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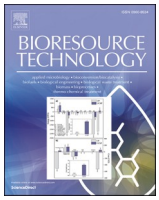

Molding the future: Optimization of bioleaching of rare earth elements from electronic waste by *Penicillium expansum* and insights into its mechanism

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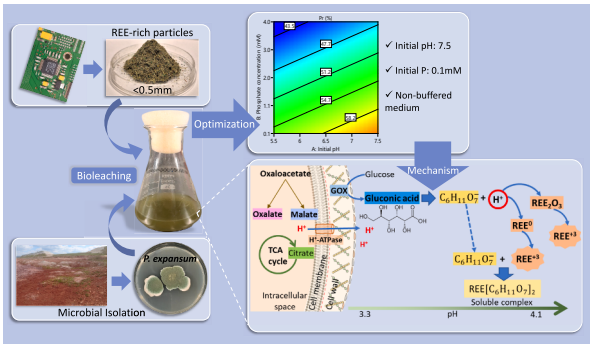
HIGHLIGHTS

- *P. expansum* isolated from contaminated land exhibited bioleaching capabilities.
- Initial pH and phosphate concentration were fundamental to optimize REE bioleaching.
- A gluconic acid-rich biolixiviant was produced by *P. expansum*.
- Bioleaching of ~ 70 % Pr, Nd, Gd, 50 % Tb, and 40 % La was achieved in only 24 h.
- Direct use of crude cell-free biolixiviant could simplify large scale applications.

GRAPHICAL ABSTRACT



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ABSTRACT

The recovery of rare earth elements (REE) from electronic waste is crucial for ensuring future demand security, as there is a high supply risk for this group of elements, and mitigating the environmental impacts of conventional mining. This research focuses on extracting REE from waste printed circuit boards through bioleaching, addressing the limited attention given to this source. A strain of *Penicillium expansum* demonstrated efficient bioleaching under optimal conditions of 7.5 initial pH, 0.1 mM phosphate concentration, and excluding a buffering agent. The study achieved significant improvements in La and Tb extraction and enhancements in Pr, Nd, and Gd recovery, approaching 70 % within 24 h. Fungal mechanisms involved in REE extraction included fungal pH control, organic acid biosynthesis, phosphate bioavailability, and potential fungal proton pump involvement. This approach offers a promising solution for sustainable REE recovery from e-waste, contributing to resource security and circular economy.

1. Introduction

The rapid development of new technologies, particularly in information and telecommunication technology, has improved the quality of life. However, this progress also escalated the proliferation of electronic devices that rapidly became obsolete. Electronic waste (e-

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waste) is the fastest-growing waste stream in the world, projected to approach almost 75 million tonnes by 2030; with formal recycling rates remaining extremely low (17 %) (Forti et al., 2020). Waste Printed Circuit Boards (WPCBs) represent a significant component of e-waste, constituting approximately 4 to 7 % of the total waste mass. While hazardous materials within WPCBs pose threats to the environment and human health, the presence of valuable and critical metals such as Cu, Au, Pd, and the less explored rare earth elements (REE), makes this type of e-waste economically attractive for recycling. Despite efforts in resource recovery from e-waste, the recycling of critical materials, particularly REE, remains overlooked. The need to recover REE from waste streams is underscored by their potentially irreplaceable role in current and future technologies, especially for the transition to low-carbon economies, which is crucial for realizing the UN Sustainable Development Goals and aligning with governmental net-zero strategies. The global high risk of supply disruptions for REE, and the environmental concerns associated with the extraction of REE from natural resources (Dushyantha et al., 2020), highlight the need for exploring alternative sources.

Pyrometallurgy is the most common industrial practice for the extraction of metals from WPCBs, and research on hydrometallurgical methods is of growing interest (Hsu et al., 2019). However, these technologies still represent a risk to the environment due to their high chemical and energy demands. In contrast, bioleaching offers an eco-friendly alternative by using microorganisms to extract metals, making use of processes like those occurring naturally in biogeochemical cycles (Brandl, 2001). While chemoautotrophic bacteria, such as *Acidithiobacillus* species, have been extensively studied for bioleaching of metals from various e-waste streams (Baniasadi et al., 2019), research on REE bioleaching has received less attention until now. Recent research has focused on the bacterium *Gluconobacter oxydans* for extraction of REE (Rasoulnia et al., 2022). Nevertheless, REE recovery rates from e-waste in existing research literature remain relatively low, ranging from 10 % to 50 %, often necessitating a minimum of two weeks for effective REE extraction (Priya and Hait, 2020) and extensive pH adjustments to highly acidic conditions (Auerbach et al., 2019).

The present study aimed to address such challenges by adopting a holistic approach, where a newly isolated *Penicillium expansum* strain with superior bioleaching capabilities was used to extract REE from the complex and untapped resource of WPCBs. Through a comprehensive optimization strategy, with the novel inclusion of metabolite production as a response variable, significant improvements to the bioleaching rates were achieved. This allowed for the identification of correlations between metabolite secretion and bioleaching efficiency. Furthermore, the research examined the kinetics of the bioleaching process and its comparison to abiotic settings to identify distinctions between these approaches, providing new insights into the mechanisms that drive the leaching of REE. The study demonstrated the efficiency of fungal heterotrophic bioleaching as a promising venue for the sustainable recovery of REE from WPCBs, within a remarkably short period of just 24 h, under near-neutral initial pH conditions.

2. Materials and methods

2.1. Sourcing and preparation of waste printed circuit boards

The WPCBs material used in this study was previously characterized and prepared as described in Gonzalez et al. (2022). Mechanically pre-treated (comminuted) WPCBs were provided by three e-waste recycling companies from the UK, originating from Information and Communications Technology equipment (ICT). Dry sieving was performed to fully characterize the material, and each size fraction was analysed for metal content.

2.2. Microbial isolation and identification of novel bioleaching microorganisms

Three soil samples were collected in April 2019 from an area affected by the large spillage of red mud waste from the Ajka alumina plant in 2010 (Kolontár, Hungary, 47°05′16.9″N 17°29′34.2″E). The isolation of microorganisms from the soil samples was carried out following the serial dilution and spread plate method (Donegan et al., 1991) on Potato Dextrose agar (PDA). From an initial group of 79 isolates, 8 fungal strains were screened and selected based on *i)* their ability to solubilize inorganic phosphate, as an indicator of organic acid production (Natarajan, 2015), assessed via the Pikovskaya (PVK) agar assay (Nautiyal, 1999); and *ii)* their capability to produce siderophores, evaluated using the Chrome azurol S agar assay (CAS) (Schwyn and Neilands, 1987). All media was obtained from Oxoid, Thermo Fisher Scientific (see supplementary material). Based on preliminary bioleaching investigations following the bioleaching method described in section 2.3., the fungal strain with the highest REE recovery rates was selected from the 8 isolates for the current study.

