Penicillium chrysogenum as a fungal factory for feruloyl esterases

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Abstract

Plant biomass is a promising substrate for biorefinery, as well as a source of bioactive compounds, platform chemicals, and precursors with multiple industrial applications. These applications depend on the hydrolysis of its recalcitrant structure. However, the effective biological degradation of plant cell walls requires several enzymatic groups acting synergistically, and novel enzymes are needed in order to achieve profitable industrial hydrolysis processes. In the present work, a feruloyl esterase (FAE) activity screening of *Penicillium* spp. strains revealed a promising candidate (*Penicillium rubens* Wisconsin 54–1255; previously *Penicillium chrysogenum*), where two FAE-ORFs were identified and subsequently overexpressed. Enzyme extracts were analyzed, confirming the presence of FAE activity in the respective gene products (PrFaeA and PrFaeB). PrFaeB-enriched enzyme extracts were used to determine the FAE activity optima (pH 5.0 and 50–55 °C) and perform proteome analysis by means of MALDI-TOF/TOF mass spectrometry. The studies were completed with the determination of other lignocellulolytic activities, an untargeted metabolite analysis, and upscaled FAE production in stirred

tank reactors. The findings described in this work present *P. rubens* as a promising lignocellulolytic enzyme producer.

Key Points

• Two *Penicillium rubens* ORFs were first confirmed to have feruloyl esterase activity.

• Overexpression of the ORFs produced a novel *P. rubens* strain with improved activity.

• The first in-depth proteomic study of a *P. rubens* lignocellulolytic extract is shown.

Keywords : Fungi,·*Penicillium rubens, Penicillium chrysogenum*, Lignocellulose, Feruloyl esterase, Extracellular proteome

Introduction

Approximately 500–1000 million years ago, fungi diverged from a common ancestor with animals. This event marked the beginning of evolutionary processes that have enabled fungi to colonize and thrive in markedly different environments, becoming one of the major eukaryotic lineages (Muggia and Grube 2018; Stajich et al. 2009). Although some unicellular forms exist, fungi are typically characterized by a filamentous vegetative form and absorptive, heterotrophic nutrition (McLaughlin et al. 2009). Fungi play important roles in practically all known ecosystems. Their symbiotic relationships are pivotal in the expansion and colonization of new habitats. Saprotrophic fungi are key players in nutrient cycles, whereas pathogenic fungi affect other fungi, bacteria, plants, and animals, including human beings (Lutzoni et al. 2004). Besides, fungi have a negative impact on human societies and economies, being responsible for economic losses in fields such as health and agriculture (Bhatnagar et al. 2002; Cleveland et al. 2003; De Lucca 2007; Mitchell et al. 2016; Wagacha and Muthomi 2008). Fortunately, their positive impact is also noteworthy, since they are some of the most relevant producers of pharmaceutical secondary metabolites such as antibiotics, only surpassed by Actinobacteria (Barreiro et al. 2012a). This is an important trait, given the current threat to global health caused by microbial antibiotic resistance (Barreiro and Barredo 2021). Moreover, fungi produce useful primary metabolites, such as organic acids and fatty acids, and they have applications in the food industry (e.g., bread or fermented beverages) (Ward 2012).

Due to their powerful enzymatic machinery, fungi are interesting hosts for the production of heterologous and endogenous enzymes (Gifre et al. 2017; Mäkelä et al. 2014). The global industrial enzyme market is constantly growing and obtained an estimated value of 8.2 billion USD in 2015 (Kumar et al. 2014; Manisha 2017). The website Marketresearch.com (https://www.marketresearch.com/) valued the global enzymes market in 2019 at 8.6 billion USD, and it could reach 14.5 billion USD by 2027. Nowadays, more than 700 commercial products in over 40 industry sectors (e.g., agriculture,

food) are commercially available. Among them, applications in the food and beverage industries stand out, reaching up to 2.0 billion USD in 2016 (Arnau et al. 2020; Chapman et al. 2018; Patel et al. 2016). Particularly, fungal lignocellulolytic enzymes are capable of degrading lignocellulose, which is the main component of dry plant biomass and the most abundant renewable organic resource on the planet (Jäger and Büchs 2012). Around 140 billion tons of biomass are produced from the global agricultural sector every year that are considered as a by-product or waste (Tripathi et al. 2019; Zuin and Ramin 2018). The hydrolysis of lignocellulose releases fermentable sugars, platform chemicals, precursors, biopolymers, and bioactive molecules with industrial applications (Guerriero et al. 2016). Among the latter, hydroxycinnamic acids, such as caffeic, p-coumaric, sinapic, and ferulic acid, are of special interest. Ferulic acid is a bioactive compound with multiple properties, namely, antiinflammatory, antihepatotoxic, antioxidant (protecting the skin against UV-mediated oxidative damage), anticarcinogenic, antimicrobial, antiviral, and antithrombotic. Besides, it can be used as a natural food preservative and as a precursor in the production of industrially interesting substances, such as the flavoring agent vanillin (Mathew and Abraham 2004). However, to release these compounds, plant cell wall hydrolysis is required, a process that is complex due to the recalcitrance of this structure (Lynd et al. 2002). To achieve this hydrolysis, physicochemical pretreatments (acid, alkali, hydrothermal, oxidizing agents) and biological treatments (lignocellulolytic enzymes) are used. The bio-logical treatments need milder reaction conditions, causing fewer side reactions, being safer, less energy consuming, and environmentally friendlier (Pérez et al. 2002). Among lignocellulolytic enzymes, feruloyl esterases (FAEs, EC 3.1.1.73) are a subclass of the carboxylic acid esterases (EC 3.1.1) that cleave ester bonds between hydroxycinnamic acids and carbohydrates of the plant cell wall (Fazary and Ju 2007). This rupture affects the stability of plant cell walls and, thus, assists in their deconstruction (Faulds 2010). Taking into account recent studies on the prevalence of FAEs in fungal genomes, there are still many fungal FAEs to be discovered and characterized (Dilokpimol et al. 2016). Considering the multiple applications of FAEs in industries such as textile, pulp and paper, pharmaceutical, food, feed, and biofuels (Fazary and Ju 2008), further research to find novel FAEs is needed.

Penicillium rubens (formerly, *Penicillium chrysogenum*) (Houbraken et al. 2011; Thom 1910) is a fungal species belonging to phylum Ascomycota, subphylum *Pezizomycotina*, order *Eurotiales*. *Penicillium* species are widely distributed in nature, occurring in soils, decaying vegetable matter, animal skin, food products, etc. (Houbraken et al. 2011). They act as decomposers of organic materials and can synthesize a wide range of secondary metabolites, including mycotoxins and antibiotics, such as penicillin, produced by *P. rubens* (Fierro et al. 2022; Visagie et al. 2014). Given the economic and social significance of these secondary metabolites, different omics analyses have been applied to unravel the metabolic machinery of *P. rubens* (García-Estrada et al. 2020). The full genome of one of the classical antibiotic-producing strains (*P. rubens* Wisconsin 54–1255) has been published (van den Berg et al. 2008) as well as studies on transcriptomes (Terfehr et al. 2017; van den Berg et al. 2008), intracellular, extracellular, and microbody proteomes (Domínguez-Santos et al. 2017; Jami et al. 2010a,

b, 2018; Kiel et al. 2009). Their strong degradative enzymatic machinery has made it a suitable genus for the screening of novel lignocellulolytic enzymes, such as xylanases, cellulases, or feruloyl esterases (Castanares et al. 1992; Chávez et al. 2006; Donaghy et al. 1998; Kroon et al. 2000; Phuengmaung et al. 2019; Terrasan et al. 2016).

The present work aimed to discover, overexpress, and characterize novel FAEs with industrial applications, produced by *P. rubens* strains. Moreover, proteomic techniques and metabolite analyses were carried out to study the extracellular proteome of this fungus when cultured under FAE production conditions, using the lignocellulosic substrate sugar beet pulp (SBP) as a carbon source and inducer of the production of lignocellulolytic enzymes.

