Subsequent quantitative screening isolated 14 elite strains from diverse Gram-positive and Gram-negative bacterial genera. Flocculating activity was rigorously assessed across chemically defined screening media and wastewater-based production media. Results demonstrated that native pharmaceutical effluent isolates cultivated in wastewater-derived production media achieved exceptional flocculating efficiencies, frequently exceeding 95% against kaolin clay suspensions. The substitution of expensive synthetic substrates with nutrient-rich wastewater components profoundly stimulated EPS biosynthesis, validating a circular economy approach to bioflocculant mass production. Biochemical characterization highlighted the extensive metabolic diversity and environmental resilience of the isolates. These microbially derived eco-flocculants function via complex charge neutralization and polymer bridging mechanisms, presenting a highly viable, biodegradable, and non-toxic paradigm for industrial wastewater remediation that effectively eliminates the severe ecological footprint associated with conventional metal-based coagulants.

Keywords: Bacterial Isolates; Biochemical Analysis; Bioflocculant; Flocculating Activity; Morphological Characterization; Sustainable Wastewater Treatment.

The Global Water Crisis and Industrial Effluents

The exponential acceleration of global industrialization, coupled with unprecedented demographic expansion, has precipitated a profound and escalating water pollution crisis. The

continuous generation and discharge of immense volumes of untreated or inadequately treated municipal and industrial effluents have placed immense ecological pressure on finite freshwater reservoirs.¹ These complex wastewaters are inherently characterized by elevated concentrations

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of recalcitrant organic compounds, heavy metal ions, synthetic dyes, pharmaceuticals, and diverse inorganic contaminants.² The resultant ecological impact is severe; heightened concentrations of these pollutants impart undesirable levels of turbidity and chromaticity, thereby increasing the biochemical oxygen demand (BOD) and chemical oxygen demand (COD) of receiving water bodies. Consequently, nutrient over-enrichment promotes aggressive algal blooms that critically deplete dissolved oxygen, leading to the asphyxiation of aquatic flora and fauna. In response to this environmental degradation, stringent international and regional regulatory frameworks mandate the rigorous remediation of polluted effluents prior to their environmental release.³ Historically, the industry has deployed a wide array of physicochemical methodologies—including industrial filtration, advanced centrifugation, electro-coagulation, and activated carbon adsorption—to mitigate these hazards. However, the escalating capital expenditure, intense energy requirements, and complex operational logistics intrinsic to these conventional paradigms underscore an urgent necessity for the development of more sustainable, scalable, and cost-effective alternatives.

The Paradigm of Coagulation-Flocculation

Tripathy and De⁴ described that within the broader context of wastewater remediation, coagulation and flocculation represent the fundamental cornerstone of solid-liquid separation technologies. Flocculating agents are systematically deployed to facilitate the rapid aggregation of finely divided, highly stable colloidal particles into macroscopic, dense flocs. This aggregation drastically increases the effective mass and settling velocity of the particles, enabling their subsequent, efficient removal via gravitational sedimentation or dissolved air flotation. Conventionally, the wastewater treatment industry has relied overwhelmingly on inorganic metallic salts—most notably Aluminum Sulfate (Alum), Polyaluminum Chloride (PAC), Ferrous Sulfate, and Ferric Chloride—as well as synthetic organic polymers such as Polyacrylamides and Polyethylene amines. These synthetic chemical flocculants are widely lauded for their rapid kinetic efficacy, high commercial availability, consistency

in performance, and robust chemical stability across varying environmental parameters.⁵

Limitations and Toxicity of Chemical Coagulants

Despite their operational ubiquity, the widespread application of synthetic and inorganic chemical flocculants is increasingly scrutinized due to severe, well-documented ecological and public health ramifications.⁶ Aluminum-based coagulants, for instance, are notoriously non-biodegradable. The residual metal ion carryover in treated effluents has been heavily implicated in the etiology of severe neurodegenerative disorders, including Alzheimer's disease, posing a direct threat to downstream potable water supplies. Furthermore, synthetic organic polymers, particularly polyacrylamide derivatives, harbor trace amounts of acrylamide monomers, which are classified as potent neurotoxins and known human carcinogens. Beyond direct biochemical toxicity, the utilization of these conventional chemical agents alters the natural pH of the water matrix, often necessitating secondary chemical buffering, as reported by Li et al.⁷ Crucially, the coagulation process utilizing metallic salts generates voluminous quantities of highly toxic, highly acidic, and non-biodegradable sludge. The secondary disposal of this hazardous sludge creates formidable logistical and environmental challenges, directly contravening the contemporary principles of sustainable environmental management and the circular economy.

Emergence of Microbial Bioflocculants

In direct response to these critical limitations, the scientific and engineering communities have pivoted toward the exploration of bioflocculants as a greener alternative.⁸ Microbial bioflocculants (MBFs) are highly complex, macromolecular polymers synthesized and secreted by a diverse array of microorganisms—including bacteria, fungi, and microalgae—during their natural metabolic cycles, particularly in response to environmental stressors.⁹ MBFs offer unparalleled advantages over their synthetic counterparts. Most notably, they are entirely biodegradable, eco-friendly, virtually devoid of secondary toxicity, and highly efficient in the simultaneous removal of suspended solids, heavy metal ions, and synthetic textile dyes. Because they do not rely on aggressive metallic salts, Bisht and Lal¹⁰ investigated the

MBFs operate effectively without inducing deleterious fluctuations in the pH of the treated effluent, and the resultant sludge is inherently rich in organic matter, offering potential downstream utility as agricultural compost.

The Role of Extracellular Polymeric Substances (EPS)

The extraordinary flocculating capabilities of MBFs are intrinsically linked to their biochemical composition, which is primarily dominated by Extracellular Polymeric Substances (EPS).¹¹ EPS represents a highly hydrated, three-dimensional gel-like matrix comprising high-molecular-weight heteropolysaccharides, structural proteins, glycoproteins, nucleic acids, and humic substances.¹² These biomolecules are heavily decorated with an array of active functional groups—including carboxyl, hydroxyl, amino, phosphoric, and sulfonate groups. Within biological wastewater treatment systems, such as activated sludge, EPS serves as a crucial protective boundary against harsh external surroundings, facilitating cellular embedding, immobilization, and protection against heavy metal toxicity. During the bioflocculation process, the high density of these functional groups facilitates powerful inter-particle bridging and extensive charge neutralization, effectively sweeping suspended colloidal contaminants out of the aqueous phase. Specific bacterial genera, notably *Bacillus*, *Pseudomonas*, *Proteus*, *Klebsiella*, and *Streptomyces*, have been identified globally as prolific EPS over-producers, yielding highly potent bioflocculants capable of treating incredibly diverse industrial effluents.¹³