2.2.1. Genomic characterization of the fungal isolate

Genomic DNA was extracted from the fungal mycelia using the DNeasy PowerSoil Pro Kit (Qiagen). The extracted DNA was purified using magnetic-bead separation with AMPure XP reagent (Beckman Coulter) and quality and quantity assessed using a TapeStation (Agilent). The Rapid Sequencing kit (SQK-RAD004; Oxford Nanopore) and DNA Prep (Illumina) kits were used to prepare libraries for Next-Generation sequencing. These libraries were sequenced on a MinION and iSeq100, respectively. The Nanopore sequence reads were assembled using Flye v2.8.3 (Kolmogorov et al., 2019). The contigs were subsequently polished using the Illumina data and the software Pilon v1.24 (Walker et al., 2014). The completeness of the polished genome assembly was assessed with BUSCO v5.2.2 (Manni et al., 2021) using the Fungi_odb10 dataset and compared to that of all other publicly available *P. expansum* genome sequences and five outgroups. BUSCO_phylogenomics was used to generate a supermatrix from the BUSCO output. This supermatrix was then used to infer phylogenetic relationships using IQ-TREE (Nguyen et al., 2015) and the JTT + F + R4 model that was selected using ModelFinder (Kalyaanamoorthy et al., 2017). Branch support was assessed using 1000 ultrafast bootstrap approximations and 1000 SH-aLRTs.

2.3. Bioleaching experiments

The modified Czapek Dox (CZ) medium was used as standard leaching medium, comprising 2 g/L $NaNO_3$, 0.5 g/L KCl, 0.5 g/L (=2.4 mM) $C_3H_7MgO_6P$, 0.01 g/L $FeSO_4$, 0.35 g/L K_2SO_4, and 30 g/L sucrose. Spore suspensions of 10^7 spores/mL were prepared from a 7-day culture of the *P. expansum* strain in CZ agar slants and inoculated into the leaching medium. Conical flasks containing 50 mL of the leaching medium were inoculated with 500 µl of the microbial suspensions (1 % v/v) (Vakilchap et al., 2016). Uninoculated medium was used as a control. Experiments were done in triplicate. A two-step bioleaching approach was developed due to its advantage in reducing the direct toxic effects of e-waste on the microbial culture, as evidenced in preliminary observations. The first step consisted of an initial incubation of 3 days, at 25 °C in an incubated shaker at 150 rpm (JeioTech). Following first incubation, in the second step, 0.5 g of the comminuted WPCB material (<0.5 mm particle size) was added to each flask (1 % w/v pulp density) and incubated for 4 days. The resulting leaching solutions were centrifuged at 4,750 x g for 10 min, and microbial biomass was separated by gravity filtration using Whatman filter paper (>11 µm retention). The filtrates were filter-sterilized using a 0.22 µm syringe filter (Merck Millipore) and kept at −20 °C for elemental analysis and examination of metabolites.

2.4. Optimization of the bioleaching process by response surface methodology

Response surface methodology (RSM) was applied for the optimization of the bioleaching process, to maximize the leaching of REE by *P. expansum*. The base elements Cu, Fe and Ni were included for overall analysis. Based on preliminary screening and existing literature (Santhiya and Ting, 2005; Arshadi et al., 2020), initial pH (5.5 to 7.5) phosphate concentration (0.1 to 4.0 mM), and the addition/exclusion of a buffering agent in the leaching medium were the three parameters selected for systematic evaluation through RSM. In this research, an I-Optimal design method was selected due to its proven capability to minimize prediction variance within the experimental region (Jones and Goos, 2012). A set of 22 experiments were subsequently created using Design-Expert v13.0 software (Table 1). The buffered medium was prepared using 0.1 M of the biological buffers MES (2-(N-morpholino) ethanesulfonic acidethanesulfonic acid) for the pH range 5.5 to 6.5, and MOPS (3-(N-morpholino) propanesulfonic acid) for the pH range 6.5 to 7.5. Such biological buffers were chosen due to the absence of phosphate in their structures. The initial pH of the medium was adjusted with either 0.1 M H_2SO_4 or 0.1 M NaOH. Buffered and non-buffered uninoculated sterile medium were used as controls, and results from such experiments were subtracted from the inoculated experiments (overall metal recovery from controls <1 %). The two-step bioleaching process described in section 2.3 was applied.

2.5. Comparison of bioleaching and abiotic leaching

Bioleaching and abiotic leaching experiments were conducted under optimized conditions determined through RSM, in 100 mL of medium, with fungal spore suspensions (1 % v/v) and pulp density (1 % w/v) as described in section 2.3. Additionally, *P. expansum* was cultivated without the addition of WPCBs as a control to determine any alterations in metabolic activities when the fungus interacts with e-waste.

2.5.1. Kinetics aspect of bioleaching and abiotic leaching process

Abiotic studies were carried out in sterile leaching medium using commercial organic acids (Fisher Scientific and Merck) at similar concentrations that were found during the fungal bioleaching (when WPCBs were added, on day 3). The mixture of organic acids was prepared in the same sterile medium used for fungal bioleaching and included gluconic (13 mM), oxalic (0.02 mM), acetic (1 mM) and fumaric (0.006 mM) acids, as measured by HPLC. Subsamples of 1.5 mL were taken daily prior to the addition of WPCBs, 3 and 6 h after WPCBs addition, followed by daily sampling for subsequent analysis of organic acids and metal content. The pH was also measured at the same intervals.

2.5.2. Bioleaching, spent medium and abiotic leaching

In additional experiments, fungal biomass was removed from the bioleaching medium (at day 3) to test the spent medium containing the microbial metabolites. Abiotic experiments included a mixture of organic acids at similar concentrations found in the bioleaching solutions. Because gluconic acid was the principal acid produced by the fungal strain, this acid was further tested in abiotic leaching on its own, at similar and higher concentrations as found in the bioleaching settings (16 mM and 32 mM, as measured in HPLC). Such experiments were limited to a 24-hour duration, as this timeframe for exposing the fungal system to WPCBs was found effective in extracting the majority of available REE (section 3.4.2). The pH was measured at time 0, as well as 1, 3, 6, and 24 h, following WPCBs addition.

2.6. Analytical techniques

2.6.1. Elemental analysis

Elemental analysis of the WPCBs material was performed as described by Gonzalez et al. (2022). Briefly, comminuted WPCBs underwent microwave-assisted aqua regia digestion, followed by metal analysis through Inductively Coupled Plasma Mass Spectrometry (ICP-MS ™iCAP ™RQ, Thermo Scientific) and Optical Emission Spectroscopy (ICP-OES, iCAP 6000 series, Thermo Scientific). The metal content of bioleaching solutions was determined following acidification to 1 % HNO_3 of the liquid samples. The precipitate observed in abiotic leaching experiments underwent elemental and molecular analysis through Scanning Electron Microscopy / Energy-Dispersive Spectroscopy (SEM-EDS) and Fourier-Transform Infrared Spectroscopy (FTIR). The precipitate was mounted on aluminium stubs with carbon adhesive tabs (Ted Pella) and analyzed with EDS using a silicon drift detector (SDD) for 60 s. Additionally, FTIR was conducted on the precipitates using an Agilent Technologies Cary 630 FTIR with Attenuated Total Reflection (ATR). Infrared spectra (60 scans) were acquired over the range of 400 to 4000 cm − 1 with 2 cm-1 resolution, and data analysis utilized Ominc v7.0 software from Thermo Scientific.