Materials and methods

Substrates and reagents

Ferulic acid, p-coumaric acid, xylan from birchwood, 3,5-dinitrosalicylic acid (DNS), ethyl 4-hydroxy-3-meth-oxy-cinnamate (ethyl ferulate, EF), 4-nitrophenol, 4-nitro-phenyl β-D-glucopyranoside (pNPG), 4-nitrophenyl β-D-cellobioside (pNPC), 4-nitrophenyl β-D-xylopyranoside (pNPX), D-(+)-gluconic acid δ-lactone, and carboxym-ethylcellulose sodium salt (CMC) were purchased from Sigma-Aldrich (Burlington, MA, USA). Methyl 4-hydroxy-3-methoxy-cinnamate (methyl ferulate, MFA) was acquired from Alfa Aesar (Haverhill, MA, USA).

The natural lignocellulosic substrate processed milling oat fiber powder (OAT) was purchased from a local provider. SBP as pellets was kindly provided by Sociedad Cooperativa General Agropecuaria ACOR (Valladolid, Spain). Pellets were ground before use, obtaining thick dust with a diameter of particles of 1–5 mm.

Strains and cultivation

Escherichia coli DH5α (Hanahan 1983) was grown in Luria–Bertani medium (Miller 1972) supplemented with 100 μg/mL of ampicillin and employed for plasmid maintenance and propagation.

Five penicillin-producing fungal strains were used: *P. rubens* NRRL 1951, *P. rubens* Wisconsin 54–1255 (ATCC 28089), *P. rubens* AS-P-78, *P. rubens* AS-P-99, and *Penicillium notatum* NRRL-1249. Sporulation of mycelia was achieved by growth on Power medium (Casqueiro et al. 1999) at 28 °C for 5 days. To initiate seed cultures, spores were scraped, inoculated in 500-mL non-baffled flasks containing 100-mL PMMY defined medium adjusted to pH 6.0 (Jami et al. 2010a) and grown at 28 °C, 250 rpm for 24 h (seed culture). Submerged cultures were initiated after inoculating 10% (v/v) seed culture in 500-mL non-baffled flasks with 100 mL of SBP medium (M-SBP; 2.0-g/L NH_4NO_3; 1.0-g/L K_2HPO_4; 0.5-g/L $MgSO_4$ $7H_20$; 0.5-g/L KCl; 1.0-g/L soy peptone; 1.0-g/L D-glucose; 18.7-mg/L $FeSO_4$, pH 5.0; 20-g/L SBP) or oat medium (M-OAT; same composition as M-SBP, but 20 g/L OAT, instead of SBP). Cultures were incubated at 28 °C, 250 rpm, for up to 1 week. Three independent biological replicates were used per strain. Bioreactor cultivations of the fungal strains were carried out

on M-SBP medium at 28 °C and 0.8 vvm (vessel volume per minute) air flow rate, using 10% (v/v) of submerged cultures in PMMY medium grown for 24 h as the seed culture. The shaking rate was adjusted so the dissolved oxygen values remained above 60% throughout the cultivation. pH, sterile air flow rate, dissolved oxygen, shaking rate, and temperature were monitored.

Molecular biology techniques

gDNA extraction and Southern blotting

Genomic DNA (gDNA) from *P. rubens* Wisconsin 54–1255 was extracted from mycelium grown for 24 h, at 25 ℃ and 250 rpm, in PMMY medium, following the protocol described by Casqueiro and co-workers (Casqueiro et al. 1999). gDNA samples were digested with the endonucleases *Hind*III and *Apa*I (New England Biolabs) and separated using 0.7% (w/v) agarose gel electrophoresis. Gels were subsequently blotted onto nitrocellulose uncharged Hybond-N membranes (Amersham, GE Healthcare Biosciences) and UV-fixed. Membranes were hybridized with specific DNA-based digoxigenin-labeled probes, corresponding to the *faeA* and *faeB* gene sequences labeled using the DIG-High Prime kit (Roche). For detection, membranes were incubated with alkaline phosphatase and an appropriate substrate (CDP-Star; Roche). Hybridization chemiluminescent signals were recorded on X-ray films and developed employing Kodak chemicals.

Plasmid design and construction

Plasmids **p*Sfae*A** and **p*Sfae*B** were constructed to overexpress PrFaeA and PrFaeB, respectively, in *P. rubens* Wisconsin 54–1255. The *faeA* gene was amplified from *P. rubens* Wisconsin 54–1255 gDNA, using Phusion High-Fidelity DNA Polymerase (New England Biolabs) and oligonucleotides *faeA*-F and *faeA*-R (Table 1). For the *faeB* gene, *faeB*-F, and *faeB*-R oligonucleotides were used (Table 1). These primers include the *Bgl*II and *Stu*I restriction sites. The PCR products (933 bp for *fae*A and 1643 bp for *fae*B) were cloned in the *Bgl*II-*Stu*I site of plasmid pIBRC43BglII (Kosalková et al. 2009), controlled by the strong *Aspergillus awamori gdh* gene promoter and ended by the *Saccharomyces cerevisiae cyc1* transcriptional terminator, generating plasmids p*Sfae*A and p*Sfae*B, respectively.

Cloning and expression

P. rubens protoplasts were transformed according to a method described previously (Ullán et al. 2007). The parent strain *P. rubens* Wisconsin 54–1255 was cotransformed with either p*Sfae*A or p*Sfae*B, plus plasmid pJL43 (Gutiérrez et al. 1991), the latter carrying a phleomycin resistance cassette. Selection was carried out in a solid Czapek minimal medium supplemented with sorbitol (1 M) as an osmotic stabilizer and phleomycin (35 μg/mL) as the selection agent.

Quantitative PCR (RT-qPCR)

Total RNA was extracted from the parent strain *P. rubens* Wisconsin 54–1255 and the two transformants B13 and A78, at 24, 48, and 72 h of cultivation in M-SBP medium, with the RNeasy Mini Kit (Qiagen).

Total RNA was treated with RQ1 RNase-Free DNase (Promega) and RNase-free DNase (Qiagen), following the manufacturer's protocols. Complementary DNA (cDNA) was obtained using the SuperScript III One-Step RT-PCR with Platinum *Taq* DNA polymerase (Invitrogen), with 2.5 µg of DNase-treated RNA as a template. Amplifications were carried out using 20-µL SYBR Premix Ex Taq (TaKaRa), according to the following pro-gram: 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 34 s at 62 °C. Two biological replicates and three technical repli-cates were used. Oligonucleotides (Table 1) were designed to produce PCR products close to the mRNA 5' end, by means of the Primer3 software (Rozen and Skaletsky 2000). Green fluorescence from SYBR was normalized with the passive reference dye ROX (carboxy-X-rhodamine). The resulting data were processed according to the Pfaffl method (Pfaffl 2001), normalizing the results using the constitutive *act*A gene as a reference. Amplification efficiency (E) was calculated using triplicates of six standard 1:5 serial dilutions of gDNA. E value was determined by the equation E = -1 + 10(-1/slope) (Rasmussen 2001), with a coefficient of correlation R2 > 0,99.

FAE activity screening

FAE activity was evaluated following the method described by Donaghy and McKay (Donaghy and McKay 1994) modified as follows. Selected fungal strains were grown on Power medium plates for 5 days at 28 °C, until sporulation. Triplicate plugs (10-mm diameter) from these plates were inserted in Petri dishes containing a 1% (w/v) Bacto agar (BD) sterile solution (50 °C) supplemented with 1.5-mL of filtered EF diluted in ethanol (0.05 g/mL ethanol). EF plates containing the fungal plugs were incubated for 12 days at 28 °C. Controls were set up on Bacto agar plates without EF. After incubation, plates were stained with a 0.04% (w/v) bromocresol green solution, for 15 min, and washed twice for 5 min using NaCl 0.9% (w/v). FAE activity was identified as a yellow halo against a blue background, due to the pH decrease produced by the release of ferulic acid.