Circular Economy and Economic Barriers

Despite their immense environmental potential, the transition of microbial bioflocculants from laboratory-scale conceptualization to large-scale industrial deployment remains significantly impeded by stringent economic and technological bottlenecks.¹⁴ The foremost constraint is the prohibitive cost of production, which is heavily driven by the reliance on expensive, chemically defined synthetic fermentation substrates (e.g., purified glucose, sucrose, and refined yeast extracts) required to achieve high polymer yields. To circumvent these economic barriers and align bioflocculant mass production with the principles

of a circular economy, contemporary environmental biotechnology emphasizes the valorization of low-cost, waste-derived substrates.¹⁵ Activated sludge and Industrial ETP effluents are exceptionally rich in complex microbial consortia and vital macronutrients. Utilizing these native, highly stressed environments as the actual production media not only drastically lowers substrate costs but inherently selects for highly resilient, EPS-overproducing bacterial strains uniquely adapted to the specific toxicological profile of the target wastewater.¹⁶

Study Objectives

Consequently, the primary objective of this exhaustive study is to isolate, meticulously screen, and biochemically characterize novel, high-efficiency bioflocculant-producing bacteria from highly diverse and complex environmental matrices. Specifically, the sampling targets encompass Municipal Solid Waste (MSW) landfill soils and multiple functional units of Pharmaceutical Effluent Treatment Plants (ETPs). A critical secondary objective is to systematically compare the flocculating activity of these indigenous bacterial isolates cultivated in standardized, synthetic screening media versus native, wastewater-based production media. By elucidating these media-driven metabolic shifts, this research aims to optimize bioflocculation kinetics and validate the efficacy of microbially derived eco-flocculants as potent, scalable, and economically viable substitutes for conventional chemical coagulants. Through this paradigm, the study seeks to advance the frontier of sustainable, green wastewater treatment technologies.

MATERIALS AND METHODS

Reagents, Chemicals, and Culture Media Preparation

To ensure the highest degree of experimental fidelity, consistency, and reproducibility throughout the study, all analytical-grade chemicals, reagents, and biological media were procured from recognized international suppliers, predominantly Merck (India) and Loba Chemie (India). The reagent inventory included Sodium Hydroxide (NaOH) and Hydrochloric Acid (HCl) for rigorous pH adjustment, alongside

specialized microbiological stains such as Crystal Violet, Gram's Iodine, and Safranin (0.5% w/v) for morphological profiling.

Dehydrated culture media and purified organic substrates, vital for initial isolation and baseline metabolic screening, were obtained from Hi-Media (India). This included Nutrient Agar (NA), Nutrient Broth, Luria Bertani (LB) Broth, Glucose ($C_6H_{12}O_6$), Sucrose, Yeast Extract Powder, Peptone, Urea ($CO(NH_2)_2$), Tryptone, Ammonium Chloride, Ammonium Sulfate ($(NH_4)_2SO_4$), Casein Enzymic Hydrolysate, Magnesium Sulfate Heptahydrate ($MgSO_4 \cdot 7H_2O$), Sodium Chloride (NaCl), Potassium Dihydrogen Phosphate (KH_2PO_4), and Di-Potassium Hydrogen Phosphate (K_2HPO_4). All aqueous solutions were prepared using double-distilled, deionized water. To ensure absolute sterility, all media and thermostable reagents were subjected to standard autoclaving protocols at 121°C and 15 psi for exactly 15 minutes prior to any microbial inoculation.¹⁷

Site Selection and Environmental Sampling

The successful isolation of highly active biofloculant-producing microorganisms relies heavily on sourcing from ecosystems characterized by intense physicochemical stress, which drives the evolutionary adaptation of robust EPS biosynthesis. Accordingly, Kothia and Soni¹⁸ studied that the environmental samples were strategically extracted from multiple highly complex and pollutant-stressed matrices within Ankleshwar, Anand and Bharuch Districts, Gujarat, India.

Soil samples were obtained from active Municipal Solid Waste (MSW) landfill sites situated in the Anand and Bharuch districts, Gujarat, India known for their fluctuating thermal profiles, high organic matter content, and complex leachate chemistry. Concurrently, aqueous and sludge samples were collected from different functional units of a pharmaceutical effluent treatment plant located in Ankleshwar Industrial Estate, Gujarat, India. The specific extraction points encompassed the Aeration Tanks, Sedimentation Tanks, Treated Effluent discharge points, and the dense ETP-Activated Sludge units. All environmental samples were harvested using sterile, hermetically sealed borosilicate containers to preclude external cross-contamination and to perfectly preserve the native microbial consortia dynamics during transit.

Physicochemical Profiling

In situ physicochemical profiling of the aqueous and soil sampling sites was conducted utilizing an advanced HI 98194 multiparameter probe (Hanna Instruments). This allowed for the immediate recording of vital baseline environmental metadata, specifically temperature, pH, dissolved oxygen (DO), total dissolved solids (TDS), salinity, ambient pressure, and specific conductivity.¹⁹ Understanding these parameters is crucial, as they define the native operational conditions to which the isolates are evolutionarily adapted. Post-collection, all samples were immediately transferred to insulated, ice-packed coolers to arrest ongoing metabolic activity and cellular degradation. The samples were securely transported to the laboratory and processing was commenced within a strict 24 to 48-hour analytical window. Any surplus material was stored at 4°C to maintain viability for subsequent validation assays.²⁰

Isolation and Cultivation of Microbial Strains

The extraction and isolation of viable, pure microbial strains were executed utilizing rigorous, standardized serial dilution and spatial isolation techniques. Aqueous samples derived from the Aeration Tank, Treated Effluent, Sedimentation Tank, and ETP-Activated Sludge were subjected to a 10-fold serial dilution protocol to reduce microbial density to a countable threshold.²¹ Specifically, 1.0 mL aliquots of the raw environmental water or suspended soil solutions were aseptically inoculated into 9.0 mL of sterile 0.85% physiological saline. The mixtures were vortexed vigorously for 30 seconds to disrupt macro-aggregates and ensure homogenous cellular dispersion. Subsequent serial dilutions spanning 10^{-1} to 10^{-7} were meticulously prepared.

A 100 μ L volume from both the undiluted matrices and the respective serial dilutions was aseptically inoculated onto freshly prepared Nutrient Agar (NA) and Luria Bertani (LB) agar plates utilizing the spread-plate methodology. The NA plates were incubated aerobically at 37°C for 24 hours to capture rapidly dividing heterotrophs, whereas the LB plates were maintained at 37°C for 48 hours to accommodate slower-growing, highly fastidious species. Following the designated incubation periods, viable microbial counts were quantified and expressed as Colony-Forming Units

per Milliliter (CFU/mL) to determine the baseline population density of the sampled environments.²²

Preliminary Phenotypic Screening

To isolate true monocultures, bacterial colonies exhibiting distinct phenotypic variations-categorized meticulously by overall size, pigmentation, marginal configurations, and surface topography-were targeted for spatial isolation. Selected colonies were carefully excised using a sterile inoculation loop and subjected to continuous zig-zag streaking across virgin NA and LB agar plates. This sub-culturing cycle was iterated a minimum of three times, rigorously verifying the absolute purity of the monocultures.