2.6.2. Organic acids analysis

The analysis of low molecular weight organic acids was done using a Shimadzu Prominence HPLC system, connected to a photodiode array detector. Gluconic, oxalic, and succinic acids were separated with a Luna® 5u C18 column (Phenomenex, 150 x 4.6 mm, 5 μm). Malic, citric, acetic, and fumaric acids were analyzed with a Synergi 4u Hydro-RP column (250 x 4.6 mm, 4 μm). Chromatographic analysis utilized a 20 μL sample injection, 0.5 mL/min flow rate, 30 °C, and a 10-minute acquisition time with isocratic elution of the mobile phase (94 % KH_2PO_4, 12.5 mM, and 6 % acetonitrile, pH 2.45). Detection was done at 220 nm and analyzed with LabSolutions software. Calibration curves were prepared from organic acid standard solutions (Fisher Scientific and Merck).

Table 1
Design of experiments based on I-Optimality for maximizing the bioleaching rates of rare earth elements (REE) from waste printed circuit boards (WPCBs).

	Factor A	Factor B	Factor C		Factor A	Factor B	Factor C
Run	Initial pH	Phosphate concentration (mM)	Buffer*	Run	Initial pH	Phosphate concentration (mM)	Buffer*
1	5.5	2.5	NO	12	5.5	0.1	NO
2	7.5	0.1	NO	13	6.5	2.0	YES
3	5.5	2.0	YES	14	6.8	2.6	NO
4	6.1	1.3	NO	15	5.5	4.0	YES
5	5.5	4.0	YES	16	5.5	2.5	NO
6	6.5	2.0	YES	17	7.5	4.0	NO
7	7.5	4.0	YES	18	7.5	4.0	YES
8	6.5	2.0	YES	19	5.5	0.1	YES
9	6.3	4.0	NO	20	6.3	4.0	NO
10	7.5	1.6	NO	21	6.5	0.1	YES
11	6.7	0.1	NO	22	7.5	0.1	YES

*Buffered medium was prepared with 0.1 M MES (pH 5.5 – 6.5) and 0.1 M MOPS (pH 6.5 – 7.5) biological buffers.

2.7. Statistical analysis

The statistical software Design-Expert v13 was used for the design of experiments and RSM in the optimization study. Significance and adequacy of the resulting models were assessed through ANOVA. Data from the different bioleaching experiments was analysed using ANOVA followed by multiple comparison Dunnett's test (GraphPad Prism v10). All statistical analyses across the software platforms were conducted at a 95 % confidence level ($p < 0.05$).

3. Results and discussion

3.1. Elemental composition of waste printed circuit boards

Particle size fractions below 0.5 mm were selected for investigation in this study due to their elevated concentrations of REE (Gonzalez et al., 2022). The elemental composition of the WPCBs material is detailed in Table 2.

3.2. Genomic characterization of the fungal isolate

The isolated *P. expansum* strain was characterized by whole genome sequencing. This resulted in the construction of a genome assembly of 32.9 Mb, consisting of 34 contigs with an N_{50} contig length of 2.8 Mb. Sequence Read Archive (SRA) data and whole-genome sequence are respectively available under accession numbers PRJNA1046705 and JAXGGN000000000 (NCBI). The BUSCO analysis for genome completeness estimated a level of completeness at 98.4 %, which is comparable to other *P. expansum* genome sequences. The genomes of ten established *P. expansum* strains, previously published in the GenBank database (NCBI, 2023), in addition to five well-known *Penicillium* species (serving as outgroups), were employed for comparative analysis against the fungal isolate. Of the 758 BUSCOs searched, 628 were single copy in all species. The protein sequences were concatenated, resulting in a final alignment of 431,219 amino acids that was used for phylogenetic analysis. The analysis positioned the isolated *P. expansum* at the base of a distinct evolutionary branch within *P. expansum* (see supplementary material). In addition, the fungal isolate clustered with a *P. expansum* strain previously isolated from a windfall apple (Clemmensen et al., 2022). *P. expansum* is commonly found in soil worldwide, but widely known for its role in fruit decay, particularly in apples, which is a cause of concern for food safety due to the presence of the mycotoxin patulin (Mccallum et al., 2002). While *P. expansum* has been associated with economic impacts on the food industry, this study highlights its potential contribution to the recycling and metal recovery sectors. Although beyond the focus of this study, the genome sequence of this microorganism can serve to explore its genetics and metabolic pathways, which holds the potential for advancing the understanding of microbial bioleaching properties.

3.3. Optimization of bioleaching of rare earth elements by p. Expansum

Experimental results from the I-optimal design were analysed and a model was generated for each desired response. The empirical models generated by the RSM approach for the bioleaching of REE were either linear (Pr, Dy) or two-factor interaction (La, Nd, Gd, Tb, Er) models, suggesting that no curvature effects were detected in the relationship between the factors and the response variables (see supplementary material). Although quadratic models are more frequently generated in RSM studies, linear models can also serve to accurately determine the response surface (Naseri et al., 2023).

3.3.1. Effects of initial pH, phosphate concentration and buffering of the medium in the bioleaching of rare earth elements

Amongst the three evaluated variables, phosphate concentration had the most pronounced negative effect on the bioleaching of REE. The lowest level of phosphate (0.1 mM) was linked to the highest recovery rates of Pr, Nd and Gd, as shown in the contour plots in Fig. 1, and for other REE (see supplementary material). Increasing the initial pH had a positive effect on Pr recovery, but it was not significant for all other REE. Buffering the leaching medium had a positive effect on Pr and Dy bioleaching but was inconsequential for most REE. In contrast, Cu and Ni recovery was hindered by the presence of the buffered medium.

The goal for the optimization was to maximize the recovery of the selected REE (La, Pr, Nd, Gd, Tb, Dy, Er) simultaneously and maintain factor levels within range. This target was introduced to the software Design-Expert v13.0 using the optimization tool, and optimal conditions were determined as: 7.5 initial pH, 0.1 mM phosphate concentration and absence of buffer in the leaching medium. Under such conditions, the fungal isolate *P. expansum* achieved maximum recovery of REE as presented in the validation experiments, Table 3. The models were found suitable to predict the bioleaching of the selected metals. Nevertheless, the bioleaching of Gd and Cu was not accurately predicted by the empirical models. These discrepancies could be due to the complex and heterogeneous nature of the WPCBs matrix, which can lead to higher variations (than predicted) in the results.

3.3.2. Effects of initial pH, phosphate concentration and buffering of the medium on microbial organic acid production

The production of organic acids by *P. expansum* was analysed during the RSM (see supplementary material). However, the metabolites were not selected as targets for maximization. Gluconic acid was identified as the predominant organic acid produced by the fungal strain. Concentrations of gluconic acid by the end of the bioleaching experiments (day 7) were found above 70 mM, followed by acetic (1 – 8 mM), citric (1 – 3 mM), succinic (0 – 5 mM), malic (0 – 4 mM), and fumaric (<0.02 mM) acids. Gluconic acid production in *P. expansum* is facilitated by glucose oxidation to D-glucono-δ lactone via glucose oxidase (GOX) and subsequent generation of gluconic acid, either spontaneously or mediated by gluconolactonase (GNL). Unlike other acids, gluconic acid is produced in

Table 2
Elemental composition of the waste printed circuit boards (WPCBs) material.