Protein recovery from cultures

Secreted protein samples were obtained from cultivation broths recovered by filtration through Nytal 45-µm pore membranes. The flow-through was centrifuged twice at 3,220 × g, 30 min at 4 °C, and the remaining pellet (fungal biomass) was dried at 80 °C, for 7 days, to determine dry weight. The resulting supernatant was cleared using 0.22-µm pore Stericup™ filtering devices (Millipore®). Soluble protein in the filtrate was recovered by precipitation with ammonium sulfate at 100% saturation at 4 °C, according to the EnCorBio ammonium sulfate calculator (Wingfield 1998). The ammonium sulfate saturated solution was stirred gently for at least 12 h. After precipitation, proteins were pelleted by centrifugation at 10,400 × g, 4 °C, 1 h. Pellets were dried for 5 min and resuspended in acetate buffer 0.02 N pH 5.0 [100-fold concentration (v/v)]. These soluble protein samples are the "enzyme extracts" employed in the proteomic techniques, activity assays, and enzyme kinetics. All steps were performed on ice or in a cold room at 4 °C, to minimize proteolysis.

Quantitative FAE activity assays

FAE activity was assessed quantitatively by HPLC–UV, measuring the release of ferulic acid after hydrolysis of the synthetic substrate MFA, and 10 µL of enzyme extract was added to 190 µL of a 0.05% (w/v) MFA solution in acetate buffer 0.1 N pH 5.0. Reactions were performed at a standard temperature (37 °C) for 20 min and terminated by boiling for 5 min. Samples were recovered by centrifugation at 16,168 × g for 5 min and filtered through 0.22-µm syringe filters (Fisherbrand). The released hydroxycinnamic acids were measured using a Mediterranean Sea C18 reversed phase column (4.6 × 150 mm, 3 µm, Teknokroma) thermostated to 20 °C, according to previously described HPLC methods (García-Calvo et al. 2018). Quantitation was performed by interpolating from linear standard curves. FAE activity was expressed in units (U), defined as the amount of enzyme required to release 1 µmol of product per min under the tested conditions. All measurements were run in triplicates and controls were set up using acetate buffer 0.1 N pH 5.0 instead of enzyme extract.

Determination of optimal FAE activity conditions

Reactions were set by adapting the protocol described in the section above ("Quantitative FAE activity assays"). To determine the pH optimum, reactions were run at the standard temperature of 37 °C, using citrate–phosphate 0.1 M pH 3.0–7.0 instead of acetate buffer 0.1 N pH 5.0.

Temperature optimum was evaluated by means of FAE activity reactions performed in acetate buffer 0.1 N pH 5.0, at temperatures ranging from 37 to 60 °C. All reactions were run in triplicates for 20 min and stopped by boiling for 5 min.

FAE activity against natural substrates

FAE activity against natural lignocellulosic substrates was determined through reactions performed at 175 rpm, 50 °C, for 1–5 h, employing 2% (w/v) SBP in acetate buffer 0.1 N pH 5.0 as a substrate. Reactions were terminated by boiling for 5 min and filtered through 0.22-µm syringe filters, before measuring the release of ferulic acid by means of HPLC. FAE activity was expressed as microgram (µg) of ferulic acid released per milliliter (mL) of the reaction mixture.

Cellulose and xylan degrading activity assays

To determine cellulase activity, the three main enzymatic activities included in this group were analyzed: endoglucanase (EC 3.2.1.4), exoglucanase (cellobiohydrolase; EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). The ability of *P. rubens* B13 enzyme extracts to efficiently degrade xylan was evaluated through the determination of two of the main enzymatic groups involved: endoxylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37) (Chávez et al. 2006).

To analyze *β-glucosidase, cellobiohydrolase,* and β-*xylosidase* activities, an assay based on the release of 4-nitrophenol after the hydrolysis of synthetic substrates was used (Li et al. 2016; Terrasan et al. 2016). Specific activities were expressed in units (amount of enzyme required to release 1 µmol of 4-nitrophenol per min under the tested conditions) per mg of total protein in the enzyme extract. Reactions were set using 10-µL enzyme extract in a final volume of 550 µL substrate solution, incubated at 50 °C

and stopped by the addition of 550 µL 2% (w/v) Na_2CO_3. Substrate solutions were (i) for β-glucosidase, 4-mM pNPG; (ii) for cellobiohydrolase, 4-mM pNPC supplemented with 1-mg/mL D-glucono-1,5-δ-lactone; (iii) for β-xylosidase, 5-mM pNPX. All substrates were diluted in acetate buffer 0.1 N pH 5.0. The sample's absorbance was measured at 405 nm, and the release of 4-nitrophenol was determined by interpolating from a linear standard curve, prepared in triplicates under the same conditions.

Endoglucanase and *xylanase* activities were analyzed by means of the DNS assay for reducing sugars (Ghose 1987; Mattéotti et al. 2012; Xiao et al. 2005). Reactions were performed by adding 10 µL enzyme extract in a final volume of 500 µL substrate solution. Substrates were carboxymethylcellulose (CMC) for endoglucanase and xylan from birchwood for xylanase, diluted 1% (w/v) in acetate buffer 0.1 N pH 5.0. Reactions were performed at 50 °C and terminated by the addition of 500 µL of DNS reagent [1.4% DNS, 0.28% phenol, 0.07% sodium sulfite, 28% Na–K-tartrate (Rochelle salt), and 1.4% NaOH]. Reactions were boiled for 5 min to develop the color, which was stabilized by cooling down the tubes in an ice-water bath, for 5 min. The amount of released reducing sugars was calculated through the absorbance of a linear standard curve at 540 nm for glucose (endoglucanase)/ xylose (xylanase) prepared in triplicates under the same conditions. Specific endoglucanase/xylanase activity was expressed in units per gram of total protein in the enzyme extract.

Saccharification assays

Enzyme extracts were added to a 2% (w/v) SBP solution in acetate buffer 0.1 N pH 5.0. Saccharification reactions were run for 24 h at 50 °C and 175 rpm. Samples were boiled for 5 min and filtered through 0.22-µm syringe filters prior to HPLC analysis. The released mono and disaccharides (sucrose, glucose, fructose, arabinose, and galactose) were determined by means of HPLC–UV (Waters), using an 8-µm Transgenomic CARBOSEP COREGEL 87P column (300 × 7,8 mm) thermostated to 90 °C and following an isocratic method described elsewhere (García-Calvo et al. 2018).

Enzyme extract treatment for proteomic analyses

Enzyme extracts were recovered as detailed in the "Protein recovery from cultures" Material and Methods section and treated prior to proteomic analyses. Proteins were precipitated at − 20 °C, overnight, by addition of an equal volume of prechilled 20% (w/v) TCA/acetone with 0.14% (w/v) DTT (Jami et al. 2010b). Proteins were recovered by centrifugation at 3,220 × g, 10 min, 4 °C, and washed twice with prechilled 0.07% (w/v) DTT in acetone and once with 80% (v/v) acetone/distilled water. The precipitate was dried briefly and resuspended in sample buffer [8 M urea, 2% (w/v) CHAPS, 0.5% (v/v) IPG buffer 3–11 NL (GE Healthcare), 100- mM DTT, and 0.002% bromophenol blue], gently shaken at 25 °C, for 1 h. Soluble protein extracts were quantified through the Bradford assay (Bradford 1976) and used immediately or frozen in aliquots at − 80 °C.

