Potential of *Bacillus pumilus* and *Bacillus licheniformis* in Bio-Based Flotation Reagent Development

T. B. Z. Baloyi, W. Nheta, E. Fosso-Kankeu and M. Tlou

***Abstract*—**Metal-laden process water and declining ore grades reduce the selectivity of xanthate collectors, which also pose environmental risks. This study screened *Bacillus pumilus* and *Bacillus licheniformis* as candidate sources of bio-based flotation reagents. In Luria–Bertani broth, *B. pumilus* reached a stationary-phase OD600 of about 2.1 and *B. licheniformis* about 1.3–1.4. Sanger sequencing of the 16S rRNA gene identified one isolate as *B. licheniformis* (99.84 % identity); the second sample, *B. pumilus*, did not grow and remains to be sequenced. Crude solvent extracts of both strains contained alkaloids, flavonoids, saponins, steroids and tannins. Minimum inhibitory concentrations were about 31 mg/L for Ni, Zn and Ag in both strains and for Cu in *B. pumilus*, whereas *B. licheniformis* tolerated Cu up to 62 mg/L, indicating moderate metal tolerance. The strains merit compound characterisation and micro-flotation testing.

Keywords*—Bacillus licheniformis*, *Bacillus pumilus*, bio-based flotation reagents, metal tolerance

I. Introduction

Froth flotation is the principal method for concentrating base and precious metal sulphides, and its performance depends heavily on the reagent suite used. Collectors render target mineral surfaces hydrophobic, depressants suppress unwanted minerals, and frothers stabilise the bubbles that carry the hydrophobic particles to the concentrate. Xanthates have dominated sulphide flotation for decades because they are cheap and effective. They are, however, toxic: only about half of the xanthate added is consumed in the process, and the remainder reports to tailings and plant effluent, where it degrades to hazardous products such as carbon disulphide [1].

The challenge is intensified by declining ore grades, finely disseminated and complex sulphide mineralogy, and growing reuse of process water. Recycled water accumulates dissolved metal ions (for example Cu, Ni and Zn), which adsorb on or activate mineral surfaces, consume reagent and reduce collector selectivity, and microbial activity in the water can further change how collectors interact with mineral surfaces [1], [2]. Reagents that remain functional in metal-rich liquors, and are less hazardous than synthetic collectors, are therefore attractive.

Microorganisms offer one route to such reagents. Whole cells, biosurfactants, extracellular polymeric substances and nucleic acids have been tested as collectors, depressants and frothers, and surface-active microbial metabolites lower surface tension and promote foaming [3], [4]. Members of the genus *Bacillus* feature prominently: *B. subtilis* LN8B recovered up to 72.8 % of pyrite and 84.6 % of chalcopyrite in seawater [5]; *B. licheniformis* has been proposed as a bio-collector for barite–quartz separation [6]; *B. pumilus* has been reported to depress pyrite [7]; mineral-adapted *B. subtilis* separated sphalerite from galena [8]; surfactin from *B. subtilis* acted as a collector for magnesite [9]; and halophilic bacteria have been proposed as pyrite bio-depressants [10]. Reviews note, however, that most of this work is still at laboratory scale, that production costs are high, and that few studies report whether the strains are non-pathogenic [3], [11]. Strains that tolerate dissolved metals are particularly interesting, because the reagents they produce are more likely to remain effective in metal-laden flotation circuits.

This paper reports the first stage of a bio-reagent development programme. Two *Bacillus* strains, *B. pumilus* and *B. licheniformis*, were characterised with respect to (i) growth kinetics in a standard medium, (ii) molecular identity by 16S rRNA gene sequencing, (iii) the classes of secondary metabolites present in solvent extracts of the culture supernatant, and (iv) tolerance to selected base metals and silver by a broth microdilution assay. The aim was to establish whether the strains have the biological and chemical attributes needed to justify subsequent flotation testing.