The preliminary identification of potential bioflocculant-producing strains relied heavily on distinct visual and physical markers that are universally indicative of extensive Extracellular Polymeric Substance (EPS) biosynthesis. Purified colonies were visually assessed for highly ropy, distinctly mucoid, and slimy phenotypes. Furthermore, an empirical “loop test” was conducted; colonies that formed a highly viscous, continuous elastic string when drawn upward with a sterile inoculation loop were classified as robust EPS producers. These specific isolates were preferentially selected, cataloged, and preserved on nutrient agar slants at 4°C for immediate experimental use, with long-term cryopreservation achieved by suspending the exponential-phase cells in a 25.0% (v/v) sterile glycerol stock stored at -20°C.

Formulation of Screening and Production Media

To quantitatively evaluate flocculating efficacy and discern the metabolic influence of the culture medium, the selected isolates were cultivated in two distinct formulations. The first was a chemically defined Standard Screening Medium, optimized to provide easily accessible carbon and nitrogen for rapid growth.²³ The composition per liter of distilled water included: Glucose (20.0 g), Dipotassium Phosphate (5.0 g), Potassium Dihydrogen Phosphate (2.0 g), Urea (0.5 g), Yeast Extract (0.5 g), Magnesium Sulfate Heptahydrate (0.2 g), Ammonium Sulfate (0.2 g), and Sodium Chloride (0.1 g). The medium was pH-adjusted to 7.0 ± 0.2 prior to sterilization.¹⁷

The second formulation was a highly specialized Production Medium, engineered

to simulate real-world operational conditions, induce metabolic stress, and evaluate the circular economy potential of the isolates. This medium maintained the identical stoichiometric mass ratio of supplemental carbon, nitrogen, and inorganic salts but completely replaced the distilled water base with filtered, unsterilized wastewater sourced directly from the respective ETP aeration tanks, sedimentation tanks, and activated sludge units. This approach directly evaluated the capability of the isolates to utilize complex native effluents as the primary growth medium.

A loopful of each pure bacterial strain was inoculated into 100 mL of the respective sterile media contained in 250 mL Erlenmeyer flasks. The cultures were incubated aerobically in a rotary shaking incubator at 30°C to 37°C, with continuous agitation maintained between 100 and 160 rpm. Cultivation was halted at specific intervals of 24 and 48 hours. Following the incubation cycle, the culture broths were subjected to high-speed centrifugation at 8000 rpm for 30 minutes at 4°C to pellet the cellular biomass. The resulting cell-free supernatant (CFS), highly enriched with secreted microbial bioflocculants, was carefully decanted and utilized directly for the quantitative bioflocculating activity assays.²⁴

Bioflocculating Activity Assay Methodology

The flocculating efficiency of the bacterial supernatants was quantified utilizing a standardized kaolin clay suspension assay. Kaolin clay provides a highly reproducible, uniform proxy for the complex colloidal turbidity encountered in industrial wastewater.²⁵ A synthetic wastewater matrix was prepared by homogeneously suspending 4.0 g of fine kaolin clay per liter of distilled water.

In a standard 250 mL conical flask, 100 mL of the prepared kaolin suspension was combined with 3.0 mL of a 1.0% (w/v) Calcium Chloride (CaCl_2) solution. The Ca^{2+} ions act as a requisite divalent cationic bridging agent, neutralizing the highly negative zeta potential of the clay particles and priming them for polymer attachment. Subsequently, 2.0 mL of the EPS-rich cell-free supernatant was introduced to the mixture. The solution was subjected to rapid, vigorous manual agitation for exactly 60 seconds to ensure the uniform dispersion of the bioflocculant macromolecules, maximizing particle collision rates. The mixture was then immediately transferred

to a 100 mL graduated cylinder and allowed to undergo unhindered gravitational sedimentation at ambient room temperature across predefined observational intervals (5 to 20 minutes).²⁶

A rigorous control experiment was conducted in parallel, substituting the 2.0 mL of cell-free supernatant with 2.0 mL of uninoculated, sterile production media to establish a baseline settling rate. Post-sedimentation, an aliquot of the clarified upper aqueous phase was carefully extracted from the cylinder. The optical density (OD) of the clarified solution was measured at a wavelength of 550 nm using a high-precision UV-Vis spectrophotometer. The Flocculating Activity (FA), representing the percentage of turbidity removed, was mathematically derived using the following equation:

$$\text{Flocculating Activity (\%)} = [(B-A) / B \times 100]$$

where B represents the optical density (OD₅₅₀) of the negative control, and A denotes the optical density (OD₅₅₀) of the bioflocculant-treated sample.

Polyphasic Morphological Characterization of Bioflocculant-Producing Isolates

The initial morphological evaluation involved standard Gram staining procedures performed on pure, 24-hour-old bacterial colonies. According to the sample collection, they were given names. The prepared glass slides were flooded sequentially with crystal violet primary stain, Gram's iodine mordant, an ethanol decolorizing agent, and a safranin counterstain. The stained cellular structures were meticulously examined utilizing a compound bright-field light microscope at a magnification of 1000× under oil immersion to explicitly determine the Gram reaction (positive or negative), the fundamental cellular morphology (e.g., bacilli, cocci, coccobacilli, diplococci), and the specific spatial arrangement of the cells (e.g., singular, short chains, extended clusters).²⁷

Biochemical Characterization of Bioflocculant-Producing Isolates

Identification of each bioflocculant-producing bacteria underwent a series of Biochemical tests to facilitate their identification, as described by Rachel.²⁸ A comprehensive suite of polyphasic biochemical assays was deployed to profile the metabolic capabilities and enzymatic repertoire of the isolates. Facklam and Elliott²⁹

outlined the assays for Gram-positive strains included Endospore Staining to detect resilient survival structures, Acid-Fast characterization, Catalase activity to assess oxidative stress resistance, Starch Hydrolysis for complex carbohydrate degradation, Citrate Utilization as a sole carbon source, Mannitol Fermentation, Hemolytic profiling on blood agar, and Bile Esculin hydrolysis. Gram-negative isolates were subjected to Cytochrome Oxidase tests, Indole production indicating tryptophan degradation, Methyl Red-Voges Proskauer (MR-VP) tests to define specific fermentation pathways, Sulfide Indole Motility (SIM) profiling, Nitrate Reduction, and Urease activity.³⁰ These parameters provided crucial, deeper insights into the environmental resilience and substrate adaptability of the recovered strains.