Rare earth elements (g/ton)								
La	Pr	Nd	Gd	Tb	Dy	Er		
23.4 ± 1.9	692.2 ± 107.5	4048.1 ± 625.1	5.1 ± 0.8	21.4 ± 2.7	169.0 ± 16.3	6.5 ± 1.8		
Base and trace metals (g/ton)								
Al	Cu	Fe	Ni	Pb	Sn	Zn	Cr	As
21757.1 ± 1960.9	139492.6 ± 17786.1	67500.2 ± 6644.1	8014.8 ± 733.1	7862.5 ± 1154.6	21238.7 ± 3446.9	7339.3 ± 786.9	642.4 ± 107.4	8.1 ± 1.2
Precious metals (g/ton)								
Au	Ag	Pd	Pt					
319.2 ± 31.5	1531.3 ± 499.6	38.5 ± 8.1	0.6 ± 0.2					

Mean values ± SD, n = 5.

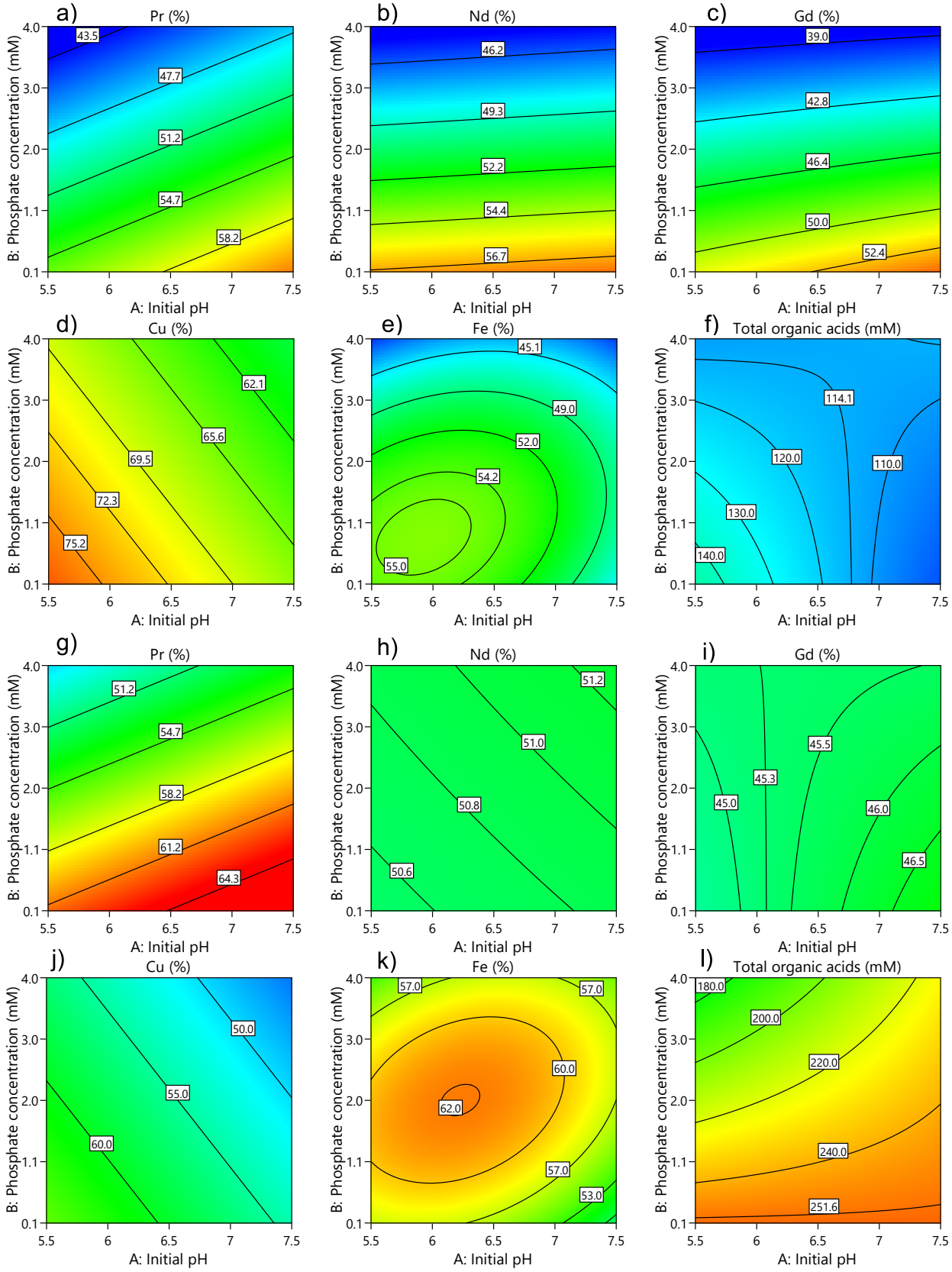

Fig. 1. Contour plots of rare earth elements and base metals recovery (%) from waste printed circuit boards by fungal bioleaching, and total production of organic acids (mM). Phosphate concentration in Y axis and initial pH in X axis. Non-buffered medium (a-f) and buffered medium (g-l).

Table 3
Prediction and validation of the responses (% recovery) by the models at optimal conditions.

Response (% recovery)	Prediction (% recovery)	Confirmation (% recovery)	95 % PI Low	95 % PI High
La	41.3	40.4	34.8	47.6
Pr	61.3	63.4	55.3	67.1
Nd	57.6	61.5	53.0	62.1
Gd	54.0	69.0	49.1	58.9
Tb	46.4	50.4	42.0	50.6
Dy	25.5	27.4	23.6	27.4
Er	20.6	22.2	18.6	22.5
Cu	66.9	76.8	57.3	76.5
Fe	45.6	52.6	38.4	52.7
Ni	9.6	11.9	2.9	16.4

PI: Prediction Interval at 95 % confidence level ($p < 0.05$), n = 3. Grey areas indicate outside intervals.

the fungal extracellular space, with GOX and GNL enzymes in the cell wall (Kubicek et al., 2011). Buffering conditions significantly influenced metabolite secretion, as shown in contour plots for total organic acid production (Fig. 1f,l), with maximum levels attributed to buffering. However, this did not result in higher bioleaching rates.

Phosphate concentration also affected organic acid production, with lower concentrations leading to higher acid amounts. Organic acid secretion in fungi is induced under nutrient-deficiency conditions. In fact, during industrial fermentation of organic acids, the concentration of phosphate is maintained low, typically between 1 to 5 mM (~0.2 to 1 g/L magnesium glycerophosphate). This would restrict biomass growth and enhance accumulation of the acids (Kubicek et al., 2011). This study evaluated 0.1 mM phosphate as minimum concentration (~0.02 g/L magnesium glycerophosphate), and further reduction was not explored because *i)* the chosen concentration was already significantly lower than usual recommendation for fungal organic acid production; and *ii)* further reductions would likely approach levels close to contamination by trace phosphate, making this unpractical with additional issues of measurement accuracy. Increasing initial pH above 5.5 did not significantly impact total organic acid production, highlighting the advantage of fungal bioleaching, which operates effectively at pH levels close to neutrality without requiring intensive adjustments.