II. Materials and Methods

A. Microbial Strains and Culture Preparation

LB broth was prepared by dissolving 10 g tryptone, 5 g yeast extract and 10 g NaCl in 1 L distilled water, followed by autoclaving at 121 °C for 1 h 15 min. After cooling, the broth was inoculated with a single colony of each strain (*B. pumilus* and *B. licheniformis*, previously isolated from passive clumps) and incubated at 37 °C and 120 rpm for 24 h. The active cultures were streaked onto nutrient agar plates (8 g nutrient agar powder in 250 mL distilled water, autoclaved and poured into sterile Petri dishes) for colony isolation. All work was done in

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a sterilised laminar flow environment. Plates were sealed with parafilm, incubated at 37 °C for 24–48 h and then stored at 4 °C until needed.

B. Growth Studies

Isolated colonies were transferred with a sterile loop into 50 mL conical flasks containing 20 mL LB broth under aseptic conditions, with two flasks per strain. Flasks were incubated at 37 °C and 120 rpm for 24 h. Every 2 h from 0 h, 1 mL of culture was transferred to a cuvette, and the optical density was measured at 600 nm (OD600) with a spectrophotometer. Duplicate flasks were used for each strain to limit experimental error.

C. 16S rRNA Gene Amplification and Sequencing

Molecular identification was outsourced to Inqaba Biotechnical Industries (Pretoria, South Africa), which received two bacterial cultures with no deviations from the method [12]. Genomic DNA was extracted with the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research). The 16S rRNA gene was amplified with primers 16S-27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 16S-1492R (5′-CGGTTACCTTGTTACGACTT-3′) using NEB OneTaq 2X MasterMix with Standard Buffer, 10–30 ng/µL genomic DNA, 10 µM of each primer and nuclease-free water. Thermal cycling comprised 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 68 °C for 1 min, a final extension at 68 °C for 10 min and a hold at 4 °C. Amplicons were checked on a 1 % agarose gel stained with EZ-vision Bluelight DNA dye, using the NEB Fast Ladder as size standard, purified enzymatically by the ExoSAP procedure and sequenced in the forward and reverse directions (BrilliantDye Terminator Cycle Sequencing Kit v3.1, ABI 3730xl Genetic Analyzer). The reads were assembled into a consensus sequence in CLC Main Workbench and compared with the NCBI database by BLASTn using default parameters, with a match accepted at 99 % query coverage and 99 % identity [12].

D. Secondary Metabolite Extraction and Phytochemical Screening

Overnight cultures were centrifuged at 4,000 rpm for 5 min (Hettich Zentrifugen Rotofix 32A) to separate biomass and supernatant. Each clarified supernatant was mixed with ethyl acetate and chloroform in a 1:1:1 ratio (aqueous phase : ethyl acetate : chloroform) and agitated on an orbital shaker until a homogeneous emulsion formed. After phase separation in a separatory funnel, the organic layer was collected and concentrated on a rotary evaporator (water bath 40 °C, 120 rpm) until no visible solvent remained. The crude extract was recovered with a sterile loop. Twenty milligrams of each extract were dissolved in 20 mL HPLC-grade methanol to give a 1 mg/mL stock, and 1 mL aliquots were tested for alkaloids (three drops of Dragendorff's reagent; orange-reddish precipitate or turbidity), flavonoids (three drops of 0.1 M NaOH; intense yellow colour), saponins (three drops of olive oil with vigorous shaking; persistent froth or emulsification), steroids (1 mL chloroform plus three drops of concentrated H_2SO_4, a Liebermann–Burchard type reaction; colour change at the interface) and tannins (three drops of $FeCl_3$; blue-black or greenish colour). Results were compared with positive and negative controls prepared in methanol.