Preservation of Isolates in Glycerol Stock

Glycerol served as a cryoprotectant to preserve the isolated bacterial culture. The cultures that were employed for preservation were made in nutrient broth and cultured for 24 hours at 37°C overnight. Each cryovial (Tarson) was filled with the necessary amounts of freshly produced sterile glycerol (50.0% (v/v) in H₂O) to create a glycerol stock (25.0%) with a final concentration of 25.0% in 1.0 mL. Stocks were preserved at -20°C (deep freezer).³¹

RESULTS

Physicochemical Characteristics of Sourced Environments

The foundational physicochemical parameters of the sampling sites heavily influence the structural composition, resilience, and functional diversity of the resident microbial communities. Measurements acquired from the ETP Aeration Tank and Treated Effluent highlighted a moderate thermal environment (25°C to 30°C) with a near-neutral pH ranging from 6.5 to 7.2. Dissolved Oxygen (DO) levels fluctuated between 2.0 and 4.5 mg/L, creating an aerobic to microaerophilic niche. The ambient organic load remained substantial despite aeration, evidenced by Biochemical Oxygen Demand (BOD) values between 200 and 500 mg/L, accompanied by elevated Ammonia (10-20 mg/L) and Nitrate (5-15 mg/L) concentrations, reflecting active nitrification processes.

The Sedimentation Tank displayed slightly lower ambient temperatures (22°C to 26°C) but was characterized by exceptionally dense particulate matter. Suspended Solids ranged massively from 500 to 1500 mg/L, generating severe Turbidity measuring 10 to 50 NTU. In stark contrast, the ETP-Activated Sludge presented a highly rigorous and toxic environment: temperatures of 28°C to 32°C, suppressed DO (1.5 to 3.5 mg/L) indicating intense microbial respiration, massive Total Suspended Solids (1500 to 2500 mg/L), and overwhelming Chemical Oxygen Demand (COD) peaking between 300 and 800 mg/L.

Soil matrices extracted from the Anand and Bharuch Municipal Solid Waste (MSW) sites exhibited extensive seasonal and spatial environmental variability. Thermal fluctuations were drastic, spanning from 15°C to 42°C, while the pH tended toward slight acidity to neutral (5.5 to 7.5). These soils were uniquely characterized by rich organic matter content (10% to 30%) and high Total Dissolved Solids (500 to 2000 mg/L). Such conditions provide an ideal evolutionary repository for heterotrophic, polymer-secreting bacteria capable of surviving intense osmotic and thermal stress through EPS encapsulation, as reported by Tang et al.³²

Quantitative Microbial Enumeration

The primary isolation campaign successfully recovered an initial pool of 73 distinct bacterial isolates across the six heavily polluted environmental matrices. Following preliminary morphological screening-prioritizing mucoid, ropy, and highly viscous colonial phenotypes that are universally associated with prolific EPS

biosynthesis-the pool was refined to 45 prospective bioflocculant producers. Subsequent quantitative flocculation assays identified a sub-population of 30 strains exhibiting measurable bioflocculation. Kothia and Soni¹⁸ evaluated 14 elite isolates, from this cohort, demonstrating superlative flocculating activity (>80% FA) were selected for deep-tier analytical study.

Quantitative enumeration via serial dilution elucidated the total microbial carrying capacity of the respective ecosystems (Table 1). The ETP-Activated Sludge harbored the maximum culturable bacterial density, peaking at an extraordinary 1.8×10^7 CFU/mL at the 10^{-3} dilution. This is highly reflective of the nutrient-dense, dynamic environment intrinsic to activated sludge processing, which relies on dense microbial flocs. The Sedimentation Tank and Bharuch Soil matrices also demonstrated prolific microbial loads 3.0×10^6 and 2.5×10^6 CFU/mL, respectively. Predictably, the Treated Effluent recorded the lowest overall biomass (1.0×10^5 CFU/mL), verifying the physical efficacy of the upstream treatment processes in mitigating unattached cellular abundance, as reported by Kothia and Soni.¹⁸

Flocculating Efficacy in Chemically Defined Screening Media

The central empirical tenet of the study evaluated the bioflocculating efficacy of the bacterial cell-free supernatants under rigorously controlled kinetic intervals. Initial visual observations during the standard kaolin clay assay confirmed that while the negative controls maintained a highly stable, dispersed, and optically

Table 1. Quantitative Enumeration of Bacterial Load (CFU/mL) Across Selected Environmental Sources

Isolation Source	Un-Diluted (CFU/mL)	10^{-1} (CFU/mL)	10^{-3} (CFU/mL)	10^{-5} (CFU/mL)	10^{-7} (CFU/mL)
Aeration Tank	TN	TN	2.0×10^6	1.0×10^4	5.0×10^2
Treated Effluent	TN	TN	1.0×10^5	1.0×10^4	5.5×10^1
Sedimentation Tank	TN	TN	3.0×10^6	1.5×10^4	7.5×10^2
ETP Activated Sludge	TN	TN	1.8×10^7	1.0×10^5	5.0×10^3
Soil (Municipal Solid Waste Site-Anand)	TN	TN	1.6×10^6	1.4×10^4	4.0×10^2
Soil (Municipal Solid Waste Site-Bharuch)	TN	TN	2.5×10^6	1.2×10^4	6.0×10^2

Note: TN = Too Numerous to Count, CFU = Colony Forming Units

Table 2. Morphological Profiles and Gram Staining Properties of Elite Bacterial Isolates

Isolates	Isolation Source	Colony	Configuration	Margin	Elevation	Surface	Opacity	Colour	Gram Staining	Shape
ASW15	Soil (Municipal Solid Waste)	Sticky, Slimy	Rhizoid	Rhizoid	Convex	Rough	Opaque	Cream	Gram-negative	Rod-shaped bacilli
ASW17	Soil (Municipal Solid Waste)	Slimy, Mucoid	Irregular	Lobate	Pulvinate	Smooth	Opaque	LightPink	Gram-negative	Round-shaped cocci (spheres in clusters)
ASW9	Soil (Municipal Solid Waste)	Shiny	Irregular	Curled	Raised	Rough	Translucent	Cream	Gram-negative	Round-shaped cocci
ASW3	Soil (Municipal Solid Waste)	Slimy, Sticky	Irregular	Undulate	Flat	Rough	Opaque	Cream	Gram-positive	Cocco-bacillus (short rods)
ASW5	Soil (Municipal Solid Waste)	Ropy, Mucoid	Filamentous	Filamentous	Convex	Smooth	Opaque	LightPink	Gram-positive	Singlerod-shaped bacilli
TE1	Treated Effluent	Shiny	Circular	Entire	Flat	Rough	Transparent	Cream	Gram-positive	Cocco-bacillus (short rods)
A7	Aeration Tank	Dull	Spindle	Entire	Raised	Smooth	Transparent	Cream	Gram-positive	Cocco-bacillus (short rods)
A8	Aeration Tank	Slimy	Irregular	Curled	Flat	Smooth	Translucent	Yellow	Gram-positive	Single rod- shaped bacilli
A9	Aeration Tank	Shiny, Slimy	Spindle	Undulate	Pulvinate	Smooth	Transparent	White	Gram-negative	Diplo-cocci
A10	Aeration Tank	Dull, Mucoid	Circular	Entire	Flat	Rough	Translucent	White	Gram-positive	Cocco-bacillus (short rods)
SED11	Sedimentation Tank	Ropy	Filamentous	Filamentous	Raised	Rough	Transparent	Cream	Gram-positive	Cocco-bacillus (short rods)
SED5	Aeration Tank	Dull	Irregular	Lobate	Convex	Rough	Opaque	Cream	Gram-positive	Bacilli with short rods
A13	Aeration Tank	Mucoid	Circular	Entire	Convex	Smooth	Transparent	White	Gram-positive	Bacilli with long rod clusters
S14	ETP Activated Sludge	Sticky	Rhizoid	Rhizoid	Pulvinate	Rough	Opaque	Cream	Gram-positive	Bacilli with long chain

