Received: 7 June 2022 | Accepted: 6 November 2022

DOI: 10.1111/1751-7915.14192

MICROBIAL BIOTECHNOLOGY Applied Microbiology International

RESEARCH ARTICLE

Rio Tinto as a niche for acidophilus enzymes of industrial relevance

Abdelali Daddaoua[1] | **Consolación Álvarez**[2] | **Monika Oggerin**[3] | **Nuria Rodriguez**[4] | **Estrella Duque**[5] | **Ricardo Amils**[3,4] | **Jean Armengaud**[6] | **Ana Segura**[5] | **Juan Luis Ramos**[5]

[1]Department of Biochemistry and Molecular Biology II, Faculty of Pharmacy, University of Granada, Granada, Spain

[2]Instituto de Bioquímica Vegetal y Fotosíntesis (CSIC-US), Consejo Superior de Investigaciones Científicas and Universidad de Sevilla, CIC Cartuja, Seville, Spain

[3]Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Universidad Autónoma de Madrid, Madrid, Spain

[4]Centro de Astrobiología (INTA-CSIC), Torrejón de Ardoz, Spain

[5]Estación Experimental del Zaidín (EEZ-CSIC), Granada, Spain

[6]Département Médicaments et Technologies pour la Santé (DMTS), Université Paris Saclay, CEA, INRAE, Bagnols-sur-Cèze, France

Correspondence
Ana Segura, Estación Experimental del Zaidín (EEZ-CSIC), Profesor Albareda 1, 18008 Granada, Spain.
Email: ana.segura@eez.csic.es

Funding information
Consejo Superior de Investigaciones Científicas, Grant/Award Number: PTI-SusPlast+ ref 2021 AEP084; European Union, Grant/Award Number: FEDER funds; Ministerio de Ciencia e Innovación, Grant/Award Number: RTI2018-094370-B-I00

Abstract

Lignocellulosic residues are amongst the most abundant waste products on Earth. Therefore, there is an increasing interest in the utilization of these residues for bioethanol production and for biorefineries to produce compounds of industrial interest. Enzymes that breakdown cellulose and hemicellulose into oligomers and monosaccharides are required in these processes and cellulolytic enzymes with optimum activity at a low pH area are desirable for industrial processes. Here, we explore the fungal biodiversity of Rio Tinto, the largest acidic ecosystem on Earth, as far as the secretion of cellulolytic enzymes is concerned. Using colorimetric and industrial substrates, we show that a high proportion of the fungi present in this extremophilic environment secrete a wide range of enzymes that are able to hydrolyze cellulose and hemicellulose at acidic pH (4.5–5). Shotgun proteomic analysis of the secretomes of some of these fungi has identified different cellulases and hemicellulolytic enzymes as well as a number of auxiliary enzymes. Supplementation of pre-industrial cocktails from *Myceliophtora* with Rio Tinto secretomes increased the amount of monosaccharides released from corn stover or sugar cane straw. We conclude that the Rio Tinto fungi display a good variety of hydrolytic enzymes with high industrial potential.

INTRODUCTION

Systematic searches for life in extreme environments have allowed the identification of micro-organisms that are highly resistant to organic solvents (Inoue et al., 1991; Isken & de Bont, 1998; Ramos et al., 2002), high pressures (barophiles) (Horikoshi, 1998), high and low temperatures (thermophiles, hyperthermophiles and psychrophiles) (Atalah et al., 2019; Cavicchioli et al., 2011), high NaCl concentrations (halophiles) (Jin et al., 2019) and, low and high pH values (acidophiles and alkaliphiles) (Di Donato et al., 2018; Mamo, 2019).

Abdelali Daddaoua and Consolación Álvarez equally contributed to this paper.

Due to the fact that extremophiles are micro-organisms that live under harsh conditions, it has been hypothesized that these micro-organisms might be armed with enzymes with exceptional properties. Indeed, they have been explored in the last decades as a source of enzymes with unusual properties for biotechnological processes (Coker, 2019; Dumorne et al., 2017; Pennisi, 1997) such as detergent additives, hydrolysis of starch, polyvalent lipases, DNA modification and amplification, and biocatalysts for the production of added-value compounds (Dumorne et al., 2017; Jin et al., 2019; Kumar et al., 2019; Raddadi et al., 2015).

Lignocellulosic residues are amongst the most abundant waste products on Earth (Zhang, 2008). Therefore, in recent years, the interest in the utilization of these residues from food crops or municipal solid wastes has led to an increase in research in the so-called 2G technology for bioethanol production and for biorefineries able to produce compounds of industrial interest (Valdivia et al., 2016). In the 2G industry, the most relevant enzymes are those that breakdown cellulose and hemicellulose into oligomers and monosaccharides, allowing the utilization of non-edible plant residues for the production of goods (Godoy et al., 2021). The 2G industrial processes require pretreatment of lignocellulosic materials to remove lignin, decrease cellulose crystallinity and increase the porosity of these materials, to enhance availability for cellulolytic enzymes. One of the most efficient pre-treatments consists of soaking the lignocellulosic material in acid solutions followed by steam explosion to deconstruct the structure of lignocellulose (Nguyen et al., 2000). Upon cooling, the pH, temperature and total solids are adjusted prior to the addition of the enzymes for saccharification (Álvarez et al., 2016; Kumar et al., 2009). Most of the current commercial cellulolytic cocktails of enzymes operate efficiently at relatively high temperatures (around 55°C) and with a pH of above 5.5. These pH conditions are not restrictive for the growth of certain micro-organisms and in some cases, the growth of potential contaminant micro-organisms, particularly lactic bacteria, occurs. Hence, the identification of cellulolytic enzymes with optimum activity at a lower pH is a request of companies producing enzymatic cocktails. Some cellulolytic enzymes stable at low pH have been identified (Cannio et al., 2004; Huang et al., 2005; Sharma et al., 2012; Thanh et al., 2019): however, they function at low efficiency rates and at pH values lower than 4.5. To the best of our knowledge, no systematic search has been carried out to identify acidophilic enzymes for lignocellulose deconstruction that demonstrate their utility using industrial substrates.

Rio Tinto, with a length of 92 km, is considered the largest acidic ecosystem on Earth (Amils, 2016; Amils et al., 2011). Despite the extreme conditions, with a mean pH of 2.3, and high concentrations of heavy metals (Fe, Cu, Mn, As, Cr and Zn), an unexpected biodiversity of micro-organisms has been described (Aguilera, Souza-Egipsy, et al., 2007; Aguilera, Zettler, et al., 2007; Amaral-Zettler et al., 2011; Bashir et al., 2021; López-Archilla et al., 2001). Although, it was assumed that acidity and high concentrations of heavy metals would limit eukaryotic diversity (Amaral-Zettler et al., 2011; Gross & Robbins, 2000), this turned out not to be the case in the Rio Tinto, where eukaryotes include specimens of most of the major lineages; algae, protozoans, fungi and yeasts (Aguilera, Souza-Egipsy, et al., 2007; Aguilera, Zettler, et al., 2007; Amaral Zettler et al., 2002, 2011; López-Archilla et al., 2001) have been described. Fungi are amongst the most abundant and diverse biomass decomposers in the Rio Tinto (López-Archilla et al., 2001, 2004), and given the physicochemical characteristics of this niche, we foresee that fungi living in this harsh environment would have the capacity to degrade the dead plant material deposited in the sediments of Rio Tinto and, therefore, that these fungi may exhibit their own battery of enzymes for lignocellulose breakdown at low pHs.

In this study, we have explored the cellulolytic and hemicellulolytic capacity of a collection of fungi isolated from the Rio Tinto and characterized their secretomes by shotgun proteomics. We show that these fungi produce cellulolytic enzymes with added advantages for the 2G technology as they function at acidic pH (<4.5).

EXPERIMENTAL PROCEDURES

Growth of fungal strains and preparation of fungal secretomes

Fungal samples were obtained from water and sediments during March, June and December of 2009, 2010 and 2011 in 19 different sampling areas. In all the sampling areas the pH was below 3. The water temperature fluctuated between 13°C in winter and 24.5°C in summer. Most fungal isolates were obtained from sediments (unpublished data, M.O., C.M., N.R., N.F. and R.A.).

Fungal strains were grown in an orbital shaker at 35°C and 250 rpm for 3 days in BIM culture medium (salts solution with yeast extract [1 g L^{-1}], lactose monohydrate [27 mM], glucose [50 mM] and defatted cotton seed flour (Pharmamedia) [5 g L^{-1}] buffered to pH 7.0) for fungal growth and afterwards incubated for 7 days in BTR culture medium; salts solution with yeast extract [0.5 g L^{-1}], lactose monohydrate [13.5 mM], defatted cotton seed flour (Pharmamedia) [5 g L^{-1}] and cellulose Vitacel [20 g L^{-1}] for enzyme production, as described by Emalfarb et al. (1996). The media were buffered with 50 mM sodium citrate pH 4.5. Incubation time for enzyme production was initially determined for a representative group of Rio Tinto fungi and afterwards was used for all of them. Then, the cultures were

centrifuged at 3220 *g* for 10 min at 4°C. The supernatants were considered as the fungal secretomes and were used for all the assays (activity against commercial substrates, PSC and PSCS hydrolysis and shotgun proteomic analysis). When required, the secreted proteins were concentrated using centrifugal concentrators (Vivaspin 20 [Sartorius stedim]) at 7000 rpm for 15 min to reduce the volume. The protein concentration was measured by a bicinchoninic acid assay using the BCA AppliChem (Ref. A7787) kit following manufacturer's instructions. The secretomes of *Mycelophtora thermophila* pre-industrial strains were a gift from Abengoa Bioenergy.

Enzymatic activity assays

For the analysis of the enzymatic activities the following colorimetric substrates were used: azo-xyloglucan (Megazymes) for xyloglucanase, azo-carboxymethylcellulose (Sigma) for endoglucanase, *p*-nitrophenyl-β-D-glucopyranoside (pNGP, Sigma) for β-glucosidase, *p*-nitrophenyl-β-D-xylopyranoside (pNXP, Sigma) for β-xylosidase. pNPG and pNXP were prepared at 0.2 g L^{-1} in 200 mM of sodium acetate buffer (pH 4.5). To run the reactions, 250 μl of the enzymatic mixture (0.1–1 U) and 250 μl of the substrate were mixed and incubated at 50°C for 10 min, the reactions were stopped using 500 μl volume of sodium carbonate 1 M. The amount of *p*-nitrophenol released was measured at 410 nm (ϵ_{410} = 15.2/mM · cm). One unit of enzymatic activity was defined as the amount of enzyme needed to release 1 μmol *p*-nitrophenol per minute (Zimbardi et al., 2013). The standard assay for endoglucanase (EG) and xyloglucanase (XYGL) activity was carried out at 50°C for 10 min in 200 mM sodium acetate buffer pH 4.5 with 1% (w/v) of azo-CMC and 0.5% (w/v) of azo-xyloglucan, respectively. After incubation, ethanol (95%) was added to stop the reaction, the sample was centrifuged (3000 *g*, 10 min) and the absorbance of the supernatant measured at 590 nm. The activity of each enzyme was calculated by reference to a standard curve produced with xyloglucanase from *Paenibacillus* sp. (Megazymes) or endo-glucanase of *Aspergillus niger* (Sigma), according to the supplier of the substrate. One unit of enzymatic activity is defined as the amount of enzyme required to release one micromole of xylose/glucose reducing-sugar equivalents from the substrate. To determine cellobiohydrolase activity, 10 mg of substrate (microcrystalline cellulase, Avicell [Sigma]) in a sodium acetate buffer pH 4.5 was mixed with the enzymatic cocktail (0.1–1 g L^{-1}) supplied with 400 U ml^{-1} of commercial β-glucosidase. The mixture was incubated for 120 min at 50°C and the reaction stopped by boiling for 10 min. After centrifugation, the glucose released was determined using the D-Glucose Assay GOPOD kit (Megazyme) following the manufacturer's instructions.

The effect of the pH on the enzymatic activities was assayed using sodium acetate buffers in the range of 3–5 and sodium phosphate buffers from 6 to 7.