DIGE análisis

Difference gel electrophoresis (DIGE) was performed according to the protocol described by Martínez-Gomariz and co-workers (Martínez-Gomariz et al. 2009; Vasco- Cárdenas et al. 2013), with modifications. Soluble protein extracts were cleaned from interfering substances using the 2D Clean-Up Kit (GE Healthcare). Proteins were resuspended in 8 M urea, 4% (w/v) CHAPS, and 30 -mM Tris pH 8.5. Protein labeling was performed on ice and in the dark, with 400 pmol/µL Cy dyes (Cy2, Cy3, Cy5) per 50 µg of protein replicate. Labeling reactions were terminated by adding 10 mM lysine, for 10 min. Samples corresponding to one single gel were combined, and the final volume was completed to 350 µL with rehydration buffer [8 M urea, 60 mM DTT, 2% (w/v) CHAPS, 0.5% IPG buffer, pH 3–11 (GE Healthcare), 0.01% bromophenol blue]. Immobiline® Drystrip pH 3–10 NL 18-cm strips (GE Healthcare) were used for isoelectric focusing (IEF), according to the following protocol, at 20 ℃: 1 h, 0 V, and 12 h, 30 V (rehydration); 2 h, 60 V; 1 h, 500 V; 1 h, 1000 V; 30 min gradient, to 8000 V; up to 7 h at 8000 V until 50 kVh. Focused IPG gels went through two equilibration steps prior to the second dimension: (i) 15 min equilibration in a buffer containing 6-M urea, 50 mM Tris–HCl (pH 8.8), 30%(v/v) glycerol, 2% (w/v) SDS, 0.002% bromophenol blue, and 1% (w/v) DTT; (ii) 15 min equilibration in the same buffer, substituting DTT for 4.0% (w/v) iodoacetamide. Equilibrated IPG gels were subjected to the second dimension in 12.5% polyacrylamide SDS-PAGE in an Ettan Dalt Six apparatus (GE Healthcare), at 25 ℃, according to the following program: 45 min, 3 W/ gel; 3.5–4 h, 18 W/gel. Precision Plus Protein Standards (Bio-Rad) were employed as molecular weight markers.

Gels were imaged by means of an Ettan™ DIGE Imager (GE Healthcare) with CyDye filters and 100 µm pixel size. Image analysis was performed using the DeCyder™ differential analysis software v7.0 (GE Healthcare) and the Differential In-gel Analysis (DIA) module. The Biological Variance Analysis (BVA) module was used. For comparison, the internal standard image gel with the greatest number of spots was chosen as a master gel. The statistical analyses between groups included the determination of the average ratio, unpaired Student's *t* test, and ANOVA. FDR (false discovery rate) was applied to reduce false positives. To be considered as differentially expressed with statistical significance, protein spots needed to comply with three conditions: (i) be present in at least 70% of gel images; (ii) show at least a 1.5-fold average volume ratio, and (iii) have *p* values lower than 0.05.

Analysis of proteins in the enzyme extracts

To analyze and identify the proteins present in the *P. rubens* Wisconsin 54–1255 B13 enzyme extracts, two-dimensional gel electrophoresis was carried out (Jami et al. 2010a). Three gels corresponding to three biological replicates from independent cultures were analyzed; 400 µg of soluble proteins resuspended in sample buffer was subjected to IEF on an IPGphor apparatus, by means of 4–7 NL Immobiline™ 18-cm IPG strips (GE Healthcare). IEF and, subsequently, the second dimension were performed as described previously ("DIGE analysis" Material and Methods section). After 2-DE, gels were stained following the "blue silver" colloidal Coomassie staining method (Candiano et al. 2004; Jami et al. 2010a). Gels were scanned by means of an ImageScanner III (GE Healthcare), calibrated

using a grayscale marker (Eastman Kodak Co.), and the Labscan 5.00 (v1.0.8) software (GE Healthcare). Gel analysis was carried out with the ImageMaster™ 2D Platinum v 5.0 software (GE Healthcare). Automatic spot detection was verified manually to avoid artifacts (background noise, bubbles, streaks, etc.). To quantify and compare spots, spot normalization was carried out based on relative volumes, defined as the volume of each spot divided by the total volume of all the spots in the gel. Protein spots were matched using landmark features. Protein spots present in the three replicates were considered for identification.

Protein identification

Selected protein spots were manually excised from stained gels, enzymatically digested with trypsin (Havliš et al. 2003), and processed as detailed in previous works (Jami et al. 2010a). To analyze samples, a 4800 Proteomics Analyzer (MALDI-TOF/TOF, AB Sciex) was employed, using a 4700 Proteomics analyzer calibration mixture (Cal Mix 5, AB Sciex) as external calibration. Additionally, MS spectra were internally calibrated considering peptides from the trypsin autodigestion. MALDI-TOF/TOF analysis using the 4000 Series Explorer v3.5.3 software (AB Sciex) originated peptide mass fingerprints (PMF). Up to 65 peptides from one spot were gathered and represented as monoisotopic molecular weights with a signal-to-noise ratio (S/N) greater than 20. Known contaminant ions, such as peptides derived from trypsin and keratin digestion, were not considered for later MS/MS analyses. The 6 most intense precursors with S/N > 20 from each MS spectrum were selected for MS/ MS analysis with CID, using atmospheric gas, in 2-kV ion reflector mode and with a mass window of ± 7 Da. Mascot generic files combining MS (PMF) and tandem MS (MS/ MS) spectra were created automatically. For protein identification, Mascot generic files were employed to query a nonredundant protein database using a local license of Mascot v2.2 (Matrix Science) through the Global Protein Server v3.6 (Applied Biosystems). The following search parameters set for peptide mass fingerprints and tandem MS spectra were established: (i) taxonomy, all entries (date 2018.10.23; 8,745,091 sequences, 3,903,651,808 residues); (ii) fixed and variable modifications were considered, Cys as S-carbamidomethyl derivative and Met as oxidized methionine; (iii) one missed cleavage site was tolerated; (iv) precursor tolerance was 50 ppm and MS/MS fragment tolerance was 0.3 Da; (v) peptide charge was set to 1 + ; vi) the employed enzyme was trypsin.

Protein identifications achieved by the abovementioned method were only considered acceptable if the global Mascot score was greater than 83, with a significance level of $p < 0.05$. The global Mascot score uses probability-based scoring and indicates the probability that the observed match is a random event. A higher Mascot score corresponds to a lower probability of a random match (Perkins et al. 1999).

Metabolite analyses

Metabolite extraction was performed from 96 h M-SBP and M-OAT cultures, grown as detailed in the "Strains and cultivation" Material and Methods section. Triplicate samples of supernatant, DMSO, and methanol extracts from independent cultivations were extracted as follows: 5 mL of broth were

centrifuged twice at 3,220 × g, for 30 min, and the supernatant and pellet were separated. The supernatant was filtered through 0.22-μm syringe filters (Millipore), and the mycelium pellets were resuspended in 10-mL methanol or 10-mL dimethyl sulfoxide (DMSO) by vortexing for 30 s and extracted for 45 min in a rotating mixer at room temperature. Enzyme extracts were prepared as described in the "Protein recovery from cultures" Material and Methods section. The extracts were analyzed by LC-DAD-QTOF-MS with MS1 and MS2 following a previously described method with modifications (Degnes et al. 2017).

A Zorbax bonus RP column (2.1 × 50 mm, 3.5 μm) was employed to separate metabolites, with formic acid and acetonitrile as the mobile phase at a 0.3 mL/min flow. LC-DAD isoplots were generated with Python. Centroid LC–MS data were processed with the MassHunter Qualitative Analysis Software v. B.04.00 (Agilent Technologies) and Mass Profiler Professional software v2.1.5 (MPP; Agilent Technologies). A search for known compounds was performed against databases such as METLIN PCD (Agilent Technologies) and a PubChem database for bioactive compounds.

Results

Selection of endogenous FAE-producing strains

The first goal of this work was to select a *Penicillium* spp. strain with endogenous FAE (feruloyl esterase) activity, in order to find putative FAEs and subsequently overexpress them through genetic engineering. This species was chosen based on its high evolutionary proximity with species of the genus *Aspergillus* (van den Berg et al. 2008), known as one of the most interesting fungal enzyme producers, whose FAEs have been thoroughly studied (Dilokpimol et al. 2016, 2017; Koseki et al. 1998, 2009b; Mäkelä et al. 2018; Zhang et al. 2013).

Two wild-type *Penicillium* spp. strains (*P. notatum* NRRL-1249 and *P. rubens* NRRL-1951) and three industrial strains used in antibiotic production, well adapted to grow under industrial conditions (*P. rubens* Wisconsin 54–1255, AS-P-78 and AS-P-99), were analyzed by means of a FAE activity plate assay (Supplementary Fig. S1). The EF (ethyl ferulate) hydrolysis efficiency of each strain was expressed through the enzymatic index [EI = (diameter of the colony + diameter of the degradation halo) / diameter of the colony] (Braga et al. 2014).