Note: A: Aeration Tank, TE: Treated Effluent, SED: Sedimentation Tank, S: ETP Activated Sludge, ASW: Anand Municipal Solid Waste Site, BSW: Bharuch Municipal Solid Waste Site

Table 3. Polyphasic Biochemical and Enzymatic Characterization of Selected High-Yielding Isolates

Isolates	ASW15	ASW17	ASW9	ASW3	ASW5	TE1	A7
Gram	Gram-negative	Gram-negative	Gram-negative	Gram-positive	Gram-positive	Gram-positive	Gram-positive
Staining	Rod-shaped	Round-shaped	Round-shaped	Cocco-bacillus	Single rod-shaped	Cocco-bacillus	Cocco-bacillus
Shape	bacilli	cocci	cocci		bacilli		
Spore-Stain	+	-	+	-	+	-	+
Acid-Fast	-	-	-	+	-	-	-
Catalase Test	+	+	+	-	+	+	+
Starch Hydrolysis	-	+	-	+	-	+	-
Citrate test	+	-	-	+	-	+	-
Mannitol Fermentation	+	-	+	-	+	-	+
Hemolysis	α	β	γ	α	γ	β	α
Bile Esculin	-	+	-	+	-	+	-
Oxidase Test	-	+	-	+	-	+	-
Lactose Fermentation	+	-	+	-	+	-	+
Indole Test	-	+	-	+	-	+	-
MR-VP Test	+	-	+	-	+	-	+
Urease Test	-	+	-	+	-	+	-
Nitrate Reduction	+	-	+	-	+	-	+
Motility	+	+	-	+	-	+	-
SIM Test	+	-	+	-	+	-	+
Glucose Fermentation	+	+	+	+	+	+	+
Activity	High	Moderate	High	Moderate	High	Moderate	High
Na ⁺ Requirement	-	+	-	+	-	+	-

Table 4. Polyphasic Biochemical and Enzymatic Characterization of Selected High-Yielding Isolates

Isolates	A8	A9	A10	SED11	SED5	A13	S14
Gram	Gram-positive	Gram-negative	Gram-positive	Gram-positive	Gram-positive	Gram-positive	Gram-positive
Staining	Single	Diplo-	Cocco-	Cocco-	Bacilli	Bacilli	Bacilli
Shape	rod-shape bacilli	cocci	bacillus	bacillus	with short rods	with long rod clusters	with long chain
Spore-Stain	-	+	-	+	-	+	-
Acid-Fast	+	-	-	-	+	-	+
Catalase Test	-	+	+	+	-	+	+
Starch	+	-	+	-	+	-	+
Hydrolysis							
Citrate test	+	-	+	-	+	-	+
Mannitol	-	+	-	+	-	+	+
Fermentation							
Hemolysis	α	γ	γ	α	β	γ	$\tilde{\alpha}$
Bile Esculin	+	-	+	-	+	-	+
Oxidase Test	+	-	+	-	+	-	+
Lactose	-	+	-	+	-	+	-
Fermentation							
Indole Test	+	-	+	-	+	-	+
MR-VP Test	-	+	-	+	-	+	-
Urease Test	+	-	+	-	+	-	+
Nitrate	-	+	-	+	-	+	-
Reduction							
Motility	+	-	+	-	+	-	+
SIM Test	-	+	-	+	-	+	-
Glucose	+	+	+	+	+	+	+
Fermentation							
Activity	Moderate	Moderate	Moderate	High	High	High	High
Na ⁺	+	-	+	-	+	-	+
Requirement							

Note:

- Spore Stain: “+” indicates the presence of spores, “-” indicates no spores observed.
- Acid-Fast: “+” indicates acid-fast bacteria, “-” indicates non-acid-fast bacteria.
- Catalase Test: “+” indicates catalase-positive, “-” indicates catalase-negative.
- Starch Hydrolysis: “+” indicates positive for starch breakdown, “-” indicates no starch breakdown.
- Citrate Test: “+” indicates the ability to utilize citrate, “-” indicates inability.
- Mannitol Fermentation: “+” indicates positive for mannitol fermentation, “-” indicates negative.
- Hemolysis: “ α ” = partial (alpha), “ β ” = complete (beta), “ γ ” = none (gamma).
- Bile Esculin: “+” indicates ability to hydrolyze bile esculin, “-” indicates negative.
- Oxidase Test: “+” indicates oxidase-positive, “-” indicates oxidase-negative.
- Lactose Fermentation: “+” indicates lactose fermentation, “-” indicates none.
- Indole Test: “+” indicates indole production, “-” indicates no production.
- MR-VP Test: “+” indicates either Methyl Red or Voges-Proskauer positivity, “-” indicates negative for both.
- Urease Test: “+” indicates urease activity, “-” indicates none.
- Nitrate Reduction: “+” indicates nitrate reduction capability, “-” indicates no reduction.
- Motility: “+” indicates motile bacteria, “-” indicates non-motile.
- SIM Test: “+” indicates presence of hydrogen sulfide or indole production or motility; “-” indicates absence.
- Glucose Fermentation: “+” indicates glucose fermentation, “-” indicates none.
- Activity: General bioactivity level in flocculation, either “High” or “Moderate.”
- Na⁺ Requirement: “+” indicates requirement for Na⁺, “-” indicates no requirement.

dense colloidal state indefinitely, the precise introduction of the biofloculants triggered near-instantaneous coagulation. Massive flocs formed rapidly, precipitating out of suspension and leaving a highly clarified upper aqueous phase.²⁶

Analysis of the 14 elite strains demonstrated that maximal flocculating rates (frequently >80%) were predominantly achieved between 24 and 48 hours of uninterrupted incubation in both the Screening and Production media. Extending the incubation cycle to 72 hours resulted in a precipitous, universal decline in flocculating activity (FA). This kinetic degradation correlates flawlessly with the onset of the bacterial death phase; the rapid depletion of essential nutrients triggers massive cellular autolysis and the subsequent secretion of endogenous biopolymer-degrading enzymes (e.g., polysaccharases and proteases) that dismantle the structural integrity of the EPS matrix, as described by Kothia and Soni.¹⁸

When cultivated in the chemically defined Screening Medium, morphological traits correlated intriguingly with performance metrics. Isolates exhibiting Small Colony (SC) morphotypes displayed a highly steady, time-dependent escalation in FA over a 48-hour incubation. Isolate A4 achieved a notable 73.90% FA, closely followed by S12 (73.50%) and A2 (69.10%). Conversely, Kothia and Soni¹⁸ explained that large Colony (LC) morphotypes exhibited profound kinetic variance; isolate A5 peaked dramatically at an exceptional 75.30%, while A1 lagged significantly at merely 23.10%.

Temporal kinetic assessments across specific observational intervals (5, 10, 15, and 20 minutes) revealed highly strain-specific behavior. Kothia and Soni¹⁸ described that isolate A9 generated unparalleled results in the standard Screening Medium, achieving an FA of 97.60% almost immediately and maintaining complete structural stability across all observed temporal intervals. In stark contrast, isolates such as A10 and SED5 exhibited severe temporal instability, characterized by negative or highly erratic FA readings at earlier intervals (5–10 mins). This behavior suggests delayed polymer activation or weak initial bridging that strictly required prolonged contact times to form structurally stable macroscopic flocs.

Flocculating Efficacy in Wastewater-Derived Production Media

Cultivation within the complex, wastewater-derived Production Medium profoundly amplified the flocculating capabilities of the vast majority of the isolates. This unequivocally indicates that the heterogeneous nutrients, trace heavy metals, and intense physicochemical stressors intrinsic to real ETP wastewater act as powerful stimulants for massive EPS biosynthesis.

After 48 hours of incubation in the Production Medium, isolates SED3, SED5, A9, and A10 exhibited a sustained, highly aggressive flocculation trajectory. Kothia and Soni¹⁸ explained that these strains consistently breached the 90% FA threshold across all tests and frequently culminated in near-total clarification (>98% FA) by the 20-minute observational limit. Similarly, MSW-derived soil isolates, specifically ASW9, consistently delivered superior metrics (>95% FA) across all measured intervals, proving their profound resilience to variable substrate qualities, as reported by Kothia and Soni.¹⁸

Kothia and Soni¹⁸ also discussed about the Anand MSW strains, including ASW21 and ASW24, exhibited a highly progressive temporal escalation in the Production Medium, advancing smoothly from roughly 37% FA at 5 minutes to highly robust values nearing 67% FA by 20 minutes. Isolates sourced from the Bharuch MSW site (BSW series) displayed highly complex kinetic profiles. Isolate BSW16 emerged as the apex performer in this specific subgroup, achieving 60.98% FA at 24 hours and expanding its efficacy to 65.00% FA at 48 hours. Isolate BSW14 likewise accelerated from a moderate baseline activity to a highly robust 71.00% FA at the 48-hour mark, as studied by Kothia and Soni.¹⁸

Morphological and Phenotypic Profiles of Elite Isolates

The 14 elite biofloculant-producing isolates underwent extensive morphological scrutiny. Table 2 details the colony configurations, surface textures, physical pigmentation, and cellular morphotypes of these resilient strains. The prevailing colony architectures were uniquely characterized by highly irregular, rhizoid, or filamentous margins, coupled with robustly mucoid, slimy, or ropy surface topographies. Such structural manifestations are definitive macroscopic

indicators of copious capsular polysaccharide and extracellular biopolymer accumulation, essential traits for high-efficiency bioflocculants, as described by Kothia and Soni¹⁸ as well as Huang et al.³³

Gram staining revealed a distinct taxonomic bifurcation within the elite cohort. Kothia and Soni¹⁸ explored that ten isolates were classified as Gram-positive, predominantly exhibiting bacillus and coccobacillus morphotypes, frequently arranged in long structural chains or dense clusters (e.g., S14, A13, SED5). Conversely, four isolates (ASW15, ASW17, ASW9, and A9) were definitively Gram-negative, encompassing rod-shaped bacilli and clustered cocci/diplococci. This pronounced morphological diversity confirms that prodigious bioflocculant production is not restricted to a narrow phylogenetic clade but is rather a widely distributed metabolic adaptation to severe environmental stressors.³⁴

Polyphasic Biochemical and Enzymatic Fingerprinting

The profound metabolic versatility of the isolates was comprehensively mapped through biochemical assays, providing critical insights into their physiological resilience, enzymatic repertoire, and substrate utilization pathways (Table 3). A critical marker of long-term environmental persistence—endospore formation—was observed in seven isolates (ASW15, ASW9, ASW5, A7, A9, SED11, A13), as discussed by Kothia and Soni.¹⁸ This trait suggests highly advanced evolutionary adaptations designed to withstand prolonged desiccation, intense chemical toxicity, and the extreme thermal fluctuations prevalent in MSW landfill sites and pharmaceutical ETPs.

Catalase synthesis was nearly ubiquitous across the cohort. This enzymatic capability indicates highly robust mechanisms for helping the cells withstand oxidative stress through the detoxification of reactive oxygen species (ROS), a common metabolic byproduct in highly aerated, pollutant-heavy environments. Interestingly, carbohydrate metabolism was extremely active; 100% of the isolates successfully fermented glucose, providing the fundamental carbon skeletons and ATP required for massive EPS biosynthesis. Conversely, lactose and mannitol

fermentation varied strictly on a strain-by-strain basis, highlighting highly specific carbon assimilation preferences, as outlined by Kothia and Soni.¹⁸

Enzymatic degradation of complex natural substrates, evidenced by positive starch hydrolysis in several strains and highly variable hemolytic activity on blood agar (ranging from α to β and γ profiles), further underscored the profound metabolic plasticity required to thrive in chemically complex effluents. Notably, the absence of a strict Na^+ requirement among the majority of isolates implies that their cellular growth and bioflocculant production are not inextricably contingent upon hypersaline conditions. Hence, Kothia and Soni¹⁸ reviewed that this physiological flexibility drastically simplifies their potential commercial application across diverse freshwater or standard industrial wastewater matrices.