Enzymatic hydrolysis of lignocellulolytic substrates

Pretreated corn stover (PCS) and pretreated sugar cane straw (PSCS) adjusted to pH 4.5 were a gift from Abengoa Bioenergy (York, Iowa, USA). The insoluble fraction of the substrates, in percentage, was composed of 35.41 (PCS) and 34.52 (PSCS) of glucan, 6.50 (PCS) and 4.37 (PSCS) of xylan, 0.79 (PCS) and 0.55 (PSCS) of arabinan, 0.54 (PCS) and 0.24 (PSCS) of mannan, 21.52 (PCS) and 18.52 (PSCS) of Klason lignin and 3.7 (PCS) and 2.41 (PSCS) of ash. In the soluble fraction of PCS and PSCS, the content (in percentage) was 3.07 and 2.93 of glucose, 16.42 and 15.02 of xylose, 1.63 and 1.66 of arabinose, 0.11 and 0.25 of mannose, 1.19 and 0.61 of acetic acid, and 0.54 and 0.26 of furans, respectively (Rocha-Martín et al., 2017). The total solids were adjusted to 20% (wt vol^{-1}) and were hydrolysed with an enzymatic cocktail from *M. thermophila* C1 derived strain (7 mg of protein g^{-1} glucan for PCS hydrolysis and 9 mg g^{-1} glucan for PCSC hydrolysis) (Martín Pérez et al., 2017), supplied with purified β-glucosidase from the same organism (1 mg of protein g^{-1} glucan) and, when indicated, with 1 mg of protein g^{-1} glucan of different Rio Tinto fungal secretomes. The final enzyme loading was 9 mg of total protein per g of glucan for PCS and 11 mg of total protein per g of glucan for PCSC. Enzymatic hydrolysis experiments were carried out in 10 ml tubes and the final reaction volume was 3 ml. The samples were incubated at 50°C under orbital shaking at 150 rpm for 72 h. All the hydrolysis experiments were performed in triplicate. Due to the high density of the hydrolysate at 20% of solids, the analytes were quantified in weight/weight (g kg^{-1}). Thereafter, the glucose, xylose and xylobiose were separated by HPLC using a 4.6 × 250 mm AMINEX® HPX-87H column and the sugars were detected using a refractive index detector, as described by Kristensen et al. (2009).

Shotgun proteomics

The secretomes were treated as previously described (Rubiano-Labrador et al., 2015). Briefly, they were precipitated by adding one fifth of the volume of the sample of trichloroacetic acid prepared at 50% (wt/vol) at 4°C. After centrifugation at 16,000 *g* for 5 min, the pellets were dissolved in 50 μl of LDS1X (Invitrogen) supplemented with 5% beta-mercaptoethanol and then sonicated for 5 min in an ultrasonic water bath (VWR ultrasonic cleaner). The samples were heated

for 5 min at 99°C. A volume of 30 μl (corresponding approximately to 20 μg of proteins) of each sample was subjected to a short (4 min) electrophoresis in denaturing conditions using NuPAGE 4%–12% gradient gel operated with MES SDS as running buffer (50 mM MES ([2-(N-morpholino) ethane sulfonic acid]), 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.3). After electrophoretic separation, the gel was stained with SimplyBlue SafeStain (Thermo) and extensively washed with milliQ water. Each secretome was extracted as a single polyacrylamide band which was processed as previously described (Rubiano-Labrador et al., 2014) and then proteolysed with trypsin Gold (Promega) in 50 mM NH_4HCO_3 in the presence of ProteaseMax detergent (Promega). Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis of the peptide samples was performed using a Q-Exactive HF tandem mass spectrometer (Thermo Scientific) coupled to an Ultimate 3000 nano-LC system which was operated as described earlier (Lozano et al., 2022). Peptide samples (10 μl, corresponding to approximately 200 ng of peptides) were loaded on a reverse-phase PepMap 100 C18 μ-precolumn (5 μm, 100 Å, 300 μm i.d. × 5 mm, Thermo Fisher) and then resolved on nanoscale PepMap 100 C18 nanoLC column (3 μm, 100 Å, 75 μm i.d. × 50 cm, Thermo Fisher) at a flow rate of 0.2 μl per min using a 180 min gradient (5%–40% B) with mobile phases A (0.1% HCOOH/100% H_2O) and B (0.1% HCOOH/100% CH_3CN). The mass spectrometer was operated in data-dependent acquisition mode with MS and MS/MS acquired at a resolution of 60,000 and 15,000, respectively. Successive selection of the 20 most abundant precursor ions for fragmentation was done on doubly charged ion precursors using a dynamic exclusion of 10 s. MS/MS spectra were interpreted to peptide sequences by the PEAKS De Novo pipeline (Bioinformatics Solutions Inc) and against the NCBInr database using the MASCOT software (version 2.3.2) from Matrix Science, using standard search parameters. Datasets are available at https://doi.org/10.6084/m9.figshare.19960211.

RESULTS AND DISCUSSION

Exploration of Rio Tinto fungi for hydrolytic enzymes

A total of 182 species of fungi from a Rio Tinto collection were used to search for potential acidophilic enzymes. Of the 182 isolates, 84% belonged to the Ascomycota phylum, whilst members of other phyla, such as Zygomycota (1%) and Basiodiomycota (0.5%), were present at lower proportions. It should also be noted that 26 isolates (14% of total) remain unclassified. These proportions were similar to those reported in a representative sub-sample (355 strains) of the complete collection (Oggerin et al., submitted). Amongst the Ascomycota, Eurotiomycetes (77%) and Sordariomycetes (18%) were the most abundant classes, whilst a low proportion of Dothideomycetes (4%) and Helothiales (1%) were present (Table S1). It should be noted that, in this case, Dothideomycetes were underrepresented compared to the reported fungal collection, while Eurotiomycetes were overrepresented. At the genus level, *Penicillium, Talaromyces* and *Trichoderma* were the most abundant isolates, representing 51%, 8% and 7%, respectively, of the isolates in the collection (Table S1). Members of *Penicilium* are also the most abundant strains amongst the Eurotiomycetes, reported by Oggerin et al. (unpublished data, M.O., C.M, N.R., N.F. and R.A.).

All the isolates were grown as described in the Experimental Procedures, and upon separation of the mycelia by centrifugation and the clarification step, the amount of proteins was determined. The concentration ranged between 0.05 $g L^{-1}$ up to 1.32 $g L^{-1}$ (Table S1). The secretomes of the isolates were tested for three activities related with cellulose deconstruction (endoglucanase, cellobiohydrolase and β-glucosidase) and two enzymatic activities related to hemicellulose breakdown (β-xylosidase and xyloglucanase) using colorimetric assays. The recorded activities are shown in Table S1. As can be observed, we found a wide range of values for each of the enzymatic activities assayed. As a rule of thumb, we considered that a secretome was positive for the tested enzymatic activity if the value recorded was at least 10% of the maximum activity found for the given enzyme for the whole set of the tested secretomes. We found that 44% of the fungal strains were positive for endoglucanase activity (EG) (>4350 $U g^{-1}$ protein); 56% were positive for cellobiohydrolase (CBH) activity (>90 $U g^{-1}$ protein); 19% were positive for β-glucosidase (BGL) (>1940 $U g^{-1}$ protein); 6% positive (>1620 $U g^{-1}$ protein) for β-xylosidase (BXL) activity; and 16% positive (>10,770 $U g^{-1}$ protein) for xyloglucanase (XYGL) activity (Table S1).

To identify the best individual activities amongst the fungal isolates from Rio Tinto, we plotted the enzymatic activity values of the isolates and identified the superior outliers (Figure 1). There were 10 isolates with a high level of EG activity (>20,000 $U g^{-1}$), nine isolates with CBH activity higher than 500 $U g^{-1}$, 10 isolates with more than 5000 $U g^{-1}$ of BGL activity, six isolates with BXL activity higher than 6000 $U g^{-1}$, and 12 isolates with XYGL higher than 20,000 $U g^{-1}$. A total of 36 isolates (approximately 20% of the strains tested) presented, at least, one high activity (Table 1).

It has been reported that some, but not all endoglucanases, are able to hydrolyze the xyloglucan backbone at unsubstituted-Glcp (Eklöf et al., 2012; Grishutin et al., 2004). In our analysis, six out of the 10 strains with the highest EG activities also presented the highest XYGL activities (Table 1). However, some other isolates presented high EG activities while exhibiting

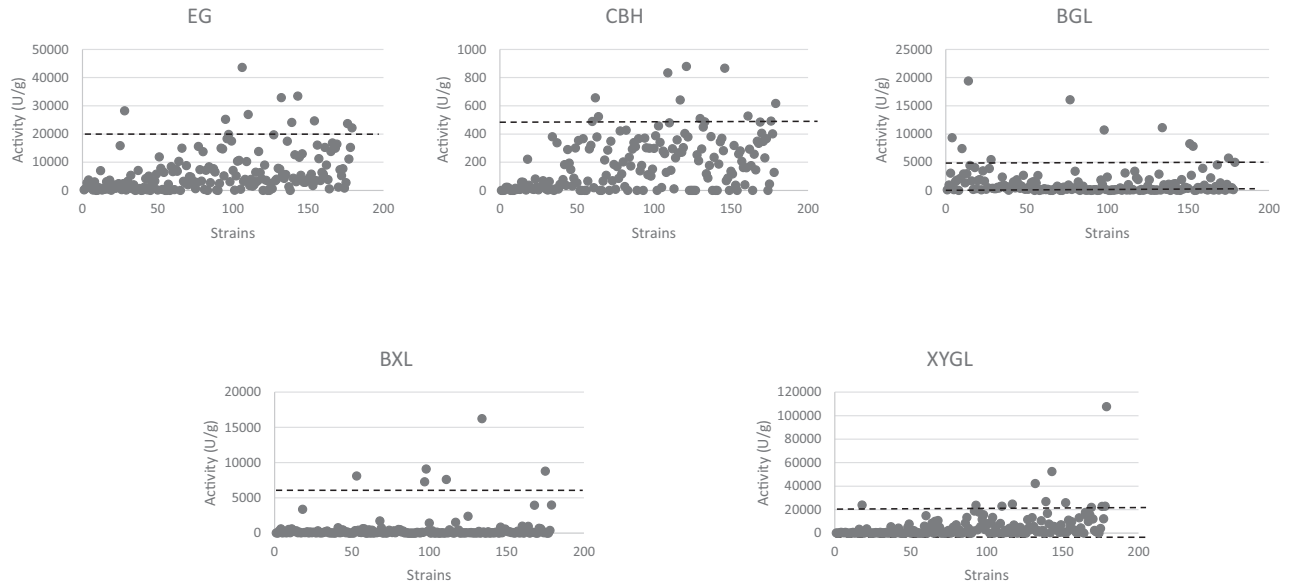

FIGURE 1 Graphical representation of the enzymatic activity ($U g^{-1}$) of the Rio Tinto fungal strains compared to the commercial substrates. The fungal strains (horizontal axis) were plotted according to their enzymatic activity (vertical axis). The superior outliers were identified and corresponded to strains with more than 20,000 $U g^{-1}$ endoglucanase (EG) activity, 500 $U g^{-1}$ cellobiohydrolase (CBH) activity, 5000 $U g^{-1}$ β-glucosidase (BGL) activity, 2000 $U g^{-1}$ β-xylosidase (BXL) activity, and 20,000 $U g^{-1}$ xyloglucanase (XYGL) activity. The dotted line indicates the arbitrary limit.

low XYGL (i.e. isolate M19046 [without taxonomical identification]) and vice versa (i.e. *Trichoderma asperellum* ID M21055, and ID M21041, *Penicillium* ssp. ID M19010, and ID M16018 and *Neosartorya glabra* ID M08026), suggesting the presence of enzymes with specific activities against the different substrates (Table 1).

Not surprisingly, species of *Penicillium*, *Trichoderma* and *Talaromyces* were the most abundant amongst those with the highest cellulolytic activities. These fungi have previously been reported as good producers of cellulolytic enzymes (Sharma et al. 2016). However, some species of *Chaetomium*, *Acidomyces* and *Neosartorya* presented extraordinarly high activity on commercial substrates. The above series of results supports the hypothesis that the Rio Tinto niche is populated with fungi that exhibit high activity against plant material and have the potential to deliver new relevant enzymes for biotechnological applications.

Effect of the pH on the hydrolytic activities of Rio Tinto fungi

The initial pH of the BTR culture medium was 4.5. However after 7 days of incubation for enzyme production, we found that the culture medium of 140 strains had experienced a decrease in the pH below 4.5; with 76 of them having acidified the medium to pH 3 or lower (Table S1). Since the pH decrease occurred with time and took place whilst the growth was sustained, we hypothesized that some of the secreted enzymes could be active in the range between pH 3.5 and 5, the pHs of interest in our study.