P. notatum NRRL-1249, *P. rubens* NRRL-1951, and *P. rubens* Wisconsin 54–1255 displayed higher FAE activity than *P. rubens* AS-P-78 and AS-P-99 (Fig. 1A). The two most promising *P. rubens* strains were further compared by means of the release of ferulic acid from SBP (sugar beet pulp) after submerged culture on M-SBP medium at 28 °C, 250 rpm, for 120 h (Fig. 1B). The results showed a similar capacity to release ferulic acid from SBP in submerged culture. Given this similar activity, *P. rubens* Wisconsin 54–1255 was selected for the enhancement of its FAE production, due to the existence of optimized genetic modification techniques, its suitability for growth and sporulation in a laboratory environment, and the availability of its sequenced genome (van den Berg et al. 2008).

Establishment of optimal growth conditions and FAE activity reaction conditions

The impact of change in cultivation conditions on FAE production was evaluated. Furthermore, the conditions for the FAE activity assay were optimized to select the optimal conditions for further experiments. SBP was used as a carbon source in these cultivations and as a natural inducer of FAEs (Benoit et al. 2006; de Vries et al. 2002; Sakamoto et al. 2005) and MFA (methyl ferulate) as a synthetic substrate for FAE activity reactions (Ralet et al. 1994; Record et al. 2003; Wang et al. 2016a).

Agitation

Both agitated and static culture conditions have previously been used to produce FAEs (Fazary and Ju 2007; Gopalan et al. 2015; Kumar et al. 2011; Topakas et al. 2007). To compare FAE production in both states, *P. rubens* Wisconsin 54–1255 was grown in M-SBP liquid medium at 28 °C with (250 rpm) and without agitation (Fig. 2A). FAE activity was determined through the amount of ferulic acid released from the substrate SBP, measured from filtered culture supernatants by means of HPLC. Cultivations shaken at 250 rpm showed higher FAE activity, particularly at the last sampling time (96 h).

Seed culture

Two types of seed cultures were tested: (i) a spore suspension obtained from Power medium plates grown for 5 days at 28 °C and inoculated directly into cultivation flasks with M-SBP medium (spores from one Power medium plate per 100 mL of M-SBP medium); (ii) 10% (v/v) of a seed culture grown from spores in plates, on PMMY medium, at 28 °C and 250 rpm for 24 h. Cultivation broth samples were recovered from cultures grown on M-SBP medium for 96 h, subsequently filtered, and precipitated using ammonium sulfate to obtain enzyme extracts according to the "Protein recovery from cultures" Material and Methods section. FAE activity was quantitatively determined through the release of ferulic acid after the hydrolysis of the synthetic substrate MFA, analyzed by HPLC. The results showed that slightly higher FAE activity was obtained when using 10% (v/v) of a submerged culture grown for 24 h on PMMY medium as seed culture (Fig. 2B).

pH and temperature optima

To determine the optimal FAE activity conditions of the enzyme extract recovered from *P. rubens* Wisconsin 54–1255 grown on M-SBP, FAE reactions against MFA were carried out at a standard temperature of 37 °C and pH ranging from 3.0 to 7.0. FAE activity was detected from pH 3.0 to 6.0 (Fig. 3A) with a pH optimum at pH 5.0. The temperature optimum was analyzed at the optimal pH (pH 5.0) and temperatures from 37 to 60 °C. The enzyme extract had high activity over a wide temperature range, with an optimum in the range of 45–55 °C (Fig. 3B); 50 °C was thus selected as the optimal temperature for further experiments, as higher temperatures lead to greater variation, with consistently reduced FAE enzymatic activity above 55 °C.

Optimal enzyme dosage and reaction time for the hydrolysis of SBP

The optimal amount of enzyme extract and reaction time for the release of ferulic acid from the natural substrate SBP were evaluated as described in the "FAE activity against natural substrates" Material and Methods section. Tests were carried out using several enzymatic doses (0, 0.1, 0.5, 1.5, and 2.1 mg of enzyme extract per g of SBP) and at two different reaction times (1 and 5 h). The results showed that the maximal absolute ferulic acid release was achieved when adding 1.5 mg of enzyme extract per g of SBP, with 5-h incubation (Fig. 4). Furthermore, the release of ferulic acid per unit of time was greater with a shorter reaction time (1-h incubation, instead of 5 h).

Cloning and overexpression of feruloyl esterases from P. rubens Wisconsin 54–1255 in the natural host

Genome search for FAE-encoding genes

Putative FAE encoding genes from *P. rubens* Wisconsin 54–1255 were identified by means of a homology search with known FAE encoding genes from *Aspergillus* spp. (AnFaeA and AnFaeB encoding genes), using the BLAST tool (Boratyn et al. 2013).

Two possible ORFs in *P. rubens* Wisconsin 54–1255 encoding putative enzymes with FAE activity (ORFs Pc20g07010 and Pc12g08300) were identified. Both sequences have a pentapeptide with conserved consensus sequence GXSXG, whose residue of serine belongs to the catalytic triad Ser-His-Asp, characteristic of the serine protease and esterase families, also identified in *A. niger* AnFaeA (Hermoso et al. 2004). These proteins were named PrFaeA and PrFaeB and their respective genes: *faeA* and *faeB,* following the nomenclature proposed by Faulds and co-workers (Crepin et al. 2004; Faulds 2010; Faulds et al. 2003).

Apart from the previously mentioned ORFs, other putative FAE encoding genes present in *P. rubens* Wisconsin 54–1255 genome have been reported in previous works, such as ORFs Pc13g15360, Pc20g11110, and Pc21g17360 (Dilokpimol et al. 2016). However, in the present work, ORFs Pc20g07010 and Pc12g08300 were selected for overexpression, as they showed the highest identity percentages with AnFaeA (99% sequence coverage, 75% identity) and AnFaeB (100% sequence coverage, 51% identity), respectively.

Cloning and expression

The PrFaeA and PrFaeB encoding sequences were cloned, separately, into the overexpression plasmid pIBRC43BglII (Kosalková et al. 2009), obtaining plasmids p*Sfae*A and p*Sfae*B, respectively. Two lines of FAE overexpressing transformants were generated: (i) line A (overexpressing gene f*aeA*) and (ii) line B (overexpressing gene *faeB*).

More than 100 transformants were obtained from each line, 48 of which were randomly selected and analyzed by means of FAE activity plate assays, as detailed in the "FAE activity screening" Material and Methods section (Supplementary Fig. S2). The 8 transformants from line A and 10 from line B with the highest FAE activity (higher EI and less deviation between replicas) were selected (line A: 1, 18,

31, 68, 71, 76, 78, and 84; line B: 1, 7, 10, 13, 17, 20, 27, 77, 83, and 88). Southern blotting verification of selected transformants Southern hybridization was carried out with the selected transformants in order to confirm the presence of the overexpression cassette (Supplementary Fig. S3). The results showed that all transformants kept the endogenous respective FAE gene and that only some had incorporated the integrative overexpression cassette (A68, A76, A78, B1, B7, B10, B13, B20, B77, B83). The complete integration of the overexpression cassette was verified by means of DNA sequencing, using the primers detailed in Table 1.

Three transformants of each line with the integrated overexpression cassette (A68, A76, A78, B13, B77, and B83) were selected to perform quantitative FAE activity assays on both natural (SBP) and synthetic substrates (MFA), at pH 5.0 and 50 ℃ (Fig. 5). Transformant A78 from line A and transformant B13 from line B stood out. B13 showed 14 times higher FAE activity than *P. rubens* Wisconsin-541255 against SBP and 90 times higher when MFA was used as a substrate. Transformants A78 and B13 were chosen for further experiments.