Synergistic Bioflocculation vs. Microbial Consortia

The study also rigorously evaluated the potential of synergistic microbial consortia, as mixed cultures frequently dominate natural wastewater systems.³⁵ Dual combinations of specific isolates yielded highly complex, time-dependent outcomes. Similarly, as discussed by Kothia and Soni,¹⁸ In a 24-hour Production Medium, the binary consortium of A5 + A4 achieved an initial rapid flocculation (52.70% at 5 minutes) but suffered a severe degradation in structural stability over time, dropping precipitously to 32.60% at 20 minutes.

However, extending the consortium cultivation to 48 hours radically altered the kinetic profile; the specific A4 + A3 combination demonstrated a powerful, sustained upward trajectory, attaining an exceptional 76.10% FA at the 20-minute mark. This suggests that specific microbial pairings can engage in complex metabolic cross-feeding or synthesize complementary polymer matrices that synergistically enhance macroscopic floc durability, as reported by Kothia and Soni.¹⁸ Interestingly, highly complex tertiary combinations (e.g., A5 + A4 + A3) resulted in severely suppressed FA, likely due to intense metabolic antagonism, direct nutrient competition, or spatial steric hindrance among competing, incompatible biopolymers.

DISCUSSION

Scientific Implications and Environmental Framework

The exhaustive empirical data generated throughout this investigation offers profound, multi-layered insights into the complex biological synthesis, physical kinetics, and immense practical applicability of microbial bioflocculants derived from highly stressed environmental ecosystems. The systematic transition from fundamental microbial isolation to the kinetic optimization of bioflocculation and the subsequent evaluation of sludge compaction dynamics establishes a highly compelling, holistic framework for replacing toxic chemical coagulants with sustainable, biodegradable alternatives in advanced wastewater treatment facilities.^{36,34}

Ecological Significance of Isolate Diversity and EPS Biosynthesis

The morphological and biochemical diversity of the recovered strains highlights the ubiquity of bioflocculation as a fundamental survival mechanism. The identification of both Gram-positive (e.g., *Bacillus* species) and Gram-negative (e.g., *Pseudomonas* and *Klebsiella* species) EPS producers is highly consistent with global literature regarding microbial diversity in activated sludge.³⁷ Strains such as *Bacillus* and *Pseudomonas* are globally recognized for their profound environmental adaptability and their capacity to secrete massive quantities of complex heteropolysaccharides and structural glycoproteins.³⁸

Recent molecular advancements suggest that the extraordinary bioflocculant yield in related genera, such as *Klebsiella*, is heavily regulated by specific biosynthetic gene clusters, including polyketide synthase I (PKS-I), polyketide synthase II (PKS-II), and non-ribosomal peptide synthetase (NRPS).³⁷ The presence of such advanced regulatory machinery allows these bacteria to dynamically alter the structural composition and molecular weight of their secreted EPS in direct response to the specific physicochemical stressors present in their immediate environment, thereby optimizing their flocculation capabilities.³⁷ The ubiquitous presence of catalase and glucose fermentation pathways across our elite isolates signifies highly evolved enzymatic mechanisms

for neutralizing reactive oxygen species (ROS) and generating the massive adenosine triphosphate (ATP) required to fuel these energy-intensive biosynthetic pathways, as described by Lu et al.³⁹

Media-Driven Metabolic Upregulation and Circular Economy Integration

A pivotal, transformative aspect of this research was the comparative evaluation of bioflocculant yield and efficacy between a highly purified, chemically defined Synthetic Screening Medium and a complex, highly variable wastewater-derived Production Medium. Historically, the widespread commercialization of MBFs has been crippled by the prohibitive economic costs associated with chemically defined fermentation substrates, such as analytical-grade glucose, yeast extracts, and peptones.⁴⁰

In this study, completely substituting the distilled water and refined carbon base with raw, untreated wastewater from Aeration Tanks, Sedimentation Tanks, and Activated Sludge significantly augmented the flocculation efficiency of the isolates. Strains such as BSW14, BSW16, ASW9, and A9 reached extraordinary FA levels (>95%) only when cultivated in the unrefined Production Medium, as explained by Kothia and Soni.¹⁸ The superior performance in this matrix can be directly attributed to the diverse array of trace minerals, complex organic carbons, and native nitrogenous compounds present in the effluent. These raw components likely function as essential enzymatic co-factors or direct metabolic precursors that upregulate the genetic pathways responsible for rapid polysaccharide and glycoprotein biosynthesis.⁴¹

From an advanced environmental engineering perspective, this represents a profound realization of circular economy principles. The utilization of highly toxic industrial effluents as free, nutrient-dense substrates to mass-produce the very biological agents required to remediate them establishes a closed-loop technological paradigm. This approach drastically offsets the massive capital expenditures associated with commercial bioflocculant production, while simultaneously providing critical secondary organic load reduction (BOD/COD removal) in the utilized wastewater during the fermentation process, in agreement with the observations of Yang et al.⁴²

Physicochemical Mechanisms of Bioflocculation: Bridging and Charge Neutralization

The remarkable capability of the extracted cell-free supernatants to rapidly coagulate fine kaolin clay suspensions underscores the highly complex physicochemical interactions orchestrated by microbial EPS.¹⁴ The fundamental mechanism of EPS-driven bioflocculation is bipartite, relying simultaneously on aggressive charge neutralization and extensive polymer bridging.³³

Fine kaolin clay particles natively possess a highly negative net surface charge (zeta potential) when dispersed in aqueous suspensions. This creates intense electrostatic repulsion between individual particles, maintaining a highly stable, turbid colloidal state that strongly resists gravitational settling.¹⁴ The introduction of the bioflocculant, strictly alongside divalent cations like Ca^{2+} or Mg^{2+} , facilitates a critical electrochemical intermediary step. The divalent cations act as potent cationic bridges, effectively neutralizing the intense electrostatic repulsion between the anionic clay particles and the negatively charged functional groups (such as carboxyl, hydroxyl, and phosphoric groups) that are distributed abundantly across the massive EPS backbone.²⁶

Once this initial charge neutralization is achieved, the massive, long-chain macromolecular structure of the bioflocculant physically adsorbs onto multiple suspended particles simultaneously. It physically ensnares them in a highly cross-linked, three-dimensional polymeric web—a purely physical process defined as polymer bridging, as described by Hadadi et al.⁴³ As observed in the highly aggressive, instantaneous kinetics of isolates like SED3 and A9, this extensive bridging leads to the rapid formation of macroscopic, dense flocs that precipitate rapidly under the influence of gravity, achieving pristine optical clarification of the aqueous phase.^{39,10}

Kinetic Constraints, Optimal Harvest Windows, and Autolytic Degradation

The temporal kinetic profiling revealed that Flocculating Activity is highly transient and strictly contingent upon the precise duration of cultivation. The optimal EPS harvest window occurred squarely between 24 and 48 hours, corresponding to the late-exponential and early-stationary growth phases of the isolates.¹⁸ Extending the biological incubation to 72 hours provoked a

severe, universal collapse in FA across virtually all tested strains.¹⁸

This severe kinetic degradation is a well-documented microbiological phenomenon wherein the total exhaustion of available carbon and nitrogen substrates triggers massive cellular autolysis.³³ The physical lysis of the cell membrane releases high concentrations of destructive intracellular hydrolases, proteases, and polysaccharases directly into the surrounding medium. These enzymes rapidly depolymerize the long-chain EPS structures into much smaller, completely ineffective monomeric or oligomeric fragments, thereby entirely obliterating their structural capacity to act as inter-particle bridging agents.⁴⁴ This empirical finding underscores the critical operational necessity of precisely timing the harvest, extraction, and stabilization of MBFs in industrial-scale continuous-flow bioreactors to permanently preserve their macromolecular integrity.

Comparative Environmental Life Cycle Assessment (LCA): Bioflocculants vs. Chemical Coagulants

The true significance of these microbial bioflocculants extends far beyond mere optical performance metrics; their absolute value is fundamentally rooted in their environmental benignity and superior Life Cycle Assessment (LCA) profile. Conventional inorganic coagulants, predominantly Aluminum Sulfate (Alum) and Ferric Chloride (FeCl_3), have historically dominated the wastewater industry due to their aggressive, brute-force charge-neutralization capabilities, as reported by Naghan et al.⁴⁵ However, the deployment of Alum routinely causes severe depression of the effluent pH, necessitating continuous, expensive chemical buffering, while simultaneously generating vast quantities of heavy, non-biodegradable, metallically contaminated sludge.⁴³ Moreover, the leaching of residual aluminum ions into downstream drinking water reservoirs poses a well-documented, severe neurotoxic threat to human populations.¹⁷

In stark contrast, the bioflocculants evaluated in this study—synthesized natively by strains such as ASW9, SED3, and BSW14—exhibited flocculation efficiencies completely parallel to, and in specific metrics superior to, those historically achieved by FeCl_3 and Alum,

without inducing any deleterious shift in the physicochemical equilibrium (pH or salinity) of the water matrix.⁴³ Cosa and Okoh⁴⁶ demonstrated that because they are inherently organic polymers, MBFs degrade safely, rapidly, and naturally in the environment without generating any secondary toxic metallic residues. Furthermore, the sludge generated via bioflocculation is entirely biodegradable and highly enriched with complex organic nutrients. This presents highly viable downstream commercial opportunities for agricultural repurposing as high-grade compost or organic fertilizer, thereby massively enhancing the holistic sustainability profile and economic viability of the entire treatment process, as reported by Kurniawan et al.⁴⁷

Synergistic Microbial Interactions and Consortium Dynamics

While pure monocultures provide essential baseline data, industrial wastewater systems operate entirely through complex microbial consortia. This study's exploration into dual-isolate combinations revealed the highly nuanced nature of synergistic bioflocculation, as described by Xu et al.⁴⁸ The exceptional performance of the A4 + A3 consortium (76.10% FA at 20 minutes) indicates that compatible strains can engage in highly beneficial metabolic cross-feeding.¹⁸ One strain may break down complex recalcitrant organics into simpler monomers, which the secondary strain then utilizes to hyper-secrete specific glycoproteins or polysaccharides, resulting in a composite, highly durable EPS matrix that resists shear forces during sedimentation.³⁵ Conversely, the failure of tertiary combinations highlights the risks of metabolic antagonism, where excessive competition for trace nutrients or the secretion of hostile bacteriocins actively degrades overall flocculant yield.⁴⁸ This suggests that industrial application requires the precise engineering of highly compatible synthetic microbial consortia rather than random poly-inoculations.

Future Perspectives: Nanotechnological Integration and Genetic Engineering

Looking forward, the integration of these highly potent microbial bioflocculants with emerging fields such as nanotechnology presents a transformative frontier in environmental remediation. Recent literature highlights the immense potential of utilizing microbial

bioflocculants MBFs as green, non-toxic capping and reducing agents for the biosynthesis of highly reactive metallic nanoparticles, such as Iron Nanoparticles (FeNPs) and Copper Nanoparticles (CuNPs).⁴⁸ When combined, the structural bridging capacity of the bioflocculant matrix, synergized with the intense catalytic and antimicrobial activity of the embedded nanoparticles, creates a highly advanced "smart" nanocomposite capable of simultaneously flocculating suspended solids while catalytically degrading dissolved organic pollutants and neutralizing resilient pathogens.⁴⁹ Ndejiko and Swalaha⁵⁰ reviewed future research must aggressively pursue these nanotechnological integrations, alongside the advanced genomic sequencing of elite isolates to identify and over-express the specific polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) gene clusters regulating EPS biosynthesis, thereby securing the ultimate commercial scalability of this critical green technology.

CONCLUSION

This comprehensive investigation unequivocally demonstrates the profound, largely untapped potential of exploiting native microbial biodiversity for the advanced, sustainable remediation of severe industrial and municipal wastewater. By meticulously isolating and evaluating 14 elite, highly resilient bacterial strains sourced directly from extreme environments—specifically pharmaceutical effluent treatment plants and volatile municipal solid waste sites—this study successfully proved that utilizing native, highly complex, nutrient-chaotic wastewater as a direct production medium drastically, and statistically significantly, enhances extracellular polymeric substance yield and overall flocculation kinetics. The microbially generated bioflocculants demonstrated an extraordinary capacity to rapidly clarify highly stable, repulsive colloidal suspensions, relying heavily on divalent cation-mediated charge neutralization and massive polymer bridging to consistently achieve phenomenal flocculating activities routinely exceeding 95%. Crucially, the strategic application of these unique microbial polymers fostered the immediate development of highly dense, heavily compacted, and rapidly settling physical aggregates. This rapid

agglomeration aggressively optimized the Sludge Volume Index (SVI), effectively mitigating the highly voluminous, water-logged, and toxic sludge generation that is the hallmark of conventional chemical coagulants like alum and ferric chloride. By generating a completely non-toxic, highly biodegradable sludge that is perfectly primed for downstream anaerobic digestion and agricultural valorization, these findings firmly position microbially derived biofloculants not merely as theoretical novelties, but as highly potent, ecologically benign, and economically sustainable alternatives to synthetic commercial polymers. The integration of these advanced biological systems offers a highly viable, technologically sound pathway toward the realization of zero-residue, circular-economy-aligned wastewater management frameworks on a global industrial scale.

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Author Contributions

Bhumi A. Kothia: Conceptualization, Methodology, Investigation, Data Collection, Formal Analysis, Funding Acquisition, Writing – Original Draft; Hiren B. Soni: Conceptualization, Supervision, Resources, Validation, Writing – Review & Editing.

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