To check this hypothesis, we randomly chose eight isolates with high BGL, CBH, EG, BXL or XYGL activities, and we determined the optimum pH for each of them. All the BGLs tested presented an optimum pH of around 4.5 (Figure 2), whilst activity at pH 6 or 7 was considerably reduced, even the strain *Penicillium glabrum* ID M15024 that had maintained the pH of the medium at 6.9 (Table S1). CBH activities were maximum at low pH values (pH 4–5) presenting 60% or less of their maximum activity at pH 6 and almost no activity at pH 7. At pH 3, two of the strains (*P. janthinellum* ID M02031 and *Talaromyces muroii* ID M19108) retained 80%–90% of the maximal CBH activity. Most of the β-xylosidases were fully active between pH 4.5 and 5 with the exception of *Trichoderma viridae* ID M03039, which showed low activity at pH 5. *Trichoderma asperellum* ID M21055, a strain with high BXL activity (3384 $U g^{-1}$), retained more than 50% of the activity at pH 3. EG and XYGL activities presented a higher variability in the pH when maximum activity was reached (Figure 2), but it was maximum in the range between 4.5 and 6 for EG and between 4 and 5 for XYLGL. Many of the assayed strains retained more than 60% of the maximal XYGL activity at pH 3 (Figure 2).

In strains in which two (*Penicillium* sp. ID M16051 for BGL and EG, *Trichoderma viridae* ID M03039 for BGL and BXL, *Trichoderma asperellum* ID M18038 for BGL and BXL, M02013 [without taxonomical identification] for XYGL and EG, and *Trichoderma asperellum* ID M21055 for BXL and XYGL) or three (*Penicillium*

TABLE 1 Isolates that exhibit high activity against cellulosic/hemicellulosic commercial substrates.

Strain	pH	Protein ($g L^{-1}$)	ITS-based identification	EG ($U g^{-1}$)	CBH ($U g^{-1}$)	BGL ($U g^{-1}$)	BXL ($U g^{-1}$)	XYGL ($U g^{-1}$)
M09022	2.6	0.23	*Penicillium daleae/singorense*	**22,204**	**616**	4960	3981	**107,738**
M19076	2.8	0.66	*Penicillium simplicissimum*	**24,116**	0	0	0	**24,293**
M16012	3.0	0.15	*Penicillium* sp.	**26,893**	479	248	9	**22,982**
M16011	2.5	0.13	*Penicillium* sp.	**23,649**	492	269	3	**22,855**
M24005	2.9	0.37	*Penicillium janthinellum*	**32,893**	449	2862	264	**42,247**
M02013	2.8	0.49	Without identification	**33,469**	344	15	140	**52,498**
M21054	2.9	0.09	*Trichoderma asperellum*	13,787	**641**	3383	1532	**24,802**
M03039	3.1	0.54	*Trichoderma viride*	3073	102	**10,692**	**9111**	15,754
M14032	2.9	0.11	*Trichoderma asperellum*	4219	118	**11,108**	**16,229**	2152
M18038	2.5	0.15	*Trichoderma asperellum*	2734	45	**5719**	**8796**	4066
M14001	5.3	0.23	*Talaromyces proteolyticus*	**28,243**	68	**5440**	337	294
M10008	3.1	0.76	*Penicillium cremeogriseum*	**25,183**	51	35	106	17,853
M19046	3.0	0.54	Without identification	**43,567**	278	122	0	4399
M19011	2.7	0.56	*Penicillium* sp.	**24,677**	nd	89	89	11,016
M19048	3.5	0.15	*Penicillium* sp.	7492	**657**	131	12	1981
M16015	3.4	0.39	*Penicillium* sp.	10,279	**523**	86	7	8466
M19052	2.9	0.08	*Penicillium* sp.	8998	**880**	174	52	421
M19108	2.8	0.11	*Talaromyces muroii*	12,944	**866**	1926	849	2627
M16019	2.6	0.1	*Penicilliium* sp.	15,248	**527**	189	10	13,068
M02031	3.0	0.13	*Penicillium janthinellum/svalbard ense/pulvillorum*	10,190	**834**	29	22	8229
M10010	2.9	0.2	*Penicillium janthinellum/svalbard ense/pulvillorum*	7823	**510**	49	22	13,224
M06026	6.7	0.41	*Talaromyces purpureogenus/alb obiverticillius*	69	16	**7416**	69	0
M15024	6.9	0.19	*Penicillium glabrum/spinulosum*	3725	22	**9317**	620	708
M02004	6.7	0.24	*Penicillium nodositatum*	839	59	**19,386**	343	620
M16051	3.3	1.02	*Penicillium* sp.	15,550	88	**16,060**	343	377
M19097	2.7	0.15	*Chaetomium aureum/Ch hispanicum/Ch aureum*	4582	117	**8269**	200	665
M15021	2.7	0.44	*Acidomyces acidophilus*	1721	37	**7827**	92	1120
M03038	3.8	0.37	*Trichoderma viride*	3265	86	2660	**8644**	5015
M18022	3.6	0.14	*Trichoderma* sp.	352	0	1375	**8105**	372
M08014	3.1	0.23	Without identification	19,843	299	1048	**7286**	11,799
M18021	3.0	0.14	*Trichoderma* sp.	1707	142	3093	**7621**	0
M21055	6.4	0.16	*Trichoderma asperellum/Hypocrea lixii*	3635	221	4030	3384	**23,928**
M19010	3.1	1.14	*Penicillium* sp.	14,687	nd	101	101	**23,783**
M21041	2.7	0.27	*Trichoderma asperellum*	5740	318	2669	200	**25,875**
M16018	2.5	0.11	*Penicillium* sp.	16,435	484	69	12	**21,957**
M08026	2.4	0.57	*Neosartorya glabra/hiratsukae*	15,229	127	134	378	**22,906**

Note: The strains shown present high activity, at least, in one of the 5 enzymatic activities tested. The threshold for the identification of the superior outliers were determined as: EG > 20.000 $U g^{-1}$; CBH > 500 $U g^{-1}$; BGL > 5000 $U g^{-1}$; BXL > 6000 $U g^{-1}$ and XLGL > 20,000 $U g^{-1}$. The activities higher than those values are in bold.

Abbreviation: nd, Not determined.

simplicissimum ID M19076 for CBH, EG and XYGL) activities were analysed, the optimum pH was around 4.5–5 for all the activities (Figure 3); it should be noted that strain *Trichoderma asperellum* ID M21055 (BXL and XYGL) did not decrease the pH of the medium (Table S1).

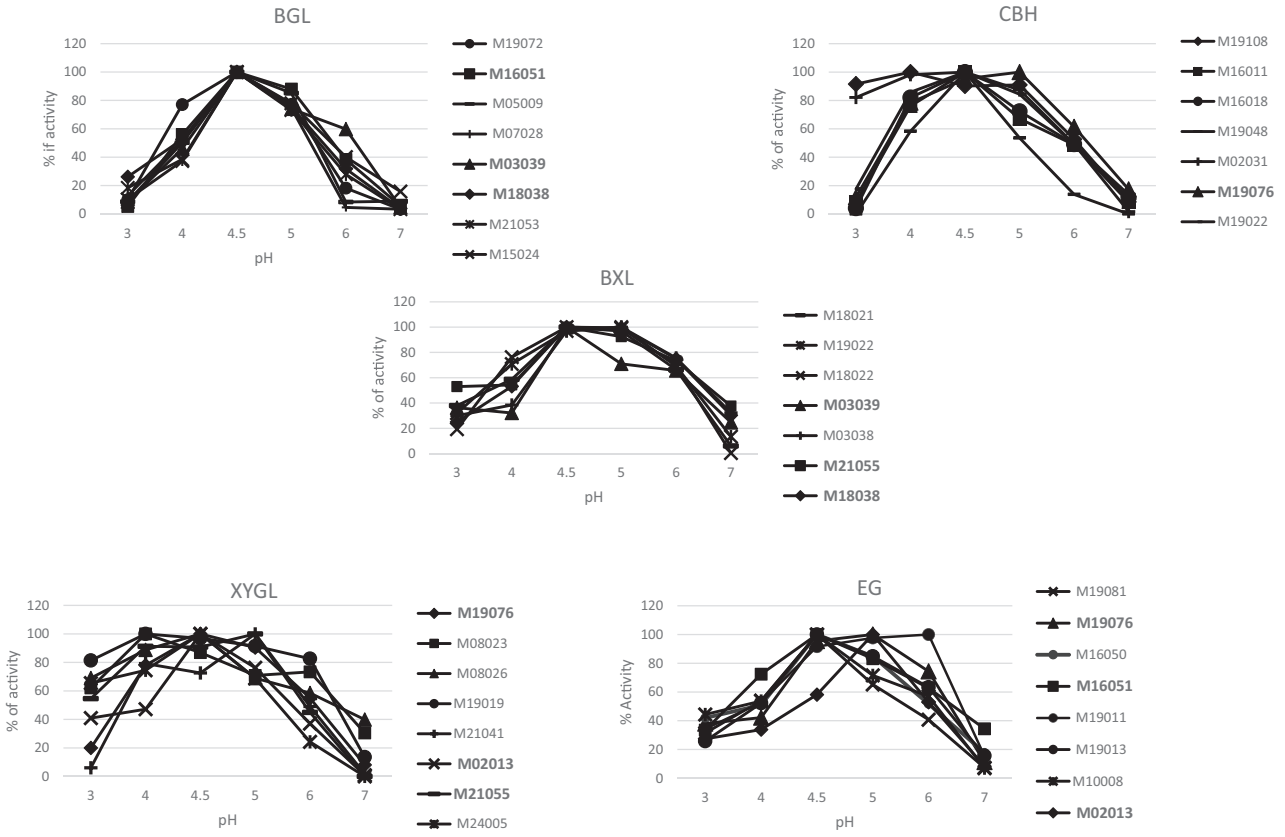

FIGURE 2 Determination of the optimum pH for BGL, CBH, BXL, XYGL and EG activities using commercial substrates. Enzymatic activities are shown as a percentage of the maximum activity (100%). Strains in which two or three activities were assayed are in bold.

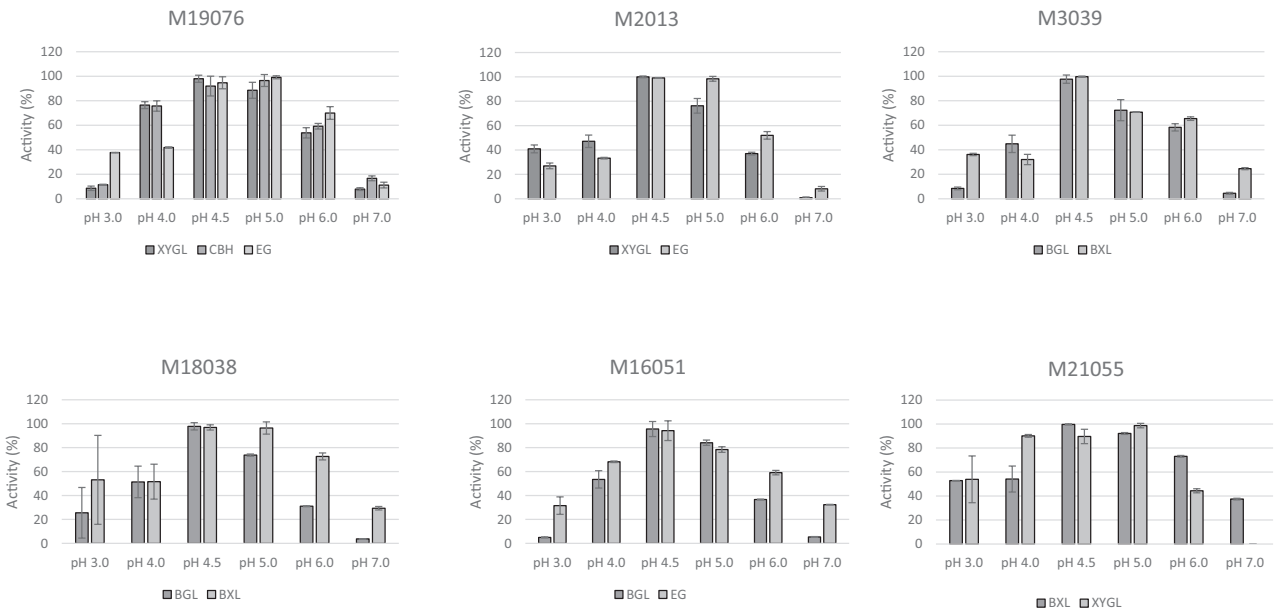

FIGURE 3 Determination of the optimum pH of two or three enzymatic activities in several strains. Enzymatic activities are shown as percentage of the maximum activity (100%). The results are the average of two independent assays.

Cellulolytic activities of *Trichoderma resei*, *Myceliophthora thermophila* and *Talaromyces cellulolyticus*, sources of some of the most commercialized cellulolytic enzymes, are routinely assayed at pH values between 5 and 6 (Inoue et al. 2014). Our results indicated that the optimum pH for most of the lignocellulolytic degradation activities of Rio Tinto fungi, which included some *Trichoderma* and

Talaromyces especies, was below 5, a result that, in general, agrees with the final pH of the growth medium and with the pH from which the environment the fungal strains were isolated. Some acidophilous fungi, mainly belonging to the *Aspergillus* or *Thielavia* genus, have been identified as producers of acidophilus cellulolytic enzymes (Sohail et al. 2009; Cai et al. 2022; Grigorevski-Lima et al. 2009); however, our results fully support that organisms that inhabit Rio Tinto, an unusually acid environment, are a broad source for acidophilus enzymes.

Proteomic shotgun analysis of a series of relevant secretomes

Taking into account the wide range of enzymatic values in the secretomes, we decided to analyse in more depth the secretomes of 16 isolates that presented at least one exceptionally very high activity, for which we analysed the peptides in these secretomes (see Table 2). For this analysis, we performed next-generation shotgun exoproteomics (Armengaud et al., 2012) using a high-resolution tandem mass spectrometer equipped with a high-field Orbitrap analyser able to give a precise partial sequence of the polypeptides. As expected, we found that most of the identified peptides (about 50% of total) in the 16 secretomes matched with sequences of hydrolases deposited in databases (Table 2). We found that, for a number of strains, the presence of high abundance peptides that matched with specific hydrolases correlated with the high enzymatic activity measured in the secretomes; that is, in the three strains with extremely high CBH activity (*Penicillium* sp. ID M19048, *Penicillium janthinellum* IDs M02031 and M10010), we found that 17% or more of the peptides matched cellobiohydrolases: in strain M08014 (without taxonomical identification) which presented high BXL activity, a high proportion of the identified peptides (10%) matched with β-xylosidases sequences deposited in databases, or in strain *P. janthinellum* ID M24005 in which 11% of the peptides mapped with endoglucanases. However, in other isolates, we could not correlate the highest activities with the highest number of peptides in the secretome. This was expected as there might be enzymes with high specific activity against certain substrates or proteins more stable than others. Furthermore, glycosyl- and glycoside hydrolases (GHs) are classified on the basis of sequence homology and therefore we could not ascribe the presence of these enzymes with any specific activity. Two examples in which we can only speculate about the functions of the most abundant proteins are *Trichoderma sp*. ID M18022 (with outlier BXL activity) *and T. asperellum* ID M18038 (with outlier BXL and BGL activities). In both strains, only 0.3% and 2.2% of the peptides matched with sequences identified as β-xylosidades (Table 2); however, 14.8% and 31%, respectively, of the peptides mapped with proteins of the GH3 family (gi|358393086 and gi|927389951, respectively) that have been adscribed to BXL. In addition, in strain M18038, 6.5% of the peptides mapped a protein of the GH3 family protein (gi|927420448) that was related with β-glucosidase activity.

In the two strains with high BGL activity, *P. glabrum* ID M15024 and *Penicillium* sp. ID M16051, around 4% of the identified peptides mapped with annotated BGL proteins. This is relevant in *P. glabrum* ID M15024, because they represented almost 20% of the peptides identified as cellulases/hemicellulases. Searches amongst the most abundant hypothetical proteins did not reveal any relevant cellulolytic enzyme. In strain M16051, the percentage of peptides that mapped with hypothetical BGL increased to 5.6% when we included peptides that mapped with hypothetical proteins or GH3 proteins with homology to known BGLs.

Another general feature in the shotgun proteome analyses of these strains is that we did not identify any peptide that matched the protein sequences identified specifically as xyloglucanases, not even in those identified as possessing high XYGL activity (i.e. *P. janthinellum* ID M24005, M02013 [without taxonomical identification], *Penicillium* sp. ID M19010, *Trichoderma asperellum* M21041, and *Neosartorya glabra* ID M08026). Xyloglucanases, are mainly included in the CAZy database as GH3, GH5, GH9, GH12, GH16, GH44, GH45 and GH74 families (Lombard et al., 2014). In the strain M21041 we identified peptides that mapped with GH3 proteins with the accession numbers gi|927389951 (8.9% of the total peptides in the secretome) and gi|927420448 (2.1%) but these proteins have similarity with putative BXL, and BGL respectively, not with XYGL; we also identified peptides that mapped within proteins of the GH16 but their relative abundance is very low (0.25% of the total petides). In strain M02013 (without taxonomical identification), we also identified peptides with homology with members of the GH3 family, but with putative functions as BXL, BGL or xylanase. We did not identified peptides with homology to any of these GHs in strains M19010, M08026 or M24005.

Overall, the analysis of the peptides in the secretome support that a high proportion of the Rio Tinto fungi possess a well-equipped machinery for cellulose and hemicelluloses hydrolysis. Furthermore, the percentage of hydrolases in the secretomes of the Rio Tinto fungal strains was only slightly lower (50%–70%) to that reported for other industrial strains such as *M. thermophila* (60%–80%) (Bukhtojarov et al., 2004; Gusakov et al., 2007), *Trichoderma reesei* (Druzhinina & Kubicek, 2017), and *Aspergillus* (Bertacchi et al., 2022).

TABLE 2 Strains selected for shotgun-proteomic analysis because they presented at least one exceptionally high activity.

Strain ID	ITS ID	Best activity (outlier)	Most abundant peptides (%)	% Hydrol	% EG	% CBH	% BGL	% BXL	% GHs	% AUX
M24005	*Penicillium janthinellum*	EG XYGL	Glycoside hydrolase family 7 gi|540130099 (18.9%) putative CBH	52	**11.4**	6.4	1.2	0.1	20.9	21.8
M02013	Without id	EG XYGL	Glycoside hydrolase family 7 gi|540130099 (12.6%) putative CBH	51.5	4.0	10.8	0.6	3.0	15.8	18.7
M18038	*Trichoderma asperellum*	BGL BXL	Glycoside hydrolase family 3 gi|927389951 (31%) putative BXL	63.7	1.9	0.5	0.5	2.2	51.0	7.7
M14001	*Talaromyces proteolyticus*	EG BGL	FG-GAP repeat protein gi|748552049 (21.4%) Endoxylanase gi|315259520 (13%)	36.4	2	1.0	2.1	0.4	3.0	14.2
M10008	*Penicillium cremeogriseum*	EG	CBH gi|729650 (20.2%)	57.1	3.2	24.2	0.7	1.6	8.5	12.7
M19011	*Penicillium* sp.	EG	Xylanase G1 gi|662532676 (15.1%) Aspergillopepsin gi|859263183 (11%)	54.2	4.3	11.1	0.7	0.8	3.5	15.4
M19048	*Penicillium* sp	CBH	CBH gi|675824120 (14.4%)	58.9	4.0	**21.5**	6.9	1.2	2.2	20
M02031	*Penicillium janthinellum/savalbardense/ochrochloron/pulvillorum*	CBH	cellobiohydrolase I, partial gi|675824120 (28.9%)	60.0	4.3	**36.2**	3.2	0.0	1.2	11.2
M10010	*Penicillium janthinellum/savalbardense/ochrochloron/pulvillorum*	CBH	cellobiohydrolase I, partial gi|67582412 (12.6%)	55.3	9.0	**17.5**	6.7	0.3	3.8	15.0
M15024	*Penicillim glabrum*	BGL	Hypothetical protein gi|924882275 (1.9%) putative peptidase Alkaline serine protease gi|237700746 (1.5%)	19.3	0.7	0.7	**3.8**	1.0	4.0	7.0
M16051	*Penicillium* sp.	BGL	Concanavalin A-like lectin/glucanases superfamily gi|700493411 (11.6%)	59.7	8.6	4.1	**3.9**	2.9	7.3	28.0
M18022	*Trichoderma* sp.	BXL	Glycoside hydrolase family 3 gi|358393086 (14.8%) putative BXL	63.8	0.5	1.4	1.5	0.3	49.8	10.1
M08014	Without id	BXL	CBH gi|675824120 (12.2%)	56.6	3.7	16.3	1.8	**10.1**	4.9	15.8
M19010	*Penicillium janthinellum*	XYGL	CBH gi|3913798 (6.7%)	50.8	3.8	10.1	1.8	1.2	3.2	30.5
M21041	*Trichoderma asperellum*	XYGL	CBH gi|42741023 (9.6%) Glycoside hydrolase family 7 gi|927397047 (8.4%) putative EG	68.6	6.3	9.8	0.2	1.5	45.6	4.5
M08026	*Neosartorya glabra*	XYGL	CBH gi|849271381 (17.3%)	75.8	10.5	21.6	7.1	5.0	1.5	25.7

Note: Strain ID and identification are given in columns 1 and 2. Column 3 shows the enzymatic activities that were above the established threshold to be considered as outliers. The most abundant peptides in the secretome are shown in column 4 with the percentage over the total of peptides identified shown in parenthesis. The percentage of peptides mapping with carbohydrate-related hydrolases (% Hydrol) are shown in column 5. The last columns show the percentage of the total peptides identified in the secretomes that mapped with different proteins; EG: Endoglucanase; CBH: Cellobiohydrolase; BGL: β-glucosidase; BXL: β-xylosidase; GHs: Glycosyl and glycoside hydrolases; aux: Galacturonases, mannosidases, chitinases, cellulases, exoglucanases, and other auxiliary enzymes. Bold values indicate the cases in which the highest enzymatic activity of the strain correlated with a high percentage of peptides, identified in the secretome,that matched with enzymes in the database with that activity.

Acidophilus enzymes for 2G technology

The above results support the fact that the Rio Tinto fungi are equipped with a set of enzymes able to degrade plant polymers at low pH, and support our hypothesis that the Rio Tinto is a niche of interest for identifing new acidophilic enzymes. Since the set of hydrolases present in the different supernatants was highly variable and the fungi were grown in a single medium, we considered that the most appropriate way to assess the industrial potential of the Rio Tinto river secretomes was to supplement available enzymatic cocktails in order to determine if they could increase the yield of monosaccharide production from pretreated corn stover (PCS) and pretreated sugarcane straw (PSCS) (Table S1). Therefore, to the *M. thermophyla* C1-derived pre-industrial enzymatic cocktail, we added 1 mg protein of a set of supernatants of the acidophilic fungal secretome

from the Rio Tinto strains, and we compared the amount of glucose and xylose released in these mixtures using as a control an equivalent dose of only the pre-industrial cocktail. As a general trend, we observed that a limited number (26 and 37 out of 94 analysed strains with PCS and PSCS respectively) of the Rio Tinto fungal isolates increased the release of glucose per kg of glucan, whilst numerous strains (70% with PCS and 84% with PSCS) were able to release more xylose than the control (Figure 4). The high number of Rio Tinto fungal supernatants that increased the release of xylose most likely reflect the relatively poor hemicellulolytic activity in the *M. thermophila* cocktails. Extracellular proteins secreted by *M. thermophila* were reported to be rich in cellulases (around 60%–80% of total proteins) with cellobiohydrolases representing 40%–55% of these cellulases, and endoglucanases representing approximately 20%–25% (Bukhtojarov et al., 2004; Gusakov et al., 2007). β-glucosidases, β-xylosidases, polysaccharide monooxygenases, xylanases, xyloglucanases, arabinofuranosidases, acetylxylan esterases, and α- and β-galactosidases are the other hydrolytic enzymes found in the secretome. In *M. thermophila*, only four β-xylosidases, belonging to the GH3 and GH43 families (Berka et al., 2011) were identified, and it has been suggested that the addition of hemicellulolytic enzymes to the commercial enzymatic cocktails could improve their performance; directly, by rending more xylose, and indirectly, by removing the hemicellulose coating increasing cellulose accessibility (García-Aparicio et al., 2007; Martín Pérez et al., 2017; Yang & Wyman, 2004).

In our screening of the supernatants of 6 isolates, *Talaromyces amestolkiae* ID M24006, *P. janthinellum* IDs M24005, M12010 and M19033, and strains M16047 and M16050 (without taxonomical identification), led to a release of, at least, 2 g more of glucose per kg of glucan than the control when PSCS was the substrate, and the secretome of 10 isolates (*Talaromyces amestolkiae* ID M24006, *Talaromyces muroii* ID M19108, *Talaromyces* sp. M18009, *Penicillium* sp. IDs M18032, M16043, M16051, M16030, and M16027, *Trichoderma asperellum* M21041, and strain M02033) released more than 2 g kg^{-1} extra of glucose when PCS was the substrate. Around 40% and 35% of the strains tested were able to increase, in at least 2 g kg^{-1}, the amount of xylose released when using PSCS and PCS respectively (Table 3). The release of 2 additional grams of glucose/xylose per kilogram of glucan/xylan is considered of industrial relevance as this represents an increase of 8%–15% in the amount of the initial substrates available for the fermentation and production of ethanol or other products.

The secretomes of strains *Penicillium* sp. ID M16051 and *Trichoderma asperellum* ID M21041 (that released more than 2 g kg^{-1} of xylose from PCS and PSCS and

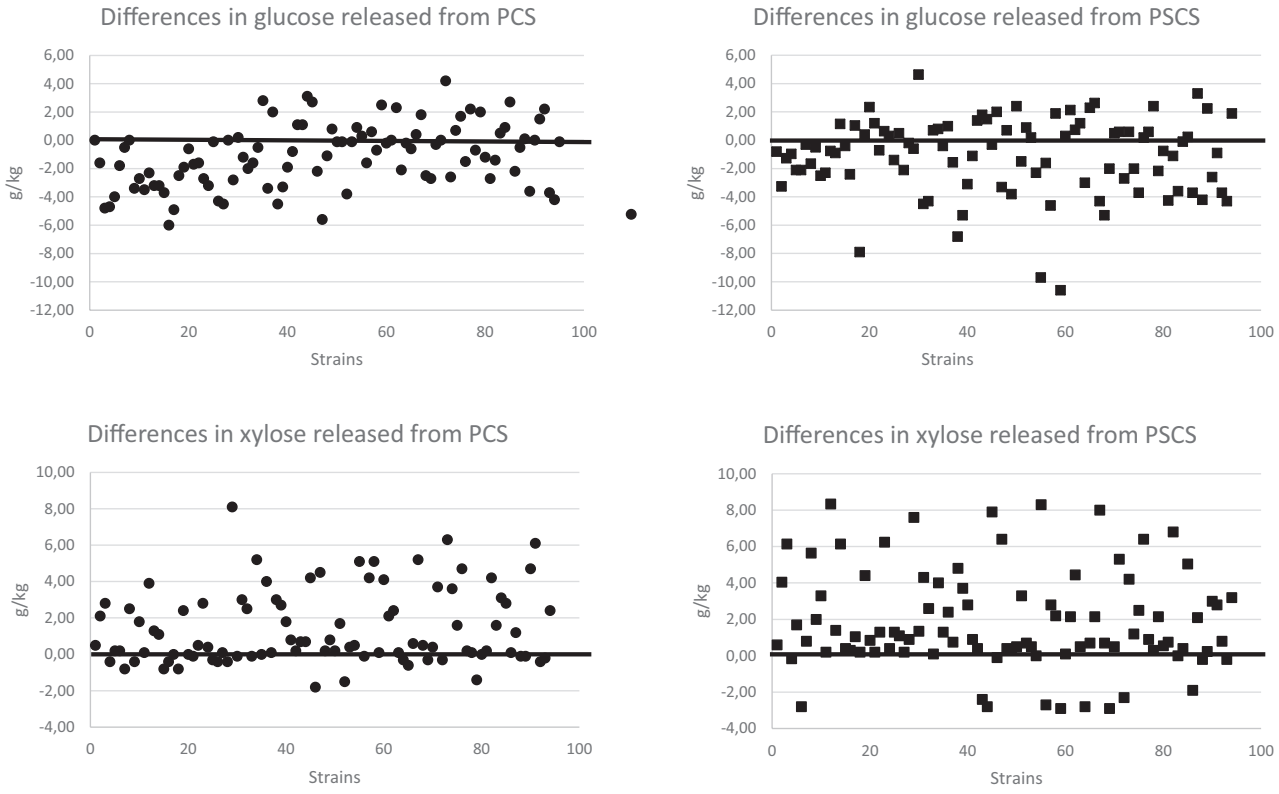

FIGURE 4 Graphical representation of the differences in glucose (up) and xylose (down) released in pretreated corn stover (PCS) and pretreated sugarcane straw (PSCS) in hydrolysis when the secretome of *M. thermophila* C1-derived cocktail was supplemented with 1 mg g^{-1} of Rio Tinto secretomes. The *M. termophila* enzymatic cocktail allowed the release of glucose (≈45 g kg^{-1}) and xylose (≈14.5 g kg^{-1}).

TABLE 3 Glucose and xylose released from PSCS and PCS when secreted enzymes of acidophilus strains were added to a commercial cocktail.

Strain	ITS-based identification	EG	CBH	BGL	BXL	XYGL	PSCS Gluc (g kg^{-1})	PCS Gluc (g kg^{-1})	PSCS Xylose (g kg^{-1})	PCS Xylose (g kg^{-1})
M24006	*Talaromyces amestolkiae/purpureogenus*	P	P	P	-	-	**2.1 ±0.8**	**2.3 ±1.1**	**2.1 ±0.3**	**2.1 ±0.1**
M18032	*Penicillium* sp.	-	P	-	-	-	1.8 ±1.5	**2.5 ±1.9**	**2.2 ±0.6**	**4.9 ±0.5**
M16043	*Penicillium* sp.	P	P	-	-	-	0.8 ±0.8	**2.9 ±2.1**	**4.0 ±0.3**	**5.0 ±0.5**
M16051	*Penicillium* sp.	P	-	O	-	-	1.0 ±0.7	**2.0 ±1.8**	**2.3 ±0.6**	**3.7 ±0.5**
M19108	*Talaromyces muroii*	P	O	P	-	-	0.5 ±0.1	**4.2 ±1.0***	**5.3 ±0.5**	**3.7 ±0.6**
M21041	*Trichoderma asperellum*	P	P	P	-	O	0.2 ±0.6	**2.2 ±1.0**	**6.4 ±0.1**	**4.7 ±0.1**
M18009	*Talaromyces* sp.	P	P	-	-	-	−0.9 ±0.7	**2.2 ±1.9**	**2.7 ±0.2**	**5.9 ±1.3**
M02033	Without identification	P	O	-	-	-	−0.2 ±0.7	**2.7 ±2.2***	0.4 ±0.1	**2.8 ±0.6***
M24005	*Penicillium janthinellum*	O	P	P	-	O	**2.64**	1.8	**2.14**	0.6
M12010	*Penicillium janthinellum/svalbardense/ochrochloron/pulvillorum*	P	P	-	-	-	**2.3 ±1.4**	−1.7 ±0.6	0.9 ±0.3	0.0 ±0.1
M16047	Without identification	P	P	-	-	P	**4.6 ±0.7**	−1.2 ±1.4	1.3 ±0.4	−0.1 ±0.1
M16050	Without identification	P	P	-	-	-	**2.0 ±0.7**	−5.6 ±1.9	−0.1 ±0.2	−2.0 ±0.5
M19033	*Penicillium janthinellum/svalbardense/ochrochloron/pulvillorum*	-	P	-	-	P	**2. 2 ±1.0**	−0.0 ±0.7	0.2 ±0.3	−0.1 ±0.1
M16030	*Penicillium* sp.	-	P	-	-	-	1.4 ±0.7*	**2.7 ±1.9**	−2.9 ±0.2	0.4 ±0.5
M16027	*Penicillium* sp.	P	P	-	-	-	1.8 ±1.2	**3.1 ±1.9**	−2.4 ±0.3	0.4 ±0.5
M02025	*Penicillium dimorphosporum*	-	-	P	-	-	−2.0 ±0.5	−2.7 ±1.5	**2.0 ±0.6**	**2.2 ±1.5**
M01032	*Penicillium daleae/singorense*	P	P	-	-	-	−2.1 ±0.8*	−5.8 ±0.6 *	**2.2 ±0.3***	**3.5 ±0.1***
M21054	*Trichoderma asperellum*	P	O	P	-	O	−1.2 ±0.1	0.0 ±1.0*	**9.0 ±0.2**	**4.1 ±0.4**
M13003	*Penicillium restrictum/meridianum*	-	-	P	-	-	−3.3 ±2.5	−4.8 ±1.8	**4.0 ±0.4**	**2.0 ±0.4**
M15024	*Penicillium glabrum/spinulosum*	-	-	O	-	-	−1.3 ±1.2	−4.6 ±0.6	**6.1 ±0.4**	**2.8 ±0.1**
M12020	*Penicillium armarii/glabrum*	P	-	-	-	-	−1.7 ±0.9	−3.4 ±1.6	**5.6 ±0.3**	**2.5 ±0.2**
M21055	*Trichoderma asperellum/Hypocrea lixii*	-	P	P	P	O	−0.8 ±0.7	−3.2 ±0.6	**8.4 ±0.3**	**3.8 ±0.1**
M18031	*Talaromyces* sp.	-	P	-	-	-	0.4 ±0.7	−0.8 ±0.7	**4.4 ±0.2**	**2.4 ±0.2**
M18026	*Trichoderma harzianum/Hypocrea lixii*	-	-	-	-	-	0.6 ±0.6	−3.2 ±0.6	**6.2 ±0.3**	**2.8 ±0.2**
M18022	*Trichoderma* sp.	-	-	-	O	-	−0.7 ±1.7	0.2 ±1.9	**7.6 ±0.2**	**7.9 ±0.5**
M15009	*Acidiella bohemica*	-	-	-	-	-	−4.5 ±0.5	−2.0 ±1.0*	**4.3 ±0.2**	**2.4 ±0.7**
M18028	*Penicillium* sp.	P	P	-	-	-	−4.3 ±0.1	−1.5 ±1.0*	**2.6 ±0.1**	**2.0 ±0.7**
M15039	*Chaetomium aureum*	P	P	P	-	-	−6.8 ±0.1	−3.2 ±0.8	**4.8 ±0.1**	**3.0 ±0.1**
M15007	*Acidiella bohemica*	-	-	-	-	-	−5.4 ±0.1	−1.8 ±1.3	**3.7 ±0.1**	**2.7 ±0.2**

(Continues)

TABLE 3 (Continued)

Strain	ITS-based identification	EG	CBH	BGL	BXL	XYGL	PSCS Gluc (g kg^{-1})	PCS Gluc (g kg^{-1})	PSCS Xylose (g kg^{-1})	PCS Xylose (g kg^{-1})
M03039	*Trichoderma viride*	-	P	O	O	P	−0.3 ±0.7	−12.2 ±0.8	**7.9 ±0.2**	**4.2 ±0.1**
M12026	*Byssochlamys nivea*/*Paecilomyces saturatus*	P	P	P	P	-	−3.3 ±0.6	−1.0 ±0.8	**6.4 ±0.1**	**4.5 ±0.1**
M18021	*Trichoderma* sp.	-	P	P	O	-	−9.7 ±0.7*	−1.6 ±0.86	**8.0 ±0.5**	**5.1 ±0.22**
M18002	*Talaromyces* sp.	-	-	-	-	-	−4.7 ±0.7	−2.0 ±1.2	**2.8 ±0.1**	**2.30 ±0.3**
M12025	*Penicillium rubens*/*dipodomyicola* /*commune*	-	P	-	-	-	0.7 ±1.3	−2.0 ±0.7*	**4.4 ±0.4**	**2.4 ±0.1**
M14032	*Trichoderma asperellum*	-	P	O	O	-	−4.3 ±0.4	−2.4 ±0.9	**8.0 ±0.3**	**5.2 ±0.2**
M18012	*Talaromyces* sp.	-	P	-	-	-	0.6 ±1.0	0.7 ±1.9	**4.2 ±0.3**	**6.0 ±0.5**
M16042	Without identification	-	P	-	-	-	−1.4 ±0.7	1.7 ±2.1	**1.1 ±0.2**	**3.3 ±0.5**
M19098	*Talaromyces ohiensis*/*Talaromyces ucrainicus*	P	P	-	-	-	−1.1 ±0.1	0.5 ±1.0*	**6.8 ±0.1**	**4.2 ±0.3**
M18013	*Nectria inventa*	-	P	-	-	-	0.2 ±0.6	−2.2 ±0.6	**5.0 ±0.3**	**2.8 ±0.1**
M02039	*Talaromyces* sp.	P	P	-	-	-	−2.7 ±0.8	1.5 ±1.9	**2.9 ±0.1**	**4.5 ±0.5**
M08026	*Neosartorya glabra*/*hiratsukae*	P	P	-	-	O	0.4 ±0.7	−0.1 ±0.14	**3.2 ±0.4**	**2.4 ±0.1**
M08023	*Neosartorya glabra*/*hiratsukae*	P	P	-	-	P	1.8 ±0.4	0.1 ±0.8	**2.1 ±0.3**	1.2 ±0.2
M15027	*Acidiella bohemica*	-	-	-	-	-	−1.2 ±0.7	−3.7 ±0.6	**2.7 ±0.3**	1.0 ±0.1
M09031	*Acidiella bohemica*	-	-	-	-	-	−3.0 ±0.4	−3.8 ±2.5	**3.3 ±03**	1.6 ±0.3
M19097	*Chaetomium aureum*/*Chhispanicum*/*Chaureum*	P	P	O	-	-	−3.7 ±1.1	−1.4 ±0.8	**2.5 ±0.7**	1.6 ±0.1
M19055	*Penicillium janthinellum*/*svalbardense*/*ochrochloron*/*pulvillorum*	P	P	-	-	-	−3.2 ±0.9	−0.7 ±1.0	**2.8 ±0.2**	1.8 ±0.1
M02004	*Penicillium nodositatum*	-	-	O	-	-	−2.5 ±0.3	−3.5 ±1.0*	**3.3 ±0.1**	1.5 ±0.42

Note: Only strains that released more than 2 gkg^{-1} are shown. The letter "P" indicates that the strain was positive in this activity (EG > 4356 Ug^{-1}; CBH > 88 Ug^{-1}; BGL > 1938 Ug^{-1}, BXL > 1622 Ug^{-1} and XYGL > 10,773 Ug^{-1}); the letter "O", indicates that the activity is considered amongst the upper outliers (EG > 20,000 Ug^{-1}; CBH > 500 Ug^{-1}, BGL > 5000 Ug^{-1}, BXL > 6000 Ug^{-1} and XYGL > 20.000 Ug^{-1}). Values higher than 2 are bolded.

more than 2 g kg^{-1} of glucose from PCS), *P. janthinellum* ID M24005 (that released more than 2 g kg^{-1} of glucose and xylose from PSCS), and *P. glabrum* ID M15024, *Trichoderma* sp. ID M18022 and *Neosartorya glabra* ID M08026 (that released more than 2 g kg^{-1} of xylose from PCS and PSCS), had been previously analysed because they presented some good enzymatic activities with commercial substrates (Table 2). However, some of the best performers with natural substrates did not exhibit high activities and their secretomes were not initially analysed. The secretomes from *Chaetomium aureum* ID M15039, strain M16050 (without taxonomical identification), *P. janthinellum* IDs M19021 and M19055, *Trichoderma harzianum* IDs M18026, and, *P. rubens* ID M12025, *Talaromyces ohiensis* ID M19098 and *Neosartorya glabra* ID M08023 and *Penicillium* sp. ID M18028 also presented a high percentage of hydrolases (Table 4). Within this group of secretomes, we were able to identify xyloglucanases, as for instance in the secretome of M15039 (*Chaetomium aureum*) and M16050 (without genus identification), although the percentage of peptides that matched these enzymes was low (0.8 and 0.1% of the total peptides).

Polysaccharide monoxygenases (PMOs) or lytic polysaccharide monoxygenases (LPMO) have also been described as important enzymes in cellulose degradation, particularly crystalline forms. (Beeson et al., 2015; Quinlan et al., 2011; Tokin et al., 2020), (Vermaas et al., 2015). These copper-dependent enzymes attack cellulose through an oxidative mechanism that involves hydroxylation of cellulose at the C1 or C4 carbon, which results in an aldonolactone that is converted into gluconic acid or a 4-keto aldose (Beeson et al., 2015). Therefore, a way to analyse participation of PMOs in cellulose degradation is the production of gluconic acid. We analysed the production of this acid in the hydrolysis of PCS and PSCS with a number of acidophilic strains (Figure 5). We found that M18032, but not M24006, that released more glucose and xylose from PCS and PSCS than the control (Table 3), produced almost double the amount of gluconic acid than the control, whilst the strains M24005, M12010 and M16047, which released more glucose than the control from PSCS, showed a modest increase in gluconic acid released from PSCS when compared with the control. These results suggest the presence of

TABLE 4 Strains selected for shotgun-proteomic analysis because they released more than 2 g kg^{-1} of glucose/xylose from PCS/PSCS.

Strain ID	ITS ID	Activ.	Most abundant peptides (%)	Hydrol	EG	CBH	BGL	BXL	GHs	AUX
M15039 (XYGL)	*Chaetomium aureum*	EG CBH BGL	Glycoside hydrolase family 11 gi|367038111 (18%) putative xylanase	58.3	2.7	5.7	1.4	0	30.1	8.3
M16050	Without ID	EG CBH	Glucoamylase gi|12666724 (12%)	59.2	2.6	10.4	6.2	0.9	5.0	33.8
M18026	*Trichoderma harzianum*	none	Cellobiohydrolase gi|50400675 (15.4%)	54.7	3.2	15.7	3.9	0.1	14.7	17.4
M18028	*Penicillium* sp.	EG CBH	Extracellular beta-glucosidase gi|323473065 (8.5%)	66.4	6.7	18.5	10.1	6.6	7.7	10.3
M19055	*Penicillium janthinellum*	EG CBH	Cellobiohydrolase gi|675824120 (7.0%)	61.5	2.8	9.7	9.6	1.9	2.5	22.4
M12025	*Penicillium rubens*	CBH	Pc21g05110 gi|255953609 (7.1%)	61.8	3.8	3.3	9.2	0.8	0*	37.6
M19098	*Talaromyces ohiensis*	EG CBH	Putative catalase gi|859260569 (4.8%)	32.9	1.6	1	6.2	4.3	4.5	14.6
M08023	*Neosartorya glabra/hiratsukae*	EG CBH XYGL	probable 1,4-beta-D-glucan cellobiohydrolase B gi|849271381 (12.5%)	57.9	8.1	21.6	0.9	2.9	0.5	21.5

Note: In strain M12025, 94% of the identified peptides mapped within proteins of *Penicillium rubens* Wisconsin 54–1255. GHs have been analysed for their putative functions.

Strain ID and identification are given in columns 1 and 2. Column 3 shows the enzymatic activities considered positive (with activity higher than 10% of the highest activity of each enzymatic activity) with commercial substrates. The most abundant peptides in the secretome are shown in column 4 with the percentage over the total of peptides identified shown in parenthesis. The percentage of peptides mapping with and carbohydrate-related hydrolases (Hydrol) are shown in column 5. The last columns show the percentage of the total peptides identified in the secretomes that mapped with different proteins; EG: Endoglucanase; CBH: Cellobiohydrolase; BGL: β-glucosidase; BXL: β-xylosidase; GHs: Glycosyl and glycoside hydrolases; aux: Galacturonases, mannosidases, chitinases, cellulases, exoglucanases, and other auxiliary enzymes.

Abbreviation: nd, Not determined.

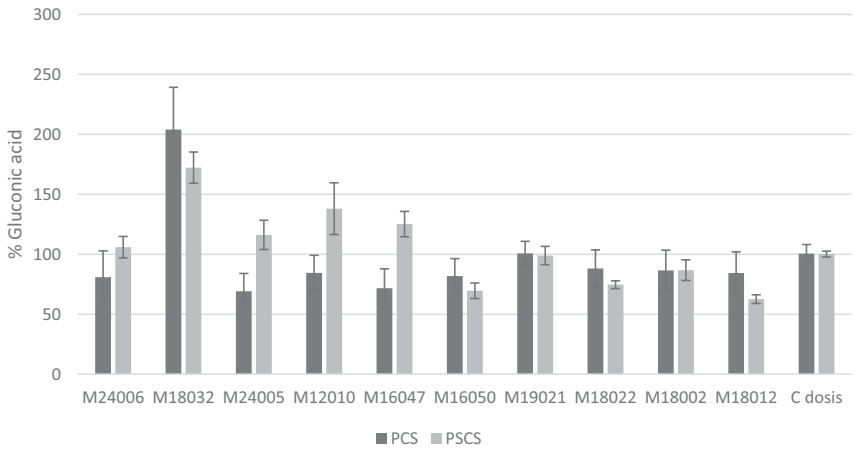

FIGURE 5 Analysis of the liberation of gluconic acid during hydrolysis of PCS and PSCS with a mixture of a pre-industrial cocktail and each of the acidophilic secretomes. The results are expressed in percentages with respect to the dose control (100%).

PMOs in the secretome of these strains. LPMOs have been included in the CAZy database as AA7 family (formerly GH61) Peptides that mapped with putative PMOs have been identified in the secretomes of *Penicillium* sp. ID M12025 and *Chaetomium aureum* ID M15039,

Although the hydrolytic activities measured with non-natural commercial colorimetric substrates do not always correlate with the activity of industrial substrates (Dashtban et al., 2010; Table S1), we cannot forget that the ability to hydrolyse PCS and PSCS is achieved by the appropriate mixture of enzymes. Therefore, the presence of a high level of a particular enzymatic activity, detected by colorimetric substrates, maybe the consequence of a high quantity of this enzyme in the secretome, even resulting in a decrease of the dose of other necessary enzymes for the hydrolysis of complex substrates. However, our results also show that they represent a useful initial screening for analysing the capabilities of fungal populations.

In addition to the cellulases and hemicellulases mentioned above, many other enzymes, such as β-furanosidases, galacturonases, mannases, β-galactosidases, acetyl-xylan esterases and others, are also required for hemicellulose processing (Reyes-Sosa et al., 2017; van den Brink & de Vries, 2011). The Rio Tinto fungi showed a great variety of these enzymes, which in our analysis, have been included under the category of auxiliary enzymes and they represent between 8% and 26% of the total identified peptides in the secretomes (Tables 2 and 4). Furthermore, enzymes such as expansin-like swollenins may play an important role in the enhancement of cellulolytic activities (Andberg et al., 2015; Liu et al., 2015; Saloheimo et al., 2002). We have identified peptides that mapped within swollenins or expansin-like modules in 16 out of the 25 secretomes analysed.

Interestingly, four strains identified as *Acidiella bohemica* presented good results in hydrolysis (increasing xylose released), although their activities with colorimetric substrates were low (Table 3 and Table S1). Currently, *Aspergillus, Trichoderma* and *Mycelliophthora* are the main fungal genera used to produce industrial cellulases (Gupta et al., 2016; Meyer et al., 2015; Singh, 2016). Several *Talaromyces* species have also been described as good cellulase producers (Maeda et al., 2013). *Talaromyces* was described as a sexually reproducing *Penicillium* species (Benjamin, 1955), although several authors have reviewed this classification, including *Penicillium* subgenus *Biverticillium* and *Talaromyces* in a monophyletic clade distinct from the other *Penicillium* sub-genera (Houbraken et al., 2020; Yilmaz et al., 2014). In species belonging to *Chaetomium*, *Neosartorya*, *Mucor*, *Byssochlamys* and *Nectria* cellulases have also been reported (Elsababty, 2015; Moo-Young et al., 1977; Morais et al., 2018; Pardo & Forchiassin, 1999; Xue et al., 2016); however, there is not much information about cellulases from the *Acidiella* species, and our results suggest that these strains may be unexplored micro-organisms for industrial exploitation.

CONCLUSIONS

Lignocellulosic material is one of the most abundant polymers on Earth. The hydrolysis of this raw material at low pH may represent a significant advance for 2G industrial processes; however, the current commercial cellulolytic cocktails perform well at a pH above 5.5. Rio Tinto, with its extraordinary environmental conditions, is an excellent niche for acidophilus microorganisms and we have demonstrated that enzymatic cocktails derived from, at least, 26% of the Rio Tinto collection strains (50% of the strains assayed in hydrolysis) improved the released of sugars from industrial substrates and therefore are suitable to be used for 2G technology.

AUTHOR CONTRIBUTIONS

Abdelali Daddaoua: Formal analysis (supporting); investigation (equal); methodology (equal); writing – review and editing (supporting). **Consolación Alvarez:**

Formal analysis (supporting); investigation (equal); methodology (equal); writing – review and editing (supporting). **Monika Oggerin:** Investigation (supporting); methodology (supporting); writing – review and editing (supporting). **Nuria Rodriguez:** Investigation (supporting); methodology (supporting). **Estrella Duque:** Investigation (supporting); methodology (supporting); writing – review and editing (supporting). **Ricardo Amils:** Conceptualization (supporting); writing – review and editing (equal). **Jean Armengaud:** Conceptualization (supporting); methodology (supporting); writing – review and editing (equal). **Ana Segura:** Conceptualization (supporting); data curation (lead); formal analysis (lead); project administration (lead); writing – original draft (lead). **Juan Luis Ramos:** Conceptualization (lead); funding acquisition (lead); writing – review and editing (lead).

ACKNOWLEDGMENTS

Work in our laboratory has been supported by FEDER Funds from the Ministry of Science and Innovation-Agencia Estatal de Investigación RTI2018-094370-B-I00; and grant from CSIC ref 2021AEP084band REC-EU/VAP-CSIC Interdisciplinary Platform (PTI+) for sustainable Plastics towards a circular economy (PTI-SusPlast+). This study was conceived under the framework of the project Extremozimas para Bioenergía (CDTI EXP-00082937/ITC-20151178) led by Abengoa Research. We thank Angela Tate for improving the use of English in the manuscript. We thank Guylaine Miotello (ProGénomix Platform, CEA-Marcoule) for help with the mass spectrometry analysis.

FUNDING INFORMATION

Work in our laboratory has been supported by FEDER Funds from the Ministry of Science and Innovation-Agencia Estatal de Investigación RTI2018-094370-B-I00; and grant from CSIC ref 2021AEP084band REC-EU/VAP-CSIC Interdisciplinary Platform (PTI+) for sustainable Plastics towards a circular economy (PTI-SusPlast+). This study was conceived under the framework of the project Extremozimas para Bioenergía (CDTI EXP-00082937/ITC-20151178) led by Abengoa Research.

CONFLICT OF INTEREST

None.

DATA AVAILABILITY STATEMENT

Proteomic data set have been send to figshare: https://figshare.com/s/e2bbded0639e63cce2cc.

ORCID

Abdelali Daddaoua https://orcid.org/0000-0002-7348-6393
Consolación Álvarez https://orcid.org/0000-0002-2893-3545
Monika Oggerin https://orcid.org/0000-0002-5130-611X
Nuria Rodriguez https://orcid.org/0000-0003-4109-4851
Estrella Duque https://orcid.org/0000-0002-4857-8974
Ricardo Amils https://orcid.org/0000-0002-7560-1033
Jean Armengaud https://orcid.org/0000-0003-1589-445X
Ana Segura https://orcid.org/0000-0001-7985-5658
Juan Luis Ramos https://orcid.org/0000-0002-8731-7435

REFERENCES

Aguilera, A., Souza-Egipsy, V., Gómez, F. & Amils, R. (2007) Development and structure of eukaryotic biofilms in an extreme acidic environment, Río Tinto (SW, Spain). *Microbial Ecology*, 53, 294–305. Available from: https://doi.org/10.1007/s00248-006-9092-2

Aguilera, A., Zettler, E., Gómez, F., Amaral-Zettler, L., Rodríguez, N. & Amils, R. (2007) Distribution and seasonal variability in the benthic eukaryotic community of Río Tinto (SW, Spain), an acidic, high metal extreme environment. *Systematic and Applied Microbiology*, 30, 531–546. Available from: https://doi.org/10.1016/j.syapm.2007.05.003

Álvarez, C., Reyes-Sosa, F.M. & Díez, B. (2016) Enzymatic hydrolysis of biomass from wood. *Microbial Biotechnology*, 9, 149–156. Available from: https://doi.org/10.1111/1751-7915.12346

Amaral Zettler, L., Gómez, F., Zettler, E., Keenan, B.G., Amils, R., Sogin, M.L. et al. (2002) Eukaryotic diversity in Spain's river of fire. *Nature*, 417, 137. Available from: https://doi.org/10.1038/417137a

Amaral-Zettler, L.A., Zettler, E.R., Theroux, S.M., Palacios, C., Aguilera, A. & Amils, R. (2011) Microbial community structure across the tree of life in the extreme Río Tinto. *The ISME Journal*, 5, 42–50. Available from: https://doi.org/10.1038/ismej.2010.101

Amils, R. (2016) Lessons learned from thirty years of geomicrobiological studies of Río Tinto. *Research in Microbiology*, 167, 539–545. Available from: https://doi.org/10.1016/j.resmic.2016.06.001

Amils, R., González-Toril, E., Aguilera, A., Rodríguez, N., Fernández-Remolar, D., Gómez, F. et al. (2011) From Río Tinto to Mars. *Advances in Applied Microbiology*, 77, 41–70. Available from: https://doi.org/10.1016/B978-0-12-387044-5.00002-9

Andberg, M., Penttilä, M. & Saloheimo, M. (2015) Swollenin from *Trichoderma reesei* exhibits hydrolytic activity against cellulosic substrates with features of both endoglucanases and cellobiohydrolases. *Bioresource Technology*, 181, 105–113. Available from: https://doi.org/10.1016/j.biortech.2015.01.024

Armengaud, J., Christie-Oleza, J.A., Clair, G., Malard, V. & Duport, C. (2012) Exoproteomics: exploring the world around biological systems. *Expert Review of Proteomics*, 9, 561–575. Available from: https://doi.org/10.1586/epr.12.52

Atalah, J., Cáceres-Moreno, P., Espina, G. & Blamey, J.M. (2019) Thermophiles and the applications of their enzymes as new biocatalysts. *Bioresource Technology*, 280, 478–488. Available from: https://doi.org/10.1016/j.biortech.2019.02.008

Bashir, A.K., Wink, L., Duller, S., Schwendner, P., Cockell, C., Rettberg, P. et al. (2021) Taxonomic and functional analyses of intact microbial communities thriving in extreme, astrobiology-relevant, anoxic sites. *Microbiome*, 9, 50. Available from: https://doi.org/10.1186/s40168-020-00989-5

Beeson, W.T., Vu, V.V., Span, E.A., Phillips, C.M. & Marletta, M.A. (2015) Cellulose degradation by polysaccharide monooxygenases. *Annual Review of Biochemistry*, 84, 923–946. Available from: https://doi.org/10.1146/annurev-biochem-060614-034439

Benjamin, C.R. (1955) Ascocarps of *aspergillus* and *Penicillium*. *Mycologia*, 47, 669–687.

Berka, R.M., Grigoriev, I.V., Otillar, R., Salamov, A., Grimwood, J., Reid, I. et al. (2011) Comparative genomic analysis of the

thermophilic biomass-degrading fungi *Myceliophthora thermophila* and *Thielavia terrestris*. *Nature Biotechnology*, 29, 922–927. Available from: https://doi.org/10.1038/nbt.1976

Bertacchi, S., Jayaprakash, P., Morrissey, J.P. & Branduardi, P. (2022) Interdependence between lignocellulosic biomasses, enzymatic hydrolysis and yeast cell factories in biorefineries. *Microbial Biotechnology*, 15, 985–995. Available from: https://doi.org/10.1111/1751-7915.13886

Bukhtojarov, F.E., Ustinov, B.B., Salanovich, T.N., Antonov, A.I., Gusakov, A.V., Okunev, O.N. et al. (2004) Cellulase complex of the fungus *Chrysosporium lucknowense*: isolation and characterization of endoglucanases and cellobiohydrolases. *Biochemistry (Moscow)*, 69, 542–551. Available from: https://doi.org/10.1023/B:BIRY.0000029853.34093.13

Cai, L.-N., Lu, T., Lin, D.-Q. & Yao, S.-J. (2022) Discovery of extremophilic cellobiohydrolases from marine *Aspergillus niger* with computational analysis. *Process Biochemistry*, 115, 118–127. Available from: https://doi.org/10.1016/j.procbio.2022.02.016

Cannio, R., Di Prizito, N., Rossi, M. & Morana, A. (2004) A xylan-degrading strain of *Sulfolobus solfataricus*: isolation and characterization of the xylanase activity. *Extremophiles*, 8, 117–124. Available from: https://doi.org/10.1007/s00792-003-0370-3

Cavicchioli, R., Charlton, T., Ertan, H., Omar, S.M., Siddiqui, K.S. & Williams, T.J. (2011) Biotechnological uses of enzymes from psychrophiles: enzymes from psychrophiles. *Microbial Biotechnology*, 4, 449–460. Available from: https://doi.org/10.1111/j.1751-7915.2011.00258.x

Coker, J.A. (2019) Recent advances in understanding extremophiles. *F1000Research*, 8, 1917. Available from: https://doi.org/10.12688/f1000research.20765.1

Dashtban, M., Maki, M., Leung, K.T., Mao, C. & Qin, W. (2010) Cellulase activities in biomass conversion: measurement methods and comparison. *Critical Reviews in Biotechnology*, 30, 302–309. Available from: https://doi.org/10.3109/07388551.2010.490938

Di Donato, P., Buono, A., Poli, A., Finore, I., Abbamondi, G., Nicolaus, B. et al. (2018) Exploring marine environments for the identification of extremophiles and their enzymes for sustainable and green bioprocesses. *Sustainability*, 11, 149. Available from: https://doi.org/10.3390/su11010149

Druzhinina, I.S. & Kubicek, C.P. (2017) Genetic engineering of *Trichoderma reesei* cellulases and their production. *Microbial Biotechnology*, 10, 1485–1499. Available from: https://doi.org/10.1111/1751-7915.12726

Dumorne, K., Cordova, D.C., Astorga-Elo, M. & Renganathan, P. (2017) Extremozymes: a potential source for industrial applications. *Journal of Microbiology and Biotechnology*, 27, 649–659. Available from: https://doi.org/10.4014/jmb.1611.11006

Eklöf, J.M., Ruda, M.C. & Brumer, H. (2012) Distinguishing xyloglucanase activity in endo-β(1→4)glucanases. *Methods in Enzymology*, 510, 97–120. Available from: https://doi.org/10.1016/B978-0-12-415931-0.00006-9

Elsababty, Z., Ali, A.M. & Houbraken, J. (2015) Cellulolytic and pectinolytic enzymes of some selected heat resistant fungi. *Journal of Microbiology & Experimentation*, 2(2), 66–69. Available from: https://doi.org/10.15406/jmen.2015.02.00042

Emalfarb, M.A., Ben-Bassat, A., Burlingame, R.P., Chernoglazov, V.M., Okounev, O.N., Olson, P.T. et al. (1996) Cellulase compositions and methods of use. United States Patent US5811381A.

García-Aparicio, M.P., Ballesteros, M., Manzanares, P., Ballesteros, I., González, A. & José Negro, M. (2007) Xylanase contribution to the efficiency of cellulose enzymatic hydrolysis of barley straw. *Applied Biochemistry and Biotechnology*, 137, 353–365. Available from: https://doi.org/10.1007/s12010-007-9064-0

Godoy, P., García-Franco, A., Recio, M., Ramos, J. & Duque, E. (2021) Synthesis of aromatic amino acids from 2G lignocellulosic substrates. *Microbial Biotechnology*, 14, 1931–1943. Available from: https://doi.org/10.1111/1751-7915.13844

Grigorevski-Lima, A.L., da Vinha, F.N., Souza, D.T., Bispo, A.S., Bon, E.P., Coelho, R.R. et al. (2009) *Aspergillus fumigatus* thermophilic and acidophilic endoglucanases. *Applied Biochemistry and Biotechnology*, 155, 18–26. Available from: https://doi.org/10.1007/s12010-008-8482-y

Grishutin, S., Gusakov, A., Markov, A., Ustinov, B., Semenova, M. & Sinitsyn, A. (2004) Specific xyloglucanases as a new class of polysaccharide-degrading enzymes. *Biochimica et Biophysica Acta - General Subjects*, 1674, 268–281. Available from: https://doi.org/10.1016/j.bbagen.2004.07.001

Gross, S. & Robbins, E.I. (2000) Acidophilic and acid-tolerant fungi and yeasts. *Hydrobiologia*, 433, 91–109. Available from: https://doi.org/10.1023/A:1004014603333

Gupta, V.K., Steindorff, A.S., de Paula, R.G., Silva-Rocha, R., Mach-Aigner, A.R., Mach, R.L. et al. (2016) The post-genomic era of *Trichoderma reesei*: What's next? *Trends in Biotechnology*, 3, 970–982. Available from: https://doi.org/10.1016/j.tibtech.2016.06.003

Gusakov, A.V., Salanovich, T.N., Antonov, A.I., Ustinov, B.B., Okunev, O.N., Burlingame, R. et al. (2007) Design of highly efficient cellulase mixtures for enzymatic hydrolysis of cellulose. *Biotechnology and Bioengineering*, 97, 1028–1038. Available from: https://doi.org/10.1002/bit.21329

Horikoshi, K. (1998) Barophiles: deep-sea microorganisms adapted to an extreme environment. *Current Opinion in Microbiology*, 1, 291–295. Available from: https://doi.org/10.1016/S1369-5274(98)80032-5

Houbraken, J., Kocsubé, S., Visagie, C.M., Yilmaz, N., Wang, X.-C., Meijer, M. et al. (2020) Classification of *Aspergillus*, *Penicillium*, *Talaromyces* and related genera (Eurotiales): An overview of families, genera, subgenera, sections, series and species. *Studies in Mycology*, 95, 5–169. Available from: https://doi.org/10.1016/j.simyco.2020.05.002

Huang, Y., Krauss, G., Cottaz, S., Driguez, H. & Lipps, G. (2005) A highly acid-stable and thermostable endo-β-glucanase from the thermoacidophilic archaeon *Sulfolobus solfataricus*. *The Biochemical Journal*, 385, 581–588. Available from: https://doi.org/10.1042/BJ20041388

Inoue, A., Yamamoto, M. & Horikoshi, K. (1991) *Pseudomonas putida* which can grow in the presence of toluene. *Applied and Environmental Microbiology*, 57, 1560–1562. Available from: https://doi.org/10.1128/aem.57.5.1560-1562.1991

Inoue, H., Decker, S.R., Taylor, H., Yano, S. & Sawayama, L.E, (2014) Identification and characterization of core cellulolytic enzymes from *Talaromyces cellulolyticus* (formerly *Acremonium cellulolyticus*) critical for hydrolysis of lignocellulosic biomass. *Biotechnology for Biofuels*, 7, 151. Available from: https://doi.org/10.1186/s13068-014-0151-5

Isken, S. & de Bont, J.A.M. (1998) Bacteria tolerant to organic solvents. *Extremophiles*, 2, 229–238. Available from: https://doi.org/10.1007/s007920050065

Jin, M., Gai, Y., Guo, X., Hou, Y. & Zeng, R. (2019) Properties and applications of extremozymes from deep-sea extremophilic microorganisms: a mini review. *Marine Drugs*, 17, 656. Available from: https://doi.org/10.3390/md17120656

Kristensen, J.B., Felby, C. & Jørgensen, H. (2009) Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. *Biotechnology for Biofuels*, 2, 11. Available from: https://doi.org/10.1186/1754-6834-2-11

Kumar, P., Barrett, D.M., Delwiche, M.J. & Stroeve, P. (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial and Engineering Chemistry Research*, 48, 3713–3729. Available from: https://doi.org/10.1021/ie801542g

Kumar, S., Dangi, A.K., Shukla, P., Baishya, D. & Khare, S.K. (2019) Thermozymes: adaptive strategies and tools for their biotechnological applications. *Bioresource Technology*, 278, 372–382. Available from: https://doi.org/10.1016/j.biortech.2019.01.088

Liu, X., Ma, Y. & Zhang, M. (2015) Research advances in expansins and expansion-like proteins involved in lignocellulose degradation. *Biotechnology Letters*, 37, 1541–1551. Available from: https://doi.org/10.1007/s10529-015-1842-0

Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P.M. & Henrissat, B. (2014) The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Research*, 42(D1), D490–D495. Available from: https://doi.org/10.1093/nar/gkt1178

López-Archilla, A.I., González, A.E., Terrón, M.C. & Amils, R. (2004) Ecological study of the fungal populations of the acidic Tinto River in southwestern Spain. *Canadian Journal of Microbiology*, 50, 923–934. Available from: https://doi.org/10.1139/w04-089

López-Archilla, A.I., Marin, I. & Amils, R. (2001) Microbial community composition and ecology of an acidic aquatic environment: the Tinto River, Spain. *Microbial Ecology*, 41, 20–35. Available from: https://doi.org/10.1007/s002480000044

Lozano, C., Kielbasa, M., Gaillard, J.-C., Miotello, G., Pible, O. & Armengaud, J. (2022) Identification and characterization of marine microorganisms by tandem mass spectrometry proteotyping. *Microorganisms*, 10, 719. Available from: https://doi.org/10.3390/microorganisms10040719

Maeda, R.N., Barcelos, C.A., Anna, L.M.M.S. & Pereira, N. (2013) Cellulase production by *Penicillium funiculosum* and its application in the hydrolysis of sugar cane bagasse for second generation ethanol production by fed batch operation. *Journal of Biotechnology*, 163, 38–44. Available from: https://doi.org/10.1016/j.jbiotec.2012.10.014

Mamo, G. (2019) Alkaline active hemicellulases. In: Mamo, G. & Mattiasson, B. (Eds.) *Alkaliphiles in biotechnology. Advances in biochemical engineering/biotechnology*, Vol. 172. Cham: Springer.

Martín Pérez, L., Benítez Casanova, L., Moreno Pérez, A.J., Pérez Gómez, D., Gavaldá Martín, S., Ledesma-García, L. et al. (2017) Coupling the pretreatment and hydrolysis of lignocellulosic biomass by the expression of beta-xylosidases. *Biotechnology and Bioengineering*, 114, 2497–2506. Available from: https://doi.org/10.1002/bit.26386

Meyer, V., Fiedler, M., Nitsche, B. & King, R. (2015) The cell factory aspergillus enters the big data era: opportunities and challenges for optimising product formation. In: Krull, R. & Bley, T. (Eds.) *Filaments in bioprocesses. Advances in biochemical engineering/biotechnology*, Vol. 149. Cham: Springer.

Moo-Young, M., Chahal, D.S., Swan, J.E. & Robinson, C.W. (1977) SCP production by *Chaetomium cellulolyticum*, a new thermotolerant cellulolytic fungus. *Biotechnology and Bioengineering*, 19, 527–538. Available from: https://doi.org/10.1002/bit.260190407

Morais, T.P.d., Barbosa, P.M.G., Garcia, N.F.L., Rosa-Garzon, N.G.d., Fonseca, G.G., Paz, M.F.d. et al. (2018) Catalytic and thermodynamic properties of β-glucosidases produced by *Lichtheimia corymbifera* and *Byssochlamys spectabilis*. *Preparative Biochemistry & Biotechnology*, 48, 777–786. Available from: https://doi.org/10.1080/10826068.2018.1509083

Nguyen, Q.A., Tucker, M.P., Keller, F.A. & Eddy, F.P. (2000) Two-stage dilute-acid pretreatment of softwoods. *Applied Biochemistry and Biotechnology*, 84-86, 561–576. Available from: https://doi.org/10.1385/ABAB:84-86:1-9:561

Pardo, A.G. & Forchiassin, F. (1999) Influence of temperature and pH on cellulase activity and stability in *Nectria catalinensis*. *Revista Argentina de Microbiología*, 31, 31–35.

Pennisi, E. (1997) Biotechnology: In industry, extremophiles begin to make their mark. *Science*, 276(5313), 705–706. Available from: https://doi.org/10.1126/science.276.5313.705

Quinlan, R.J., Sweeney, M.D., Lo Leggio, L., Otten, H., Poulsen, J.-C.N., Johansen, K.S. et al. (2011) Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 15079–15084. Available from: https://doi.org/10.1073/pnas.1105776108

Raddadi, N., Cherif, A., Daffonchio, D., Neifar, M. & Fava, F. (2015) Biotechnological applications of extremophiles, extremozymes and extremolytes. *Applied Microbiology and Biotechnology*, 99, 7907–7913. Available from: https://doi.org/10.1007/s00253-015-6874-9

Ramos, J.L., Duque, E., Gallegos, M.-T., Godoy, P., Ramos-González, M.I., Rojas, A. et al. (2002) Mechanisms of solvent tolerance in gram-negative nacteria. *Annual Review of Microbiology*, 56, 743–768. Available from: https://doi.org/10.1146/annurev.micro.56.012302.161038

Reyes-Sosa, F.M., López Morales, M., Platero Gómez, A.I., Valbuena Crespo, N., Sánchez Zamorano, L., Rocha-Martín, J. et al. (2017) Management of enzyme diversity in high-performance cellulolytic cocktails. *Biotechnology for Biofuels*, 10, 156. Available from: https://doi.org/10.1186/s13068-017-0845-6

Rocha-Martín, J., Martinez-Bernal, C., Pérez-Cobas, Y., Reyes-Sosa, F.M. & García, B.D. (2017) Additives enhancing enzymatic hydrolysis of lignocellulosic biomass. *Bioresource Technology*, 244, 48–56. Available from: https://doi.org/10.1016/j.biortech.2017.06.132

Rubiano-Labrador, C., Bland, C., Miotello, G., Armengaud, J. & Baena, S. (2015) Salt stress induced changes in the exoproteome of the halotolerant bacterium *Tistlia consotensis* deciphered by proteogenomics. *PLoS One*, 10, e0135065. Available from: https://doi.org/10.1371/journal.pone.0135065

Rubiano-Labrador, C., Bland, C., Miotello, G., Guérin, P., Pible, O., Baena, S. et al. (2014) Proteogenomic insights into salt tolerance by a halotolerant alpha-proteobacterium isolated from an Andean saline spring. *Journal of Proteomics*, 97, 36–47. Available from: https://doi.org/10.1016/j.jprot.2013.05.020

Saloheimo, M., Paloheimo, M., Hakola, S., Pere, J., Swanson, B., Nyyssönen, E. et al. (2002) Swollenin, a *Trichoderma reesei* protein with sequence similarity to the plant expansins, exhibits disruption activity on cellulosic materials: a fungal expansin homologue. *European Journal of Biochemistry*, 269, 4202–4211. Available from: https://doi.org/10.1046/j.1432-1033.2002.03095.x

Sharma, A., Kawarabayasi, Y. & Satyanarayana, T. (2012) Acidophilic bacteria and archaea: acid stable biocatalysts and their potential applications. *Extremophiles*, 16, 1–19. Available from: https://doi.org/10.1007/s00792-011-0402-3

Sharma, A., Tewari, R., Rana, S.S., Soni, R. & Soni, S.K. (2016) Cellulases: classification, methods of determination and industrial applications. *Applied Biochemistry and Biotechnology*, 179, 1346–1380. Available from: https://doi.org/10.1007/s12010-016-2070-3

Sohail, M., Siddiqi, R., Ahmad, A. & Khan, S.A. (2009) Cellulase production from *Aspergillus niger* MS82: effect of temperature and pH. *New Biotechnology*, 25, 437–441. Available from: https://doi.org/10.1016/j.nbt.2009.02.002

Singh, B. (2016) *Myceliophthora thermophila* syn. *Sporotrichum thermophile*: a thermophilic mould of biotechnological potential. *Critical Reviews in Biotechnology*, 36, 59–69. Available from: https://doi.org/10.3109/07388551.2014.923985

Thanh, V.N., Thuy, N.T., Huong, H.T.T., Hien, D.D., Hang, D.T.M., Anh, D.T.K. et al. (2019) Surveying of acid-tolerant thermophilic lignocellulolytic fungi in Vietnam reveals surprisingly high genetic diversity. *Scientific Reports*, 9, 3674. Available from: https://doi.org/10.1038/s41598-019-40213-5

Tokin, R., Ipsen, J.Ø., Westh, P. & Johansen, K.S. (2020) The synergy between LPMOs and cellulases in enzymatic saccharification of cellulose is both enzyme- and substrate-dependent. *Biotechnology Letters*, 42, 1975–1984. Available from: https://doi.org/10.1007/s10529-020-02922-0

Valdivia, M., Galan, J.L., Laffarga, J. & Ramos, J. (2016) Biofuels 2020: biorefineries based on lignocellulosic materials. *Microbial Biotechnology*, 9, 585–594. Available from: https://doi.org/10.1111/1751-7915.12387

van den Brink, J. & de Vries, R.P. (2011) Fungal enzyme sets for plant polysaccharide degradation. *Applied Microbiology and*

Biotechnology, 91, 1477–1492. Available from: https://doi.org/10.1007/s00253-011-3473-2

Vermaas, J.V., Crowley, M.F., Beckham, G.T. & Payne, C.M. (2015) Effects of lytic polysaccharide monooxygenase oxidation on cellulose structure and binding of oxidized cellulose oligomers to cellulases. *The Journal of Physical Chemistry. B*, 119, 6129–6143. Available from: https://doi.org/10.1021/acs.jpcb.5b00778

Xue, X., Wu, Y., Qin, X., Ma, R., Luo, H., Su, X. et al. (2016) Revisiting overexpression of a heterologous β-glucosidase in *Trichoderma reesei*: fusion expression of the *Neosartorya fischeri* Bgl3A to cbh1 enhances the overall as well as individual cellulase activities. *Microbial Cell Factories*, 15, 122. Available from: https://doi.org/10.1186/s12934-016-0520-9

Yang, B. & Wyman, C.E. (2004) Effect of xylan and lignin removal by batch and flowthrough pretreatment on the enzymatic digestibility of corn Stover cellulose. *Biotechnology and Bioengineering*, 86, 88–98. Available from: https://doi.org/10.1002/bit.20043

Yilmaz, N., Visagie, C.M., Houbraken, J., Frisvad, J.C. & Samson, R.A. (2014) Polyphasic taxonomy of the genus *Talaromyces*. *Studies in Mycology*, 78, 175–341. Available from: https://doi.org/10.1016/j.simyco.2014.08.001

Zhang, Y.-H.P. (2008) Reviving the carbohydrate economy via multi-product lignocellulose biorefineries. *Journal of Industrial Microbiology & Biotechnology*, 35, 367–375. Available from: https://doi.org/10.1007/s10295-007-0293-6

Zimbardi, A., Sehn, C., Meleiro, L., Souza, F., Masui, D., Nozawa, M. et al. (2013) Optimization of β-glucosidase, β-xylosidase and xylanase production by *Colletotrichum graminicola* under solid-state fermentation and application in raw sugarcane trash saccharification. *International Journal of Molecular Sciences*, 14, 2875–2902. Available from: https://doi.org/10.3390/ijms14022875

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Daddaoua, A., Álvarez, C., Oggerin, M., Rodriguez, N., Duque, E., Amils, R. et al. (2023) Rio Tinto as a niche for acidophilus enzymes of industrial relevance. *Microbial Biotechnology*, 16, 1069–1086. Available from: https://doi.org/10.1111/1751-7915.14192