

Environmental engineering Aplinkos inžinerija

BIOFILM FORMATION KINETICS AND SULFUR OXIDATION PERFORMANCE OF *ACIDITHIOBACILLUS* SPP. CULTIVATED IN BIOFILTER PACKING COMPOSED OF KOH-MODIFIED BIOCHAR AND FeCO_3 -IMPREGNATED CLC WASTE

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Abstract. This article interrogates the sulfur-oxidation kinetics and spatial biofilm architecture of *Acidithiobacillus* spp. (DSM 12475, DSM 739) supported on KOH-activated biochar and FeCO_3 -modified Cellular Lightweight Concrete (CLC) waste within a 1.0-m controlled biofilter. Pure cultures were anaerobically enriched for 96 h at 30 °C in nitrate-reducing medium, achieving $\sim 10^7$ CFU mL⁻¹ before inoculation. The reactor was operated for 144 h at 30 °C, 45–60% moisture, 0.2–1.0 L min⁻¹ flow, Empty Bed Retention Time (EBRT) 15–60 s, and inlet concentrations of 100–2000 ppm H₂S. Microscopy revealed slower, but highly stable colonization kinetics compared to *Pseudomonas* spp. Biomass increased from 7.2×10^5 to 3.1×10^6 CFU/sample over 72–96 h, with uniform distribution across all six bed layers. Extracellular Polymeric Substance (EPS) visualization confirmed maturation of cohesive biofilms under localized acidification driven by sulfate accumulation. The hierarchical porosity of biochar promoted greater cell loading, while FeCO_3 -CLC enhanced catalytic oxidation of thiosulfate and sulfur intermediates, contributing to stable redox cycling. Steady-state operation yielded Removal Efficiencies (RE) of 85–92% and elimination capacities of 14–18 g H₂S m⁻³ h⁻¹ with minimal functional decline across the 144-h cycle. The facultative anaerobic metabolism of *Acidithiobacillus* spp. enabled sustained oxidation under oxygen-limited and mildly acidic microenvironments – operational regimes in which many conventional SOB systems destabilize. Collectively, the results validate the presence of *Acidithiobacillus* spp. – engineered media systems as robust, acid-tolerant, and long-duration biocatalytic platforms suited for high-sulfur, variable-composition biogas purification.

Keywords: hydrogen sulfide removal, biofilter, biochar, cellular concrete (CLC) waste, microbial desulfurization, biogas purification.

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1. Introduction

Biogas hydrogen sulfide (H₂S) is one of the most critical contaminants in raw biogas, frequently occurring at concentrations ranging from several hundred to several thousand parts per million depending on substrate composition, digester performance, and operational conditions (Appala et al., 2022; Alkhatib et al., 2021). Its presence poses severe environmental and industrial challenges due to its high toxicity, corrosive action on metallic surfaces, and inhibitory effects on catalytic upgrading systems (Bu et al., 2021; Cortes et al., 2021). Beyond direct material degradation, the oxidative conversion of H₂S to SO_x emissions restricts its combustion-based utilization and contributes to air quality deterioration (Choudhury & Lansing, 2021; Clotas et al., 2020). As biogas upgrading technologies expand globally, the development of reliable and sustainable H₂S abatement systems has become a technological priority (Cano et al., 2021; Danila et al., 2022).

Biological H₂S removal offers an intrinsically self-regenerating, low-cost, and environmentally compatible alternative, relying on Sulfur-Oxidizing Bacteria (SOBs) to convert sulfide into elemental sulfur (S⁰), thiosulfate (S₂O₃²⁻), or sulfate (SO₄²⁻) (De Souza et al., 2021; Das et al., 2022). However, the use of biofilters (BFs) in biogas purification remains limited by several unresolved mechanistic and operational limitations (Ghimire et al., 2021; Haosagul et al., 2020). These include a) packing material instability, especially under acidic conditions generated by sulfate accumulation; b) insufficient microbial retention and limited long-term biofilm persistence on conventional substrates such as compost or woodchips; c) oxygen diffusion limitations in deeper filter zones, which suppress the activity of classical obligately aerobic SOBs (e.g., *Thiobacillus*, *Paracoccus*); and d) slow regeneration and mass-transfer bottlenecks under high-sulfide loading rates typical of real digester gases (Jiang et al., 2020; Jedynak & Charnas, 2023).