3.4. Kinetics aspect of bioleaching and abiotic leaching process

3.4.1. Organic acid content and pH variations

The presence of WPCBs significantly enhanced the secretion of gluconic, oxalic, and malic acids by *P. expansum* compared to the fungal growth control without e-waste (Fig. 2b,c,d), while the absence of WPCBs led to higher production of citric, acetic, and fumaric acids (Fig. 2e,f,g). Although succinic acid was detected in previous experiments, under optimized conditions this metabolite was not present. This could be expected, as succinic acid production was positively influenced by higher concentrations of phosphate, and the applied optimal levels require the lowest amount of phosphate. The production of gluconic acid was significantly higher than the other acids, for both biotic experiments, which is related to the intrinsic biological characteristics of this fungus (Hadas et al., 2007). Some authors have evidenced the stimulation of oxalic and malic acid production by *Aspergillus* and *Penicillium spp.*, in the presence of Mn^{+}, Zn^{+} and Cu^{+} (Sazanova et al., 2015). The toxic effects of metal ions could therefore be reduced by chelation or complexation with the produced organic acids (Qu and Lian, 2013). The increment in secretion of organic acids evidenced in this study could be the result of favourable conditions created by the introduction of the WPCBs, rather than a pure defence mechanism. As seen in Fig. 3h, phosphate was almost depleted in both biotic experiments by day-3. However, in the fungal bioleaching experiment, P was reintroduced into the system through the addition of WPCBs. Instead, the fungal culture that continued to grow in absence of WPCBs, was under stress conditions as the phosphate source was completely consumed, potentially becoming a limiting factor for microbial acid production. The notable decrease in phosphate concentration in the control medium following the addition of WPCBs might be attributed to phosphate adsorption onto metal oxy-hydroxides present in the WPCBs particles (Fe-O, Mn-O, Al-O and Si-O) (Vakilchap and Mousavi, 2022; Gonzalez et al., 2022). Metal oxy-hydroxides are known as effective sorbents of phosphate through electrostatic and ion exchange interactions, favoured at pH > 7 (Li et al., 2016).

The trends indicated that the higher oxalic and malic acid production, occurring initially within the cytoplasm, hindered subsequent citric acid production, as evident in the presence of WPCBs. Contrary, when the WPCBs material was not introduced into the medium, as cytosolic oxaloacetate activity was not favoured, citric acid secretion took place at higher levels, as seen in the fungal growth control experiment. Some authors have indeed highlighted that suppression of oxalate production can boost the synthesis of citric acid in *A. niger* (Kubicek et al., 2011). The observed pH variations in fungal growth control and bioleaching experiments (Fig. 2a) played a crucial role in regulating metabolic pathways, influencing the production of organic acids. The alkaline nature of WPCBs contributed to a sharp increase in pH in the bioleaching medium, facilitating the production of gluconic and oxalic acids. It is known that the enzymes oxaloacetate hydrolase (OAH) and GOX, which respectively mediate the synthesis of oxalic and gluconic acid, have been found at higher activity levels when pH is maintained above 4.0 (Kubicek et al., 2011). The lower pH levels (~3.0) found in the fungal growth in the absence of WPCBs would have contributed to the higher production of citric and fumaric acid, as biosynthesis of such acids is favoured through the TCA cycle when the pH is below 3.5 (Ma et al., 2022).

The unexpected malic acid increments in abiotic leaching experiments may be explained by potential contamination of WPCBs with malate salts, as reported in previous research on PCBs surface analysis (Huang et al., 2017). The decline in acid concentrations after one or two days in abiotic experiments suggested the possible precipitation of metal–organic complexes. Abiotic experiments displayed higher pH levels (>5.0), likely due to salt formation after WPCBs addition.

3.4.2. Metal leaching and precipitation

The observed pH trends correlated with the metal leaching behaviour (Fig. 3), with the lower pH (<4.1) in the fungal bioleaching experiment promoting higher metal solubilization compared to abiotic experiments with higher pH (>5.0). Leaching kinetics indicated a significant increase in REE solubilization within three hours in the fungal bioleaching experiment. In contrast, Cu dissolution showed a slower rate, likely due to its much higher content in the e-waste material (over 40 times higher than Nd, the most abundant REE). Despite the higher concentration of Cu, the fungal biolixiviant demonstrated higher efficiency for Nd leaching, with a calculated Cu/Nd ratio of 0.2 and 0.3 (μg/L Cu per μg/L Nd solubilized) in the first three and six hours, respectively. However, at 24 h, as REE bioleaching reached a plateau, the Cu/Nd ratio increased to almost 10. Cu bioleaching exhibited ongoing growth over subsequent days, suggesting that available organic acids were utilized by such reaction. Ni showed a similar trend, although with minimum leaching efficiency (results not included). Organic acids facilitate metal dissolution through acidolysis and complexolysis mechanisms.

The bioleaching medium pH was ~3.3 when WPCBs were introduced, which was below the dissociation constant (pKa) of acetic and gluconic acid (Fig. 2a), meaning that they were present mainly as acid and not as the conjugated salts. Therefore, rapid REE bioleaching was initiated through acidolysis, due to the prevalence of H^{+} (proton-promoted dissolution of the metals). The subsequent increase in medium pH above the pKa value confirms significant complexolysis of metals with deprotonated organic acid, particularly for Cu extraction occurring at higher pH levels. The enrichment of REE in fine particle size provides

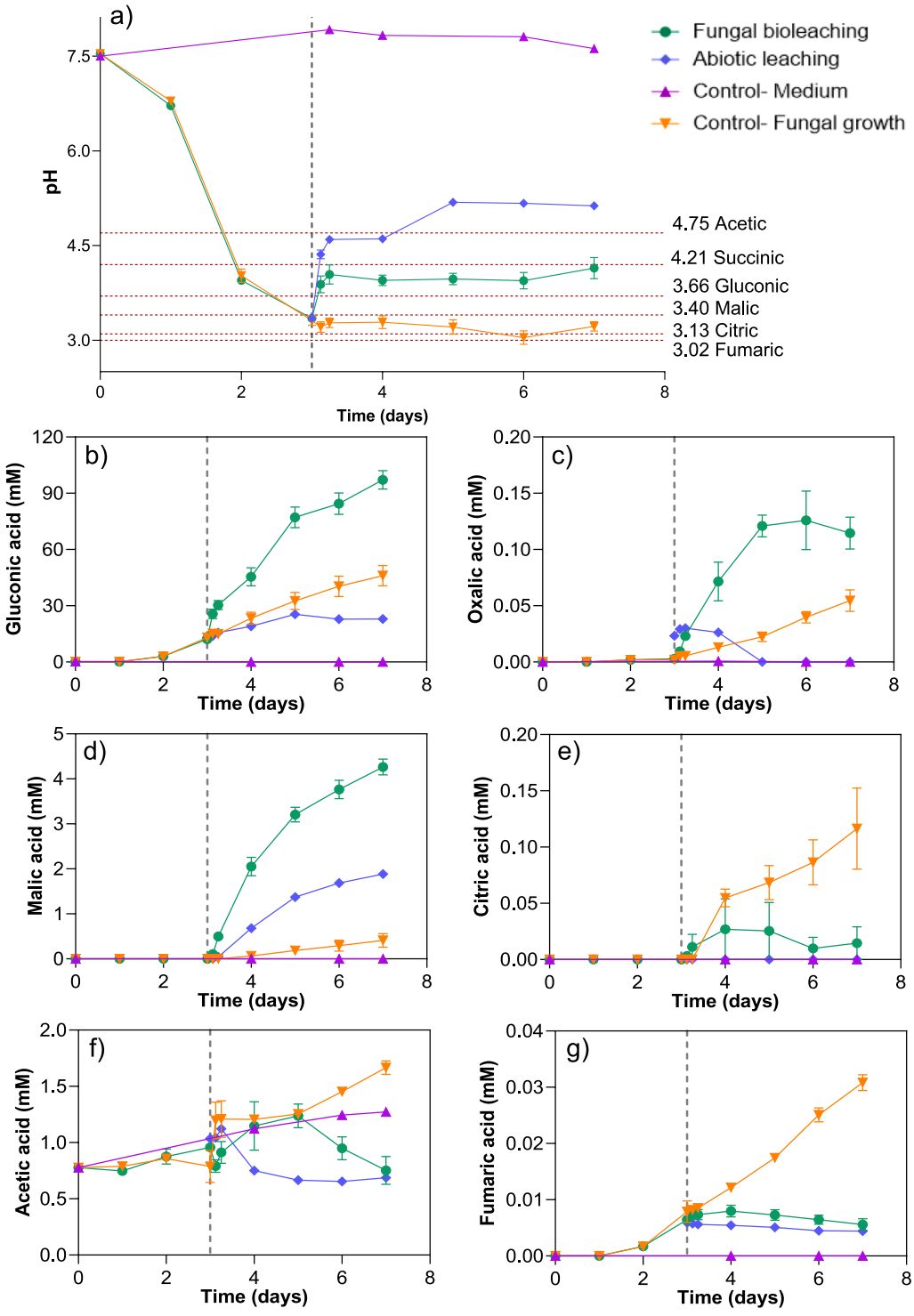

Fig. 2. Variation of pH and organic acid concentrations in leaching experiments. Changes in the pH (a), red horizontal dotted lines denote acid dissociation constants (pKa). Concentration of organic acids (b-g). Vertical grey dotted line represents introduction of waste printed circuit boards (day 3). Mean ± SE (error bars not shown for those that fall within mean symbol), n = 3.

more surface area for interactions with the leaching agent. However, as Cu solubilization increases, REE dissolution is hindered, suggesting Cu extraction dominance due to its higher content. Mixed oxides and silicates in the WPCBs matrix could further impede the extraction of REE and other metals, given the different binding energies and bond lengths of such mixtures (Bahaloo-Horeh et al., 2018), leading to challenges in metal separation.

In abiotic leaching experiments with organic acids, the solubilization of all REE decreased after 24 h (day 5), along with a similar trend for Fe and Cu dissolution after 48 h (Fig. 3). The declining trend in P concentration in abiotic experiments suggested co-precipitation. Formation of metal–organic complex was found in crystal particles collected from the abiotic experiment, with Cu-gluconate being identified (see supplementary material). Contrary to bioleaching, the elevated pH levels in abiotic solutions facilitated the precipitation of metal-gluconate complexes. As evidenced in this study, gluconate serves as an important

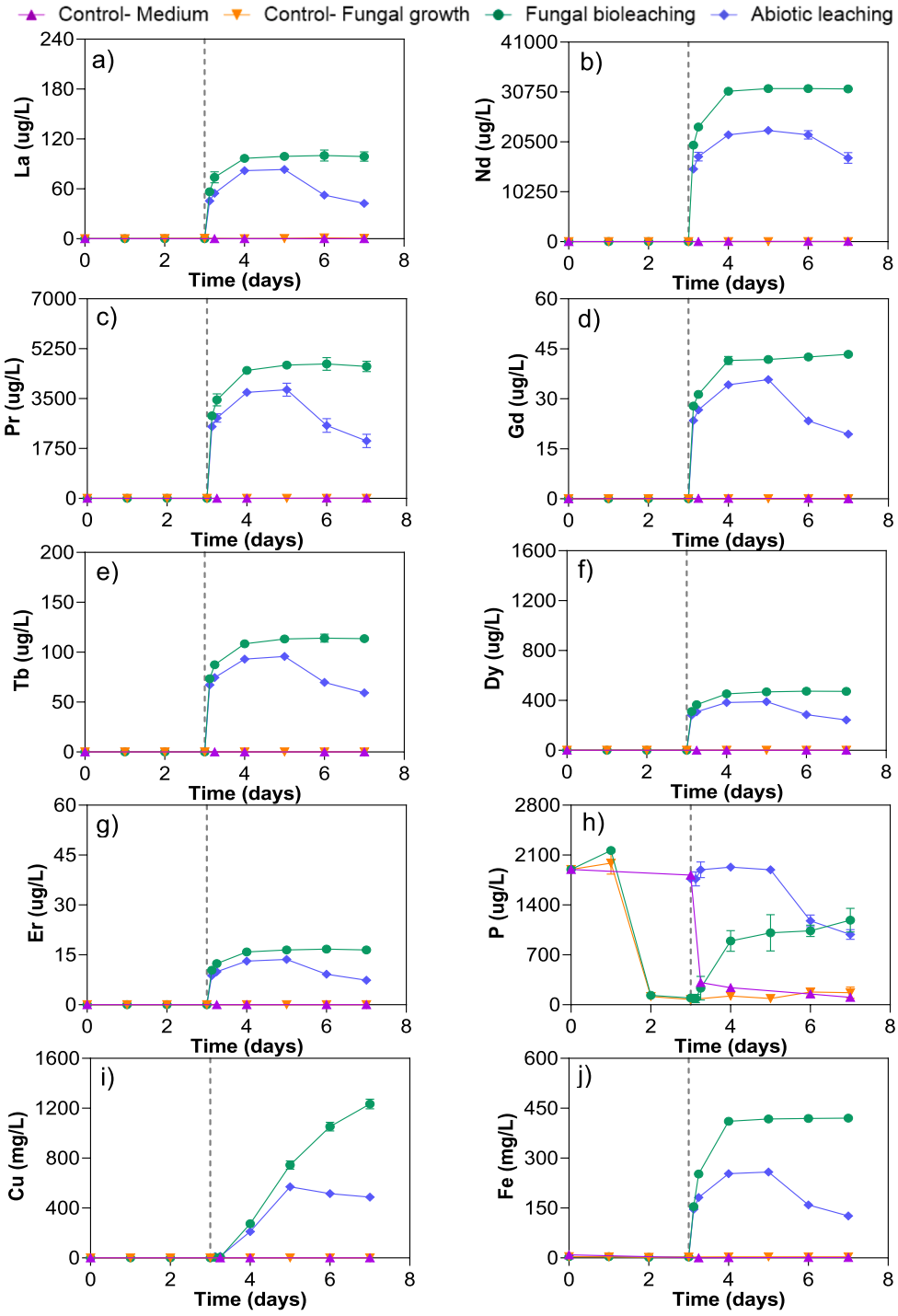

Fig. 3. Rare earth elements, base metals and phosphate concentration in bioleaching of waste printed circuit boards (WPCBs) by *P. expansum*, and abiotic leaching by organic acids. Control experiments for fungal growth and sterile medium. Vertical grey dotted line represents WPCBs introduction (day 3). Mean ± SE (error bars not shown when they fall within mean symbol), n = 3.

ligand for the extraction of REE from the complex WPCBs substrate.

3.4.3. *The bioleaching mechanism of P. expansum*

Fungal growth induced a reduction in medium pH from an initial 7.5 to 3.3 by secreting organic acids, notably gluconic acid. This acidification, recognized as a natural process during fungal growth, serves both as a competitive strategy against rival organisms (Andersen et al., 2009) and as a mechanism to release energy when microbial growth is limited by a nutrient source other than carbon (Vrabl et al., 2012). This metabolic activity facilitated the bioleaching of REE, Cu, and Fe from WPCBs. Contrary to the conventional defensive mechanism triggered by the toxicity of WPCBs, the mechanism of the *P. expansum* isolate also centred around nutrient sourcing, especially phosphate, after its depletion in the medium before WPCBs introduction. Optimizing phosphate concentration to a minimum of 0.1 mM served two crucial purposes: challenging microbial metabolite activity and reducing available phosphate in the solution, thereby preventing co-precipitation with REE and other metals, as observed in abiotic leaching. The availability of protons

in the bioleaching medium, essential for REE extraction, was attributed to organic acid production. However, it is possible that the fungal pH homeostasis mechanism, facilitated by proton pumps such as P-type adenosine triphosphatase (H + -ATPase), also plays a significant role (Kane, 2016). This is noteworthy because organic acids may not be released as fully protonated molecules; instead, they might be secreted in a partially protonated form or as anions during transportation across the fungal plasma membrane (e.g. oxalate, malate, citrate) (Vrabl et al., 2012). Although the release of gluconic acid, produced in the extracellular space, is more likely to occur in protonated form, this would also highly depend on the pH of the medium, considering the pKa of the acid. In an effort to elucidate the presence of H^+-ATPase in the current study, the genome of the *P. expansum* isolate was searched using the BLAST comparison algorithm, employing the *Saccharomyces cerevisiae* plasma membrane H^+-ATPase (PMA1) protein as a query (NCBI reference sequence: NP_011507.1). The yeast *S. cerevisiae* has been used as an important model organism for investigating the fungal proton transport system and its regulation (Kane, 2016). The search resulted in the identification of the P-type H + -ATPase protein in the fungal isolate, with a 65.1 % identity to the yeast PMA1 protein. Therefore, it is likely that the presence of such proton pumps in *P. expansum* also played a role during the bioleaching process. The schematic depiction of the bioleaching process in the presence of the fungal isolate is presented in Fig. 4.

In a bioleaching study from WPCBs using a mixture of the iron-oxidizing bacteria *Acidithiobacillus ferrooxidans* and *Acidiphilium acidophilum,* an 18-day process was required to recover 30 % REE (Priya and Hait, 2020). Contrary, a prior bioleaching investigation from hard drive magnets using *A. ferrooxidans* achieved higher (>80 %) solubilization of Pr, Dy and Nd (Auerbach et al., 2019), likely attributed to the physical and chemical composition of REE in permanent magnets. Iron-oxidizing bacteria rely on redoxolysis mechanism for metal solubilization. However, the method necessitates the introduction of iron and/or sulphur sources for microbial oxidation, and substantial pH adjustments to highly acidic conditions (pH < 2.0) (Auerbach et al., 2019). Hence, fungal heterotrophic bioleaching may present a more efficient avenue for REE solubilization from the highly challenging WPCBs material.

3.5. Bioleaching, spent medium and abiotic leaching

The leaching efficiencies achieved with the spent medium were comparable to the two-step bioleaching approach. However, utilizing the spent medium offers the advantage of avoiding potential REE biosorption. Additionally, handling the solution becomes more straightforward without fungal mycelia, enhancing process scalability. Spent medium was chosen for further comparison with abiotic experiments. Results from these experiments (Fig. 5) indicated that gluconic acid predominantly drove leaching activity in abiotic experiments for all REE and base metals, as the addition of other acids did not yield a significant difference, likely due to their trace amounts (<1 mM).

When comparing abiotic experiments to spent medium, it was found that the biolixiviant produced by the *P. expansum* isolate was significantly more effective ($p < 0.05$) at leaching REE and base metals. The pH of the spent medium remained slightly below the pH of all abiotic experiments (Fig. 5a). Leaching of the most abundant REE, Nd, was 13 % higher by the spent medium method, compared to gluconic acid. Similarly, the solubilization of Cu and Fe was 12 and 22 % higher using the spent medium. This suggests that there may be other metabolites, not analysed in this research, that could be secreted by the fungus and have a synergistic effect with the low molecular weight organic acids already identified, such as phosphatases, amino acids, and long-chain acids (e.g. fatty acids) (Fathollahzadeh et al., 2019; Brisson et al., 2020). The comparison between the spent medium and a higher concentration of gluconic acid (32 mM) revealed that the microbially produced lixiviant was as equally effective in the leaching of REE.

The industrial methods for gluconic acid production include chemical oxidation of glucose, electrolytic oxidation of glucose, and microbial fermentation. Due to environmental toxicity and rising costs, fermentation is the preferred economical application (Pal et al., 2016). However, sodium gluconate produced from fermentation requires additional purification steps, generating substantial wastewater and posing environmental and cost challenges (Ma et al., 2022). Direct use of crude biolixiviant could avoid additional processing and purification steps which would otherwise be needed in commercial gluconic acid production. Moreover, the gluconic acid-containing spent medium bioleaching offers a less hazardous and potentially more environmentally friendly alternative to conventional inorganic acid leaching.

In contrast to the corrosiveness, need for higher temperatures, and health and safety risks associated with inorganic acid leaching (Khanna et al., 2020), spent medium bioleaching operates close to room temperature (25 °C) and at pH levels between 3.3 and 4.0. This highlights the economic advantage of bioleaching over conventional metallurgy in reducing infrastructure costs and energy requirements (Arya and Kumar, 2020). In a techno-economic analysis of REE bioleaching, it was found

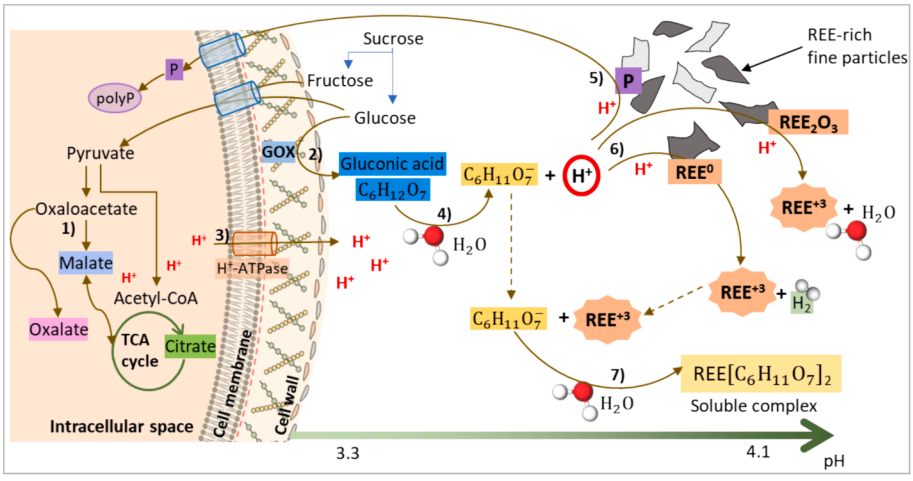

Fig. 4. Schematic representation of the bioleaching of rare earth elements (REE) from waste printed circuit boards (WPCBs) by the fungal isolate *P. expansum*. 1) stimulated production of oxalic and malic acid at the cytosol level, 2) enhanced oxidative production of gluconic acid by the gluconoxidase enzyme at the cell wall, 3) transport of protons from cytoplasm to the extracellular space by plasma membrane H + -ATPase during fungal pH homeostasis, 4) formation of protons by deprotonation of gluconic acid in contact with water molecules, 5) release of available phosphate from WPCBs by acid action, 6) release of REE from WPCBs by proton-induced oxidation of the metal and dissolution of the oxides, and 7) complexation of the REE ions in solution with organic ligand (deprotonated form of acid).

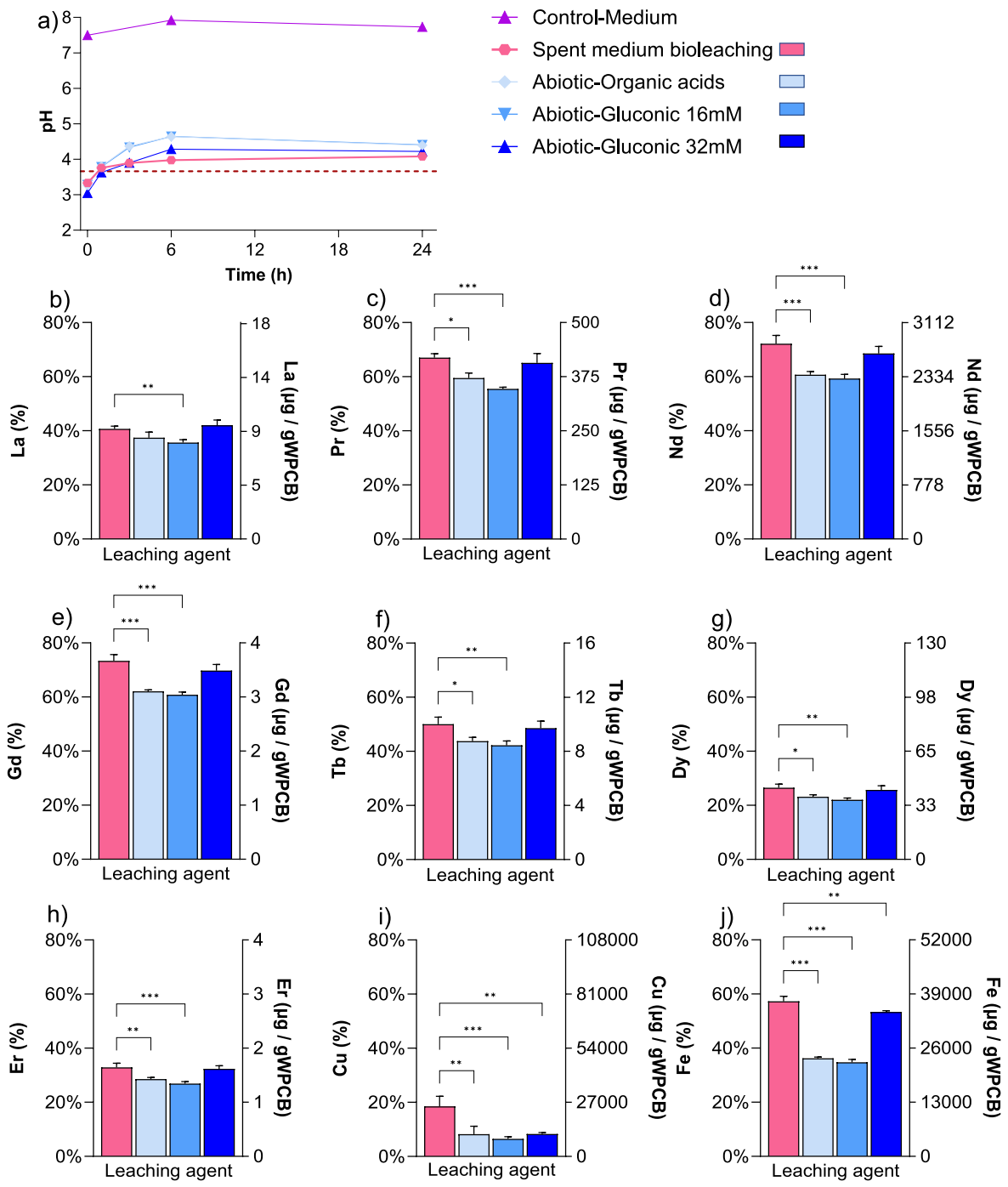

Fig. 5. Spent medium bioleaching by *P. expansum* and abiotic leaching from waste printed circuit boards. Variations of pH (a), horizontal dotted line denotes gluconic acid dissociation constant (pKa). Rare earth elements and base metals recovery (b-j). Mean ± SE, n = 3. Statistically significant differences: $*p < 0.03$ $**p < 0.002$ $***p < 0.001$ (ANOVA, Dunnett).

that bioleaching yielded slightly lower profits compared to chemical (nitric acid) leaching (Thompson et al., 2018). However, the higher carbon footprint of the chemical approach (e.g. smog, ozone depletion, and ecotoxicity), makes bioleaching a more appealing technology for large-scale development. Future research should therefore address the challenges in advancing this technology to a higher technology readiness level (TRL). Scaling up the equipment often decreases the bioleaching efficiency due to impacts on the microbial population (Yaashikaa et al., 2022), and the cost of nutrients for microbial growth is also of concern. Using feedstock such as agricultural residues, food industry by-products and municipal waste could offer cost-effective alternatives. Finally, the separation of REE from the leaching solution is particularly challenging because of their chemical similarities. Biosorption and electrical methods (e.g. electrowinning and electrodialysis) have emerged as environmentally friendlier techniques to conventional solvent extraction (Opare et al., 2021). However, further improvements are required to ensure economic viability at large scale.

4. Conclusion

Fungal bioleaching using *Penicillium expansum* under the optimized conditions, 7.5 initial pH and 0.1 mM initial phosphate concentration

without a buffering agent in the medium, has demonstrated its ability to recover REE from WPCBs, an underutilized resource. The fungal mechanism of REE bioleaching was associated to the production of a gluconic acid-rich biolixiviant and involvement of the plasma membrane proton pump. The comparable efficacy of using cell-free supernatant and the direct use of the crude biolixiviant could simplify large-scale application of bioleaching, making this approach a promising and sustainable biotechnology to recover REE from e-waste streams.

CRediT authorship contribution statement

Alejandra Gonzalez Baez: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Leonardo Pantoja Muñoz:** Writing – review & editing, Validation, Methodology, Conceptualization. **Martijn JTN Timmermans:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Hemda Garelick:** Writing – review & editing, Methodology, Conceptualization. **Diane Purchase:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2024.130750.

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