E. Minimum Inhibitory Concentration (MIC) Assay

Metal tolerance was determined by broth microdilution with an INT viability readout. Tryptone Soya Broth (TSB, 30 g/L) was autoclaved at 121 °C for 1 h 15 min. Single colonies of each strain were grown in TSB at 37 °C with shaking for 18–24 h to mid-log phase, and the cultures were adjusted to an OD600 of 0.5 (CV-10 Plus Onda spectrophotometer). In sterile flat-bottom 96-well plates, each metal (Ni, Cu, Zn and Ag) was assigned a pair of duplicate columns, together with sterility controls (TSB only) and growth controls (TSB plus inoculum, no metal). TSB (200 µL) was added to each well, and 200 µL of a 1000 ppm aqueous metal stock was added to the first row of the relevant columns. Two-fold serial dilutions were then made down the plate, and 20 µL of the adjusted inoculum was added to every well except the sterility controls. Plates were sealed with parafilm, incubated statically at 37 °C for 18–24 h, and metabolic activity was assessed by adding INT. Preliminary controls ruled out metal–dye interactions. The MIC was defined as the lowest metal concentration that prevented the INT colour change relative to the growth control. Metal-containing waste was disposed of according to institutional hazardous waste regulations.

III. Results and Discussion

A. Growth Kinetics

Both strains grew in LB broth at 37 °C and 120 rpm over the 24 h monitoring period (Fig. 1). *B. pumilus* showed rapid exponential growth and reached a stationary-phase OD600 of about 2.1 within 16–24 h. *B. licheniformis* grew more slowly and stabilised at an OD600 of about 1.3–1.4. The roughly 1.5-fold higher plateau of *B. pumilus* indicates greater biomass productivity under the same conditions.

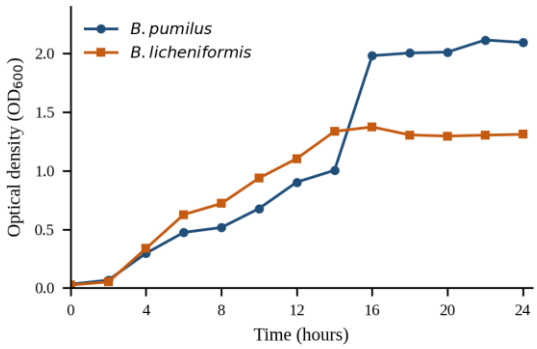


Fig. 1. Growth curves of *B. pumilus* and *B. licheniformis* (mean of duplicate flasks).

Biomass productivity is relevant to reagent production because metabolite yield per unit volume of culture generally scales with cell density, and because rapid attainment of stationary phase shortens batch times. Many secondary metabolites are formed as cultures approach or enter stationary phase, so the stationary phase reached within 24 h suggests that

metabolite harvest could be scheduled within a single day for both strains. The higher density of *B. pumilus* favours it for scale-up, while the lower density of *B. licheniformis* does not rule it out, since specific product yield may differ between the strains.

B. Molecular Identification

Two cultures were submitted for 16S rRNA gene analysis. For sample JCM 2505, the amplified 16S target region of about 1.5 kb was visualised on the agarose gel (Fig. 2), and BLASTn analysis of the consensus sequence identified the isolate as *B. licheniformis*, with 99.84 % identity over an alignment length of 1258 bp, an E-value of 0 and GenBank accession OM984666.1 (Table I). The second sample, JCM 20372, showed no growth at the sequencing laboratory and could not be analysed, so no sequence-based identification is yet available for *B. pumilus* [12]. The identity of *B. licheniformis* is therefore confirmed at the 16S level, whereas that of *B. pumilus* remains to be confirmed by repeating the analysis on a fresh culture. Since closely related *Bacillus* species can have highly similar 16S sequences, comparison with reference databases, and where necessary additional gene targets, is recommended before the species names are reported as final.

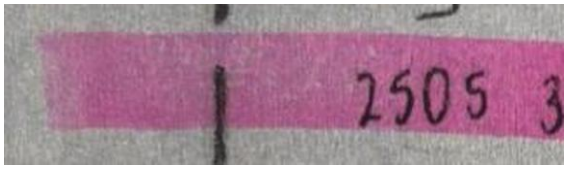


Fig. 2. Agarose gel image showing amplification of the 16S target region (~1.5 kb) for sample JCM 2505 (*B. licheniformis*) [12].

TABLE I
SEQUENCE-BASED IDENTIFICATION OF THE SUBMITTED SAMPLES

Sample	Predicted organism	GenBank accession	E-value	HSP (bp)	Identity (%)
JCM 2505	*B. licheniformis*	OM984666.1	0	1258	99.84
JCM 20372	No growth	–	–	–	–

C. Secondary Metabolites

The phytochemical screening results are summarised in Table II. Both extracts gave positive reactions for all five metabolite classes tested.

TABLE II
QUALITATIVE PHYTOCHEMICAL SCREENING OF CRUDE EXTRACTS (+ = POSITIVE)

Test	Observation	*B. pumilus*	*B. licheniformis*
Alkaloids (Dragendorff)	Turbidity / precipitate	+	+
Flavonoids (NaOH)	Intense yellow colour	+	+
Saponins (olive oil)	Persistent froth / emulsification	+	+
Steroids (Liebermann–Burchard)	Reddish-brown colour at interface	+	+
Tannins (FeCl3)	Blue-black or greenish colour	+	+

These classes have properties that are potentially relevant to flotation. Saponins and other amphiphilic molecules lower surface tension and stabilise froth, which points to frother or collector-type behaviour. Tannins and flavonoids contain phenolic hydroxyl groups that chelate metal ions and adsorb on mineral surfaces, which suggests depressant behaviour. Alkaloids and steroids may contribute to surface activity and to adsorption on mineral surfaces, although their specific functions in flotation are less well established. On this basis, the extracts may serve as bio-collectors, bio-depressants or frothing agents, depending on the compounds involved and on the mineral surface chemistry.

These tests are qualitative and show the presence of compound classes, not individual compounds or their concentrations. Colour reactions can also give false positives in a crude extract containing many components, for example when proteins, peptides or pigments interfere. Identification and quantification by chromatographic and spectroscopic methods (HPLC, LC-MS, FTIR, NMR), together with surface tension and contact angle measurements, are needed before the compounds can be linked to flotation function. Because the two strains gave identical qualitative profiles, quantitative or compound-level differences will be needed to discriminate between them.

D. Metal Tolerance

The 1000 ppm stock solutions corresponded to 17.0 mM Ni, 15.7 mM Cu, 15.3 mM Zn and 9.26 mM Ag. Because the stock was diluted 1:1 with TSB in the first row, the highest tested concentration was 500 mg/L of each metal, and eight two-fold dilutions spanned 500 to 3.9 mg/L (Table III). The MIC values read from the INT-stained plates (Fig. 3) are also given in Table III.

TABLE III
CONCENTRATION RANGE TESTED AND MIC OF NI, CU, ZN AND AG (MM, WITH MG/L IN BRACKETS)

Metal	Range tested (mM)	*B. pumilus* MIC	*B. licheniformis* MIC
Ni	8.500 – 0.066	0.531 (31.2)	0.531 (31.2)
Cu	7.850 – 0.061	0.491 (31.2)	0.981 (62.3)
Zn	7.650 – 0.060	0.478 (31.3)	0.478 (31.3)
Ag	4.630 – 0.036	0.289 (31.2)	0.289 (31.2)

Seven of the eight MIC values fall on the same dilution step, about 31 mg/L; the exception is Cu for *B. licheniformis*, which tolerated one further step (62.5 mg/L) and was therefore twice as tolerant to Cu as *B. pumilus*. Metabolic activity was retained at all lower concentrations, so both strains tolerate Ni, Cu, Zn and Ag at tens of mg/L and are inhibited above that, which is moderate and not high tolerance. Two-fold dilutions limit the resolution of the assay, so the equal MICs on a mass basis may hide differences of up to a factor of two, and replicate plates are needed to confirm the Cu difference.

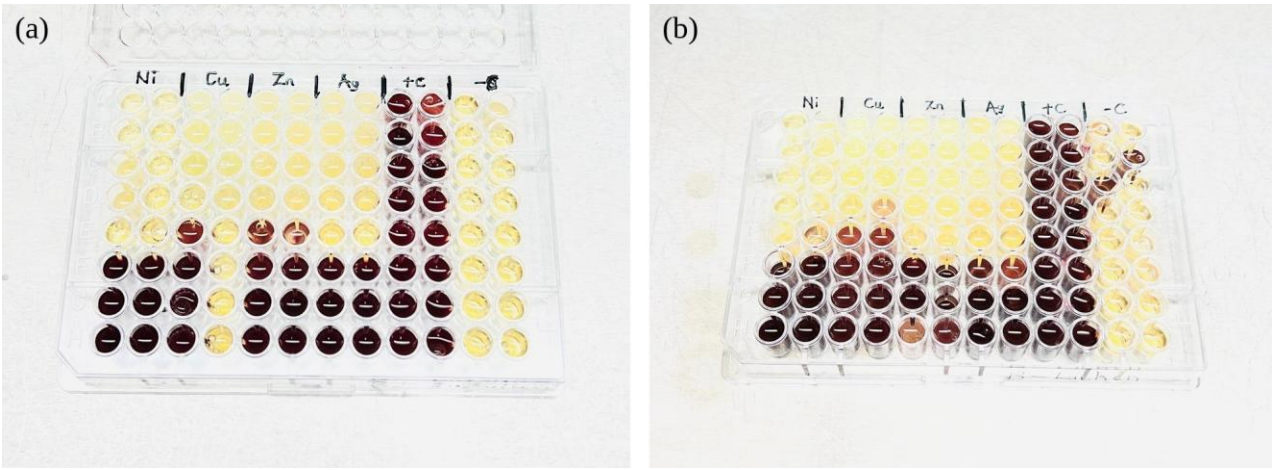


Fig. 3. INT-stained 96-well plates showing the MIC readout for (a) *B. pumilus* and (b) *B. licheniformis*.

This tolerance is relevant to flotation because process water in sulphide circuits typically contains dissolved Cu, Ni and Zn from mineral dissolution and reagent additions. It suggests that the producing organisms could be cultured in dilute metal-bearing solutions, but not in strongly metal-laden liquors. Two caveats apply. First, MIC values reflect tolerance of living cells in a nutrient-rich medium, whereas flotation reagents are cell-free metabolites, so the performance of the extracts in the presence of dissolved metals must be tested directly. Second, components of TSB (peptides and amino acids) complex metals and can raise apparent MIC values relative to defined media, which should be considered when comparing the results with other reports.

IV. Implications and Limitations

Taken together, the results show that both strains are fast-growing, metabolically diverse and moderately metal tolerant, and that the identity of *B. licheniformis* is supported by 16S rRNA gene sequencing, which are the necessary preconditions for a bio-reagent source. *B. pumilus* has the advantage of higher biomass productivity, while *B. licheniformis* showed higher Cu tolerance and belongs to a species already proposed as a flotation bio-collector [6]. The study has several limitations that define the next steps: (i) the phytochemical tests are qualitative; (ii) the identity of *B. pumilus* has not been confirmed by sequencing because its sample did not grow at the sequencing laboratory; (iii) the MIC values come from one two-fold dilution series per strain, without replicate-based statistics; and (iv) no flotation response has yet been measured. The following work is therefore proposed: repeating the 16S analysis for *B. pumilus*; fractionation and chemical characterisation of the extracts; surface tension and contact angle measurements; confirmation that the strains are non-pathogenic, since few bioflotation studies report this [3]; and micro-flotation tests on pure sulphide minerals (for example chalcopyrite, pentlandite and pyrite) in the presence and absence of dissolved metal ions, using the extracts alone and in combination with conventional reagents.

V. Conclusions

1) *B. pumilus* grew faster and to a higher cell density (OD600 about 2.1) than *B. licheniformis* (about 1.3–1.4), and both reached stationary phase within 24 h.
2) Sanger sequencing of the 16S rRNA gene identified one isolate as *B. licheniformis* (99.84 % identity, GenBank accession OM984666.1); the *B. pumilus* sample did not grow and is yet to be sequenced.
3) Crude solvent extracts of both strains contained alkaloids, flavonoids, saponins, steroids and tannins, which are classes associated with surface activity, metal chelation and adsorption modification.
4) The MIC was about 31 mg/L for Ni, Zn and Ag in both strains and for Cu in *B. pumilus*; *B. licheniformis* tolerated Cu up to 62 mg/L, showing moderate metal tolerance in both strains.
5) The strains merit further evaluation as sources of sustainable bio-collectors, bio-depressants and frothers, starting with compound characterisation and micro-flotation testing.