Recent advances have highlighted that waste-derived materials such as sewage sludge-derived bio-char

offer promising sorptive, catalytic, and structural properties suitable for SOB colonization (Khan et al., 2021; Lee et al., 2021). KOH-modified biochar can be used due to its micro-mesoporous structure, increased surface area, favorable moisture-retention capacity, and good environment for microbial activity (Lin et al., 2021; Mohammadi & Vaiškūnaitė, 2023). Mean-while, FeCO₃-modified cellular lightweight concrete (CLC) waste introduces catalytic redox-active Fe (II)/Fe (III) cycling, which can accelerate sulfur-intermediate transformations and buffer local acidity in the biofilm microenvironment (Mitchell et al., 2022).

Equally important is the selection of microbial species capable of tolerating fluctuations in oxygen presence, acidic conditions, and nutrient limitations within the operating biofilters (Nhut et al., 2020). Un-like classical obligate aerobes (e.g., *Thiobacillus thiooxidans*), anaerobic *Acidithiobacillus* spp. allow sustained sulfur oxidation under nitrate-reducing, oxygen-limited, and mildly acidic conditions-operational regimes where many traditional SOB communities become unstable (Pudi et al., 2022; Poser et al., 2023; Shi et al., 2022).

Despite growing interest, the mechanistic coupling between engineered waste-derived media and anaerobic SOBs remains insufficiently studied, particularly in terms of a) spatial biofilm architecture and vertical distribution patterns along the filter bed; b) colonization kinetics on chemically modified substrates with hierarchical porosity; c) the catalytic role of FeCO₃ in mediating sulfur intermediate oxidation under biologically relevant redox conditions; d) micro-environmental acidification effects on long-term viability of *Acidithiobacillus* spp.; and e) sustained elimination capacity under high-sulfur, fluctuating concentration loads typical of industrial biogas (Torres et al., 2020; Vaiškūnaitė, 2020).

To address these knowledge gaps, this study systematically investigates the biofilm kinetics and sulfur oxidation performance of *Acidithiobacillus* spp. strains DSM 12475 and DSM 739 supported on KOH-modified biochar and FeCO₃-impregnated CLC waste within a 1.0-m controlled laboratory biofilter operating under realistic biogas treatment conditions (100–2000 ppm H₂S; EBRT 15–60 s). By integrating fluorescence microscopy, microbial quantification, sulfur-intermediate profiling, and multilayer bed analysis, the research elucidates the spatial distribution of biomass, Extracellular Polymeric Substance (EPS) development, and the material-microbe interactions. The combined physicochemical and microbiological in-sights provide a mechanistic foundation for designing durable and high-performing biofilters that leverage waste-derived, low-cost packing materials and anaerobic sulfur-oxidizing bacteria (Wang et al., 2022; Wu et al., 2020).

2. Materials and methods

2.1. Biofilter design and experimental configuration

A laboratory-scale, vertically oriented packed-bed biofilter was constructed to evaluate the performance of



Figure 1. Schematic of the reactor, gas inlet/outlet, irrigation system, and sampling port arrangement

engineered media (KOH-modified biochar and FeCO₃-impregnated CLC waste) in supporting *Acidithiobacillus* spp. mediated sulfur oxidation (Jiang et al., 2020). Raw biochar was produced via slow pyrolysis of municipal sewage sludge at 600 °C for 2 h under oxygen-limited conditions, followed by KOH activation (Watsuntorn et al., 2020). The reactor column was fabricated from a transparent cylinder (height: 1.0 m; inner diameter: 14 cm), allowing continuous monitoring of moisture distribution, material settling, and biofilm development (1 Figure). The column was equipped with six equidistant sampling ports (every 16 cm) along its height to facilitate spatially resolved analysis of microbial colonization, physicochemical changes, and biofilm dynamics. Gas distribution was controlled by a support plate at the column base, ensuring integrated upward flow through the packed bed.

A Wi-Fi-enabled automated irrigation system maintained optimal moisture conditions (Mitchell et al., 2022). A TDR-based moisture probe (VH400, ±3 % accuracy) was installed at mid-column depth and interfaced with a solenoid pump to activate a water spray when the moisture content fell below 45% (Xu et al., 2022). The BF was thermally regulated at 30 °C using a digital heat-control unit to sustain microbial metabolic activity (Table 1) (Ying et al., 2020; Zhang et al., 2022; Zhang et al., 2021).