Gene expression analyses

Gene expression of the *P. rubens* Wisconsin 54–1255 strain and transformants A78 and B13 was then analyzed by RT-qPCR to verify that the increase in FAE activity was the result of an increase in the expression of the *faeA* and *faeB* genes in the two overexpressed lines (Supplementary Figs. S4 and S5). This assay showed that the *faeA* or *faeB* expression was increased in both transformants (A78 and B13, respectively), obtaining the maximum expression at 24 h of incubation. Particularly noteworthy was the B13 transformant, in which the expression of *faeB* at 24 h was 214 times higher than in the *P. rubens* Wisconsin 54–1255 strain.

The A78 transformant was remarkably less active against the natural lignocellulolytic substrate SBP than the B13 transformant. Given that SBP was the inducing substrate of choice in the present work, B13 was chosen for further experiments. Previous studies have indicated that FAEs belonging to category A according to the functional classification proposed by Crepin and co-workers have low or no activity against SBP, whereas type B FAEs (such as PrFaeB) are the most active (Crepin et al. 2004; Dilokpimol et al. 2016; Kühnel et al. 2012). Moreover, type A FAEs are induced by monocotyledonous plants, such as corn or wheat, and not by dicotyledonous plants, such as sugar beet (Fazary and Ju 2007; Zwane et al. 2014).

P. rubens extracellular proteome analysis

The differences in the extracellular proteome between *P. rubens* Wisconsin 54–1255 and the B13 transformant were evaluated through 2D-DIGE electrophoresis, using four biological replicates per strain. Protein samples were obtained by ammonium sulfate precipitation of broths recovered at 72 h cultivation in M-SBP medium, in Braun Biostat® B 20 L fermentors. As detailed in the "DIGE analysis" Material and Methods section, protein spots that presented a 1.5-fold or greater variation between both

strains ($p < 0.05$) were selected for identification. 10 showed significant differences and 4 were identified by means of trypsin digestion and MS analyses (MALDI-TOF/TOF). Two of them corresponded to PrFaeB (Pc12g08300), which was the overexpressed FAE in the B13 transformant. The fold change (ratio) of these protein spots was 4.49 and 4.78, indicating a higher PrFaeB production in the overexpressed transformant *P. rubens* B13 (Supplementary Fig. S6).

Besides comparing the differences in the extracellular proteins secreted by *P. rubens* Wisconsin 54–1255 and the B13 transformant, the complete extracellular proteome of the B13 transformant grown on SBP was analyzed by means of 2D electrophoresis and MALDI-TOF/TOF MS protein identification. As most proteins in the 2D-DIGE analyses were detected below pH 7.0, Immobiline® Drystrip pH 4–7 NL 18-cm strips were used for this experiment. Cultivation broths from 3 biological replicates were recovered from 72-h M-SBP cultures in Braun Biostat® C 20 L bioreactors and precipitated with ammonium sulfate. The secreted protein extract 2D reference map was obtained (Fig. 6), with 205 protein spots common to all three replicates. Out of the detected protein spots, 120 were successfully identified by peptide mass fingerprinting and tandem MS (Table 2, Supplementary File S7). From them, 124 protein identifications were obtained; 102 identifications corresponded to several protein species (isoforms) of 22 proteins, while the remaining 22 were unique protein species.

The SignalP 5.0 server was used to identify the presence of putative signal peptides in the identified proteins (Almagro Armenteros et al. 2019; Nielsen 2017; Nielsen et al. 1997). Out of the total 124 protein species, 117 (94.35%) were predicted to have a signal peptide. Of the seven remaining proteins, two protein species (1.61%) were predicted to present nonclassical secretion, according to the SecretomeP 1.0 server (Bendtsen et al. 2004), and 5 did not present any of the mentioned signals. The fact that most of the identified proteins presented putative signal peptides or nonclassical secretion indicates low presence of intracellular proteins in the analyzed samples, and, thus, supports the absence of important cell lysis processes at the selected sampling time.

Subsequently, functional annotation of the proteins identified in the enzyme extract was performed by using the COG (clusters of orthologous groups of proteins) system, which includes eukaryote organisms (KOG), via the eggNOG 5.0 online orthology resource (Fig. 7) (Huerta-Cepas et al. 2019; Tatusov et al. 2003). The main findings in these groups are summarized below.

Plant cell wall degradation (proteins in COG G)

The biological processes leading to plant cell wall degradation require different enzymatic groups that act synergistically (de Souza 2013; Wei et al. 2009). As shown in Fig. 7, the majority of the identified proteins were related to carbohydrate metabolism and transport (a total of 88 isoforms of 28 different proteins). One of the most represented enzymatic groups was the cellulases, with 4 β-glucosidases (EC 3.2.1.21), which were identified from 5 protein spots; 2 exoglucanases (cellobiohydrolases, EC 3.2.1.91), identified from 8 spots; and an endoglucanase (endocellulase, 3.2.1.4), identified from 1 spot.

The synergistic action of these enzymatic groups is required to achieve the complete hydrolysis of cellulose (Dashtban et al. 2009; Escamilla-Alvarado et al. 2017; Lynd et al. 2002).

One of the spots with higher relative volume (% volume, Supplementary File S7) in the reference map (spot 113), with 3 isoforms present (spots 111, 112, and 113), was identified as a putative pectate lyase (EC 4.2.2.2). Several other hemicellulases and pectinases were identified, including (i) α-L-arabinofuranosidases (EC 3.2.1.55), (ii) endo-1,4-betaxylanase (EC 3.2.1.8), (iii) rhamnogalacturonan endolyases (EC 4.2.2.23), (iv) exo-arabinanases, and (v) feruloyl esterases (EC 3.1.1.73). The strong presence of matrix polysaccharide degrading enzymes may be due to the carbohydrate composition of the lignocellulosic substrate which was used (SBP), as detailed in the Discussion section.

Proteins related to the fungal cell wall (proteins in COGs G and M)

In this category, 10 different proteins with 48 isoforms were identified from the analyzed partial extracellular proteome. One of these distinct proteins was a β-Nacetylhexosaminidase (EC 3.2.1.52) identified from the protein spot with the highest relative volume (spot 62; protein name Pc20g10360; Supplementary File S7), as well as 16 more spots (a total of 17 isoforms). Moreover, 5 isoforms of another putative β-N-acetylhexosaminidase (Pc22g24800), 2 and 3 isoforms of two chitinases (Pc13g09520 and EN45_103640, respectively), and 3 isoforms of 2 endo- 1,3(4)-β-glucanases (Pc16g08980 and Pc13g08730) were also identified. These enzymes are involved in the hydrolysis and reorganization of chitin, one of the major components of most fungal cell walls (Díez et al. 2005; Zhao et al. 2014), and its promoter has been patented for genetic engineering uses by Antibióticos León S.A. (patent number WO/1998/039459) (Barredo Fuente et al. 1998).

General cell metabolism (proteins in COGs E, O, C, M)

The extracellular proteome analysis permitted the identification of 5 distinct putative proteases and peptidases (protein names Pc22g15910, Pc13g09680, Pc21g14160, Pc20g09400, and Pc22g13950). Other identified proteins belonging to this functional category were a glutaminase (Pc20g02640), an amidase (Pc22g01750), 2 FAD-linked oxidoreductases (Pc21g12590, Pc18g05530), and a thioredoxin reductase (Pc22g22810).

Cell rescue, defense, and virulence (proteins in COGs C, P, Q)

An important factor in the microbial ability to degrade plant cell walls is the capacity to tolerate or remove toxic compounds derived from such degradation. In this regard, 10 isoforms of a single putative catalase (Pc16g11860) were identified. Catalases catalyze the hydrolysis of hydrogen peroxide, which can be produced as a consequence of the hydrolysis of plant cell walls, to H_2O and oxygen (Jami et al. 2010b). Catalases are also related to pathogenicity in fungal plant pathogens (Garre et al. 1998).