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References

[1] D. J. Duarte, R. P. J. Hoondert, E. D. Amato, M. M. L. Dingemans, and S. A. E. Kools, “Making waves: Xanthates on the radar – environmental risks and water quality impact,” *Water Research X*, vol. 24, Art. no. 100232, 2024. https://doi.org/10.1016/j.wroa.2024.100232

[2] N. Mhonde, M. Smart, K. Corin, and N. Schreithofer, “Investigating the electrochemical interaction of a thiol collector with chalcopyrite and galena in the presence of a mixed microbial community,” *Minerals*, vol. 10, Art. no. 553, 2020. https://doi.org/10.3390/min10060553

[3] A. Pawlowska and Z. Sadowski, “The role of biomodification in mineral processing,” *Minerals*, vol. 13, no. 10, Art. no. 1246, 2023. https://doi.org/10.3390/min13101246

[4] S. K. Behera and A. F. Mulaba-Bafubiandi, “Microbes assisted mineral flotation a future prospective for mineral processing industries: a review,” *Mineral Processing and Extractive Metallurgy Review*, vol. 38, pp. 96–105, 2017. https://doi.org/10.1080/08827508.2016.1262861

[5] D. Arias *et al.*, “Exploring the potential of the halotolerant bacterial strain *Bacillus subtilis* LN8B as an eco-friendly sulfide collector for seawater flotation,” *Journal of Applied Microbiology*, vol. 135, no. 1, 2024. https://doi.org/10.1093/jambio/lxad313

[6] R. A. Ashkavandi, E. Azimi, and M. R. Hosseini, “*Bacillus licheniformis* a potential bio-collector for barite–quartz selective separation,” *Minerals Engineering*, vol. 175, Art. no. 107285, 2022. https://doi.org/10.1016/j.mineng.2021.107285

[7] E. Sanwani, S. Chaerun, R. Mirahati, and T. Wahyuningsih, “Bioflotation: bacteria–mineral interaction for eco-friendly and sustainable mineral processing,” *Procedia Chemistry*, vol. 19, pp. 666–672, 2016. https://doi.org/10.1016/j.proche.2016.03.068

[8] B. Vasanthakumar, H. Ravishankar, and S. Subramanian, “Selective bio-flotation of sphalerite from galena using mineral-adapted strains of *Bacillus subtilis*,” *Minerals Engineering*, vol. 110, pp. 179–184, 2017. https://doi.org/10.1016/j.mineng.2017.05.002

[9] S. Koca, D. Aksoy, S. Ozdemir, P. A. Çelik, A. Çabuk, and H. Koca, “Surfactin as an alternative microbial collector to oleate in magnesite–quartz selective flotation,” *Separation Science and Technology*, vol. 58, pp. 394–405, 2023. https://doi.org/10.1080/01496395.2022.2118612

[10] G. L. Consuegra, S. Kutschke, M. Rudolph, and K. Pollmann, “Halophilic bacteria as potential pyrite bio-depressants in Cu–Mo bioflotation,” *Minerals Engineering*, vol. 145, Art. no. 106062, 2020. https://doi.org/10.1016/j.mineng.2019.106062

[11] P. Kinnunen, H. Miettinen, and M. Bomberg, “Review of potential microbial effects on flotation,” *Minerals*, vol. 10, Art. no. 533, 2020 https://doi.org/10.3390/min10060533.

[12] Inqaba Biotechnical Industries (Pty) Ltd, “Bacterial identification (16S),” Test Report, Form P-01, Rev. 02, Pretoria, South Africa, Quote no. SA2025/202950, Oct. 2025.



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