Table 1. The BF was operated under conditions simulating realistic biogas desulfurization

Parameter	Range
H ₂ S concentrations	100, 500, 1000, 1500, and 2000 ppm
Flow rate	Adjusted accordingly between 0.2–1.0 L min ⁻¹
EBRT	15, 30, 45, and 60 s
Moisture	45–60% (automatic irrigation)
Temperature	30 °C (thermostatically controlled)

Real-time H₂S concentrations were recorded at inlet and outlet ports. The Removal Efficiency (RE, %) and Elimination Capacity (EC, g m⁻³ h⁻¹) of the biofilter were calculated as follows (Zhanga et al., 2020):

$$RE(\%) = \frac{C_{in} - C_{out}}{C_{in}} \times 100; \quad (1)$$

$$EC = \frac{Q(C_{in} - C_{out})}{V}, \quad (2)$$

Q = gas flow rate ($\text{m}^3 \text{h}^{-1}$), V = bed volume (m^3), C_{in} = inlet concentration of H_2S to BF (ppm), C_{out} = outlet concentration of H_2S from BF (ppm).

2.2. Preparation and modification of packing materials

Two waste-derived materials-sewage-sludge biochar and CLC waste (at a 1:1 mass ratio (corresponding to approximately 60:40 v/v due to density differences), were engineered to enhance surface area, porosity, catalytic reactivity, and biocompatibility.

Raw biochar (particle size: 2–5 mm) was modified with KOH (1:3 mass ratio, biochar: KOH) to introduce micro-mesoporous structures and increase surface area. The mixture was heated in a muffle furnace under N_2 at 600 °C for 2 h, followed by extensive washing with deionized water until the pH reached neutrality (Torres et al., 2020; Ying et al., 2020). Expected modifications included increased BET surface area, creation of microporous networks, and improved microbial activity. CLC waste particles were sieved (3–7 mm), rinsed, and dried before being impregnated in FeCO_3 solution (0.1 M) (Wang et al., 2022). Through precipitation-impregnation, FeCO_3 formed within the CLC pore network and on external surfaces. This modification provided catalytic Fe (II)/Fe (III) cycling, enhanced oxidation of thiosulfate and S^0 intermediates, and local pH buffering.

2.3. Microscopy and biofilm assessment

Solid-phase samples (approx. 2 g) were collected from each of the six ports at different times (0, 24, 72, 96, 144 h). Samples were taken for fluorescence microscopy, microbial quantification, and EPS assessment.

Biofilm was visualized using DAPI (DNA) and SYPRO red (proteins/EPS) (Xu et al., 2022). Steps were as follows: 1) Formaldehyde fixation (3–4%, 20 min), 2) Triton X-100 permeabilization (0.1%, 10 min), 3) DAPI staining (10 $\mu\text{g mL}^{-1}$, 10 min, dark), 4) SYPRO red staining (1:500 dilution, 10 min), 5) Rinsing and drying under sterile airflow. Microscopy was performed using a Zeiss Axioscope 5 with 10 $\times$ and 40 $\times$ objectives for pattern detection, and 100 $\times$ oil immersion for cell-level visualization. Emission filters were DAPI (excitation 340–380 nm, emission at 461 nm) and SYPRO (excitation 580 nm, emission at 620 nm).

Microbial density was estimated by first counting fluorescent nuclei in randomly selected fields; second, extrapolating cell density to the total surface area of the sample; and third, calculating CFU per gram of medium (2, 3 Figure).



Figure 2. DAPI, SYPRO, and Triton solutions were used on the samples before microscopy analysis



Figure 3. A microscope is used to monitor, and samples are stained

3. Results and discussions

Fluorescence microscopy revealed that *Acidithiobacillus* spp. colonization proceeded slowly, with sparse initial adhesion and moderate EPS development, becoming evident after 72–96 h. Quantitative counts confirmed these trends: biochar supported up to 5.8×10^6 cells/sample for *Acidithiobacillus* spp. and 3.1×10^6 cells/sample for *Acidithiobacillus* spp. (Table 2).

Table 2. Number of *Acidithiobacillus* spp. on the biochar and CLC waste after 144 h

Material	Condition	Average count	Estimated total bacteria/sample
Biochar	<i>Acidithiobacillus</i> spp.	18	$\sim 7.2 \times 10^5$
Biochar	Nutrient + <i>Acidithiobacillus</i> spp.	77	$\sim 3.1 \times 10^6$
CLC Waste	<i>Acidithiobacillus</i> spp.	12	$\sim 4.8 \times 10^5$
CLC Waste	Nutrient + <i>Acidithiobacillus</i> spp.	55	$\sim 2.2 \times 10^6$

Biochar consistently outperformed CLC waste as a microbial carrier, supporting higher biomass and more cohesive biofilms due to its greater porosity and surface area. CLC waste exhibited lower densities and more spatial

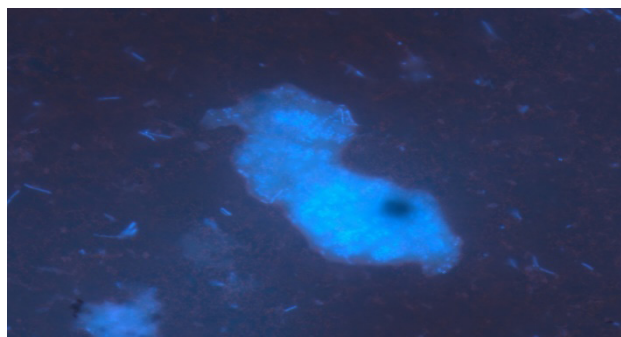


Figure 4. Image of biochar from layer 6, DAPI-stained with *Acidithiobacillus* spp., showing dense colonization (after 144 h)

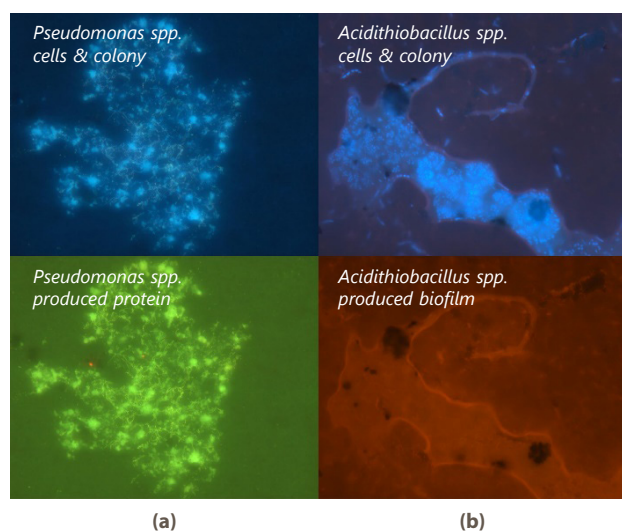


Figure 5. Comparison of biofilm intensity for biochar (a) and CLC waste (b) samples stained with *Acidithiobacillus* spp.

coverage, particularly in the upper filter zones, where moisture and nutrient availability decreased.

Acidithiobacillus spp. exhibited a uniform vertical distribution, with moderate densities ($3.9\text{--}6.8 \times 10^7$ cells g^{-1}) across all layers, reflecting its tolerance to oxygen-limited and nutrient-variable microenvironments. The produced biomass was lower than that of *Pseudomonas* spp. spp., but it remained present even in less favorable zones, indicating metabolic adaptability (4 Figure).

H_2S RE closely reflected the spatial patterns of colonization. Peak removal occurred in layers 3–4, achieving 78–81% layer-level RE, corresponding to zones of highest microbial activity. RE toward the column ends (45–47%), where both biomass and EPS intensity were lower. This correlation highlights the importance of biofilm maturity and microbial density for effective sulfur oxidation. Overall, the BF achieved steady-state system performance of 85–92% RE and $14\text{--}18 \text{ g H}_2\text{S m}^{-3} \text{ h}^{-1}$ EC, indicating stable long-term operation under variable inlet loads (5 Figure).

Afterwards, the desulfurization process resulted in the production of elemental sulfur (S^0), Thiosulfate ($\text{S}_2\text{O}_3^{2-}$), and Sulfate (SO_4^{2-}).

4. Conclusions

1. Quantitative microscopy and reactor performance data demonstrate that engineered waste-derived media significantly influence microbial colonization dynamics and biofilter efficiency. KOH-activated biochar supported consistently higher microbial densities than FeCO_3 -modified CLC waste, with cell counts reaching 3.1×10^6 cells/sample for *Acidithiobacillus* spp., compared to $\leq 3.6 \times 10^6$ cells/sample on CLC waste under equivalent conditions. These differences, characterized by a 60% higher biomass on biochar, are attributed to its greater porosity and surface roughness, which promote stronger attachment and EPS development, as confirmed by the higher SYPRO fluorescence intensity.
2. Species-specific growth kinetics strongly affected spatial biofilm distribution. *Acidithiobacillus* spp. showed slow growth but a more uniform vertical distribution, maintaining $3.9\text{--}6.8 \times 10^7$ cells g^{-1} across all layers. This broader distribution reflects the facultative anaerobic metabolism of *Acidithiobacillus* spp., enabling persistence under oxygen-limited and nutrient-depleted conditions, whereas *Pseudomonas* spp. activity declined.
3. Biofilter performance correlated strongly with microbial density and biofilm maturity. Layers exhibiting the highest biomass and EPS production achieved 78–81% layer-level H_2S removal, while layers with lower microbial counts showed reduced removal efficiencies (45–47%). At the system level, steady-state operation yielded an overall removal efficiency of 85–92% and elimination capacities of $14\text{--}18 \text{ g H}_2\text{S m}^{-3} \text{ h}^{-1}$, with minimal performance decay over 144 h, indicating statistically stable operation under variable inlet concentrations.

Author contributions

Conceptualization, K. M. and R. V.; methodology, K. M. and R. V.; validation, K. M. and R. V.; data curation, K. M. and R. V.; writing – original draft preparation, K. M. and R. V.; writing – review and editing, K. M. and R. V.; visualization, R. V.; supervision, R. V. All authors have read and agreed to the published version of the manuscript.

Disclosure statement

The authors have no competing financial, professional, or personal interests from other countries.

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BIOPLĖVELĖS FORMAVIMOSI KINETIKA IR SIEROS OKSIDACIJOS EFEKTYVUMAS KULTIVUOJANT *ACIDITHIOBACILLUS* SPP. BIOFILTRO ĮKROVOJE IŠ KOH MODIFIKUOTOS BIOANGLIES IR FECO₃ IMPREGNUOTŲ CLC ATLIEKŲ

Santrauka. Šiame straipsnyje analizuojama *Acidithiobacillus* spp. (DSM 12475, DSM 739) sieros oksidacijos kinetika ir bioplėvelės erdvinė struktūra, susiformuojanti 1,0 m aukščio biofiltro įkrovoje. Bioplėvelė buvo formuojama ant KOH aktyvuotos bioanglies ir FeCO₃ modifikuoto akytojo lengvojo betono (CLC) atliekų. Prieš mikroorganizmų įterpimą į biofiltrą grynos bakterijų kultūros buvo anaerobiškai papildomos nitratus redukuojančioje terpėje 96 valandas 30 °C temperatūroje, kol pasiekė maždaug 10⁷ CFU mL⁻¹ koncentraciją. Paskui filtras buvo eksploatuojamas 144 valandas palaikant 30 °C temperatūrą, 45–60 % drėgmę ir 0,2–1,0 L min⁻¹ dujų srautą. Biodujų buvimo trukmė biofiltre (EBRT) svyravo nuo 15 iki 60 s, o įeinanti pradinė H₂S koncentracija – nuo 100 iki 2000 ppm. Mikrobiologiniai tyrimai parodė, kad *Acidithiobacillus* spp. kolonizacijos kinetika buvo lėtesnė nei *Pseudomonas* spp., tačiau pasižymėjo dideliu stabilumu. Per 72–96 val. biomasės kiekis padidėjo nuo 7,2 × 10⁵ iki 3,1 × 10⁶ CFU mėginyje, o mikroorganizmai tolygiai pasiskirstė visuose šešiuose biofiltro sluoksniuose. Tarpląstelinių polimerinių medžiagų (EPS) struktūros stebėjimas patvirtino vientisos bioplėvelės formavimąsi ir jos brendimą. Šis procesas vyko lokaliaje rūgštėjančioje mikroaplinkoje, kuri susidarė dėl elementinės sieros kaupimosi. Daugialygė bioanglies porų struktūra sudarė tinkamas sąlygas gausesniai mikroorganizmų prisitvirtinimui ir jų biomasės kaupimuisi. Su FeCO₃ modifikuotos akytojo lengvojo betono (CLC) atliekos pagerino tiosulfato ir sieros tarpinių junginių katalizinę oksidaciją bei palaikė stabilų oksidacijos–redukcijos procesą. Veikiant pastovios būsenos režimu biofiltras užtikrino 85–92 % sieros vandenilio šalinimo efektyvumą (RE) ir 14–18 g H₂S m⁻³ h⁻¹ šalinimo pajėgumą, o per 144 val. trukmės valymo procesą nenustatyta reikšmingų sistemos veikimo sutrikimų. Fakultatyvinis anaerobinis *Acidithiobacillus* spp. metabolizmas leido palaikyti nuolatinę sieros oksidaciją net esant ribotam deguonies kiekiui ir šiek tiek rūgščiai mikroaplinkai. Tai sąlygos, kuriomis daugelis kitų sierą oksiduojančių bakterijų tampa nestabilios. Apibendrinant galima teigti, kad *Acidithiobacillus* spp. pagrindu sukurti inžineriniai biofiltro užpildai yra patikima, rūgštims atspari ir ilgalaikio veikimo biokatalizinė sistema, tinkama biodujoms valyti esant didelėms ir kintančioms sieros vandenilio koncentracijoms.

Reikšminiai žodžiai: vandenilio sulfido šalinimas, biofiltras, bioanglis, akytojo lengvojo betono (CLC) atliekos, mikrobiologinis sieros junginių skaidymas, biodujų valymas.