Unknown function (proteins in COG S)

559 Lastly, 13 isoforms of 4 different proteins whose biological function remains to be elucidated were
560 identified.

561 **Industrial applicability of *P. rubens* B13 enzyme cocktail**

562 The enzyme extract recovered from cultivations of *P. rubens* B13 grown on M-SBP showed promising
563 levels of FAE activity (14 times higher than the parent strain *P. rubens* Wisconsin 54–1255 against SBP,
564 and 90 times higher against MFA). To evaluate a possible industrial application, several studies were
565 conducted, including the determination of other lignocellulolytic activities acting synergistically, the
566 analysis of the saccharification capacity, and its suitability to be adapted to fermentation in a bioreactor
567 at higher culture volumes. Moreover, an untargeted metabolite study was performed to assess the
568 putative presence of bioactive metabolites in the enzyme extracts.

569 **Lignocellulolytic activities in the enzyme extract**

570 Commonly, commercial preparations for the hydrolysis of plant biomass include a mixture of
571 lignocellulolytic enzymes, instead of one single purified enzyme (Fazary and Ju 2008; Mäkelä et al.
572 2014). Considering this fact and the presence of a wide array of lignocellulolytic enzymes in the partial
573 extracellular proteome studied in this work, two of the most important group of activities were
574 determined: cellulase and xylanolytic activities. Reactions were performed with *P. rubens* B13 enzyme
575 extracts at pH 5.0 and 50 ºC, to characterize the synergistic activities occurring simultaneously at the
576 optimal FAE activity conditions.

577 As shown in Table 3, *P. rubens* B13 enzyme extract presented all the five cellulase and xylanolytic
578 tested activities. This finding is in accordance with the identification of β-glucosidases, exoglucanases,
579 and β-xylosidases in *P. rubens* B13 extracellular proteome ("Plant cell wall degradation (proteins in
580 COG G)" Results section). Previous works have evaluated the cellulolytic and hemicellulolytic potential
581 of *Penicillium* spp. Song and co-workers determined these enzymatic activities in the hypercellulolytic
582 mutant *P. oxalicum* JU-A10, whose β-glucosidase values were over 4.5 times lower than those of *P.*
583 *rubens* B13 (Song et al. 2016). Moreover, when compared to *P. oxalicum* JU-A10, *P. rubens* B13
584 showed more than twice as high cellobiohydrolase activity and slightly higher β-xylosidase activity,
585 which points to *P. rubens* B13 as a promising strain to produce cellulolytic and hemicellulolytic
586 preparations.

587 The β-glucosidase, cellobiohydrolase, β-xylosidase, and endoxylanase activities in *P. rubens* B13
588 enzyme extracts were higher than in an enzyme cocktail obtained from *Alternaria alternata* PDA1,
589 which, in turn, showed higher endoglucanase values (García-Calvo et al. 2018). As the
590 cellobiohydrolase activity in the *A. alternata* PDA1 enzyme cocktail was remarkably lower than in the
591 *P. rubens* B13 enzyme extract (around 50 times lower), a combination of both preparations might be
592 able to enhance the degradation of plant biomass.

593 **Sugar beet pulp saccharification**

The industrial applications of fungal enzyme extracts such as the one obtained from *P. rubens* B13 are related to their ability to hydrolyze plant cell walls and release simple sugars, a process which is known as saccharification (Jimenez- Flores et al. 2010; Menon and Rao 2012; Micard et al. 1996; Ravalason et al. 2012). The saccharification capacity of the enzyme extracts recovered from *P. rubens* Wisconsin 54–1255 and transformant B13 was measured through the release of simple sugars determined by HPLC–UV, after enzymatic reactions performed on SBP. Reactions were carried out for 24 h at pH 5.0, 50°C, and 175 rpm, adding 15 mg of enzyme extract per gram of SBP. As shown in Table 4, the analyzed extracts efficiently released limited quantities of arabinose, fructose, and glucose, none of which were found in the control reactions without enzyme extracts. Besides, sucrose, which was found in the control reactions, was not detected in the reactions employing enzyme extracts, which suggests the presence of hydrolases that are capable of degrading the aforementioned disaccharide, such as invertases (Nuero and Reyes 2002; Poonawalla et al. 1965).

Scale-up of the PrFaeB production process

In order to achieve production levels that are compatible with commercial uses, it is necessary to scale up the fermentation process. The suitability of *P. rubens* B13 to cultivation in a bioreactor was assessed by means of 5-L and 20-L cultivations in Braun Biostat® B and C fermentors, with working volumes of 3 L and 12 L, respectively, as described in the "Strains and cultivation" Material and Methods section. Two conditions were analyzed regarding cultivation pH: (i) uncontrolled pH (starting at pH 5.0) and (ii) pH 5.0, controlled by the automatic addition of NaOH 1 M and HCl 1 N. Samples were recovered every 24 h to measure total protein, cell growth (dry weight), and FAE activity against the synthetic substrate MFA.

According to the monitored cultivation parameters and the FAE activity values (Supplementary Figs. S8.A and S8.B), cultivations carried out at pH 5.0 controlled automatically were preferable to achieve higher FAE activity in the enzyme extracts. Besides, considering the monitored parameters and FAE activity values, the optimal sampling time for the recovery of enzyme extracts was 24 h, in 5-L fermentors (Supplementary Fig. S8C). In 20-L fermentors, FAE activity was slightly higher at 48 h; however, this limited increase would not justify a 24-h extension of the cultivations, considering the associated expenses and the delay in the recovery of the commercial products (Supplementary Fig. S9B). These reasons indicated that the optimal cultivation time of *P. rubens* B13 in 20-L bioreactors for FAE activity would also be 24 h.

The suitability of *P. rubens* B13 for cultivation in bioreactors (Supplementary Figs. S8.A and S9.A) as well as the earlier sampling time for optimal FAE activity observed when grown in fermentors (24 h as opposed to 96 h in flask cultivation) confirms this strain as a propitious FAE producer for industrial applications.

Metabolite analyses

Enzymes are generally considered safe for industrial uses, as indicated by the lack of adverse effects in oral toxicity and genotoxicity safety studies (Sewalt et al. 2016). However, as *P. rubens* is a well-known antibiotic producer and is also capable of synthesizing other bioactive molecules, such as mycotoxins (Fernández-Aguado et al. 2014; García-Estrada et al. 2011; Jami et al. 2010b), an untargeted metabolite analysis was performed to study the presence of putative undesirable or hazardous compounds.

Supernatants, DMSO, and methanol extracts of pellets, as well as enzyme extracts prepared from *P. rubens* B13 cultures on M-SBP and M-OAT media, were analyzed using three independent biological replicates per condition. LC-DAD isoplots were generated to give an overview of which UV-absorbing compounds were present in the different extracts (Supplementary File S10.A). For the MS data, only those metabolites present in at least two of the three replicates of each condition were considered for identification. A total of 88 masses were putatively annotated against METLIN PCD and a PubChem database for bioactive compounds. Out of the 88 metabolites, 30 were present in both M-SBP and M-OAT media, 29 were exclusive to M-SBP and 29 were only found in M-OAT. The putatively annotated metabolites included peptides; fatty acids and related compounds; sphingolipids; glycerophospholipids; vitamin D and related compounds; vitamin E and related compounds; sterols and related compounds, a mycotoxin; antibiotics; and other bioactive metabolites (Supplementary File S10.B). Among the detected metabolites were two categories that could affect the industrial applicability of the enzyme extracts: a mycotoxin (aflatoxin B1) and two antibiotics (penicillin G and penicillin V). The possible presence of penicillin was assessed by MS/MS fragmentation followed by comparison against the METLIN database and the molecular networking database GNPS (Wang et al. 2016b). This approach identified three masses with no database annotation, which were apparently related to penicillin G.

Discussion

The cleavage of the bonds that stabilize the plant cell wall, necessary for its deconstruction, is not a trivial process, and it requires an assortment of lignocellulolytic enzymes that act synergistically (Guerriero et al. 2016; Wei et al. 2009). Among these enzymatic groups, auxiliary enzymes, such as FAEs, have been the subject of an increasing number of studies in the last decades (Fazary and Ju 2007; Udatha et al. 2012). The discovery of novel FAEs with different optimal activity conditions is needed to achieve more effective industrial processes in fields such as biofuels, paper, food, and feed industries (Dilokpimol et al. 2016; Gopalan et al. 2015; Topakas et al. 2007). Moreover, FAEs catalyze the release of hydroxycinnamic acids, which are a key component in lignocellulose recalcitrance to hydrolysis and have several commercial applications, as antioxidants and precursors for the synthesis of valuable compounds like vanillin (Alam et al. 2016; de Oliveira et al. 2015; Mathew and Abraham 2006; Ou and Kwok 2004; Taofiq et al. 2017). As heterotrophic osmotrophs, fungi are natural producers of extracellular hydrolases, including carbohydrate-active enzymes (CAZymes). In this regard, the present work aimed to identify and overexpress novel fungal FAEs with potential industrial applications.

P. notatum NRRL-1249, *P. rubens* NRRL-1951, and *P. rubens* Wisconsin 54–1255 showed higher FAE activity than *P. rubens* AS-P-78 and AS-P-99 (Fig. 1A). The two strains with the highest FAE activity in the plate assay were the least genetically modified: *P. notatum* NRRL-1249 and *P. rubens* NRRL-1951. These results indicate that strain selection for antibiotic production has negatively impacted their endogenous FAE activity, presumably as a consequence of major modifications in primary and secondary metabolism (Barreiro et al. 2012b; Jami et al. 2010a, 2010b). Two ORFs found in the *P. rubens* Wisconsin 54–1255 genome encoding possible enzymes with FAE activity (Pc20g07010 and Pc12g08300) were functionally annotated according to their homology with previously described fungal FAEs (Dilokpimol et al. 2016; Udatha et al. 2011). The observed results prove for the first time that the overexpression of the aforementioned ORFs led to an increase in the release of ferulic acid from synthetic FAE substrates such as EF and MFA, which indicates that the respective encoded proteins effectively show FAE activity.

The PrFaeA sequence comprised 5 conserved domains related to lipase activity (cd00519, pfam01764, PLN02802, PLN03037 and COG3675), and the most recent FAE phylogenetic classification assigned it to subfamily 7, which includes FAEs that are related to lipases (Dilokpimol et al. 2016). On the other hand, the PrFaeB sequence included one single conserved domain (pfam07519) related to tannases and was included in subfamily 1. FAEs seem to have evolved from diverse enzymatic groups and present differences in their sequences and structural features, which can explain their distinct specificity and activity toward various substrates.

The extracellular proteome reference map from *P. rubens* Wisconsin 54–1255 has been studied in previous works in other culture conditions, such as the use of PMMY medium (Jami et al. 2010b). The number of total identified proteins in the said study was greater than in the present work (328, as opposed to 124). However, the percentage of proteins related to plant cell wall degradation was remarkably lower (11.59%, versus the 47.31% reported in our study). PMMY medium does not include plant-derived compounds, whereas the M-SBP medium employed in the present work contains the lignocellulosic substrate SBP as a carbon source. The higher presence of lignocellulolytic enzymes in the extracts recovered after cultivation on SBP indicates the influence of plant-derived carbon sources as inducers of these enzymatic groups. Previous works have found high percentages of fungal- secreted CAZymes when inducing substrates derived from plants were used (Adav et al. 2012; Dilokpimol et al. 2020; Liu et al. 2013; Song et al. 2016).

Among the proteins identified by Jami and co-workers in *P. rubens* Wisconsin 54–1255 secretome, there was PrFaeA (Pc20g07010), which was not identified in this work when analyzing enzyme extracts produced by *P. rubens* B13 grown on SBP (Jami et al. 2010b). This may be explained by the fact that the B13 transformant does not overexpress PrFaeA but PrFaeB (Pc12g08300), and the endogenous PrFaeA, being a type A FAE, is not successfully induced by SBP (Crepin et al. 2004). On the other

699 hand, PrFaeB was not identified in the study performed by Jami and co-workers, whereas 4 isoforms
700 were found in the present work, presumably because of the use of SBP and the analysis of a different *P.*
701 *rubens* strain (in this case, the B13 transformant, which is capable of overexpressing PrFaeB).

702 Some of the identified proteins involved in the degradation of plant cell walls were cellulases,
703 hemicellulases, and pectinases. The presence of enzymes capable of degrading matrix polysaccharides
704 may be due to the polysaccharide composition of SBP, which was used as an inducing substrate and has
705 a high content of hemicelluloses and pectins (Hutnan et al. 2000; Mardones et al. 2019; Micard et al.
706 1996; Oleas et al. 2017). A strong representation of these groups of hydrolases has been reported in an
707 extracellular proteome study of the fungal strain *A. alternata* PDA1, grown on exactly the same
708 lignocellulosic substrate (García-Calvo et al. 2018). The presence of several xylanolytic enzymes in
709 *Penicillium* spp. has been determined in previous studies, which have identified and characterized
710 CAZymes such as endoxylanases, β-xylosidases, arabinofuranosidases, acetyl xylan esterases, as well
711 as FAEs (the latter belonging to species such as *P. expansum*, *P. funiculosum*, *P. pinophilum*, *P.*
712 *purpurogenum*, and *P. subrubescens*). The studies on the two novel enzymes with FAE activity carried
713 out in the present work (PrFaeA and PrFaeB, from *P. rubens* Wisconsin 54–1255) contribute to the
714 understanding of the xylanolytic enzymatic system from the genus *Penicillium* (Dilokpimol et al. 2020;
715 Donaghy and McKay 1995; Kroon et al. 2000; Mardones et al. 2019; Oleas et al. 2017; Schneider et al.
716 2016).

717 The *P. rubens* B13 enzyme extracts studied in the present work were capable of catalyzing the release
718 of ferulic acid from the natural substrate SBP, extracting approximately 22.7–41.7% (w/w) of the total
719 ferulic acid content using very limited quantities of enzyme extract (Benoit et al. 2006; García-Calvo et
720 al. 2018; López Alonso et al. 2011; Micard et al. 1996). Other *Penicillium* sp. strains have demonstrated
721 the ability to release this hydroxycinnamic acid from natural substrates, such as *P. chrysogenum* 31B,
722 which showed optimum FAE conditions at 50 and pH 6.0–7.0 and extracted approximately up to 85%
723 (w/w) of the alkaline extractable ferulic acid in SBP (Sakamoto et al. 2005). In general, previously
724 described fungal FAEs show great variations in their physiochemical characteristics (molecular weight,
725 isoelectric point, pH, and temperature optima). This is likely related to the different origins of FAEs,
726 which have evolved from highly divergent families of enzymes (Dilokpimol et al. 2016). Fungal FAEs
727 are active from pH 3.0 to 10.0 and 20 to 75 °C, even though optimal activity values are usually at pH
728 4.0–7.0 and temperatures below 50–55 °C (Dilokpimol et al. 2016; Fazary and Ju 2007; Koseki et al.
729 2009a; Topakas et al. 2007; Wang et al. 2016a). To the best of our knowledge, no correlation has been
730 observed between the physiochemical characteristics of the FAEs and their optimal reaction conditions.
731 The results obtained in the present study fall within the optimal activity ranges of other fungal enzymes
732 with FAE activity consulted in the literature.

733 Although the main interest in *P. rubens* Wisconsin 54–1255 has traditionally been due to its role as an
734 antibiotic producer, its powerful mechanism for the degradation of plant biomass represents an

interesting source of lignocellulolytic enzymes. A study published by van den Brink and de Vries in 2011 indicated the putative presence of several CAZymes in the *P. rubens* Wisconsin 54–1255 genome, including as many as 158 glycoside hydrolases (GHs), 7 carbohydrate esterases (CEs), and 9 polysaccharide lyases (PLs) (van den Brink and de Vries 2011). According to the Carbohydrate Active Enzymes database as of 23rd August 2022 (CAZy; http:// www. cazy. org), the number of putative CAZymes in this fungal strain could in fact be greater, including up to 230 putative sequences of GHs, 21 CEs and 10 PLs (Drula et al. 2022). The identification of several CAZymes in the *P. rubens* B13 partial secreted proteome analysis, as well as the detected lignocellulolytic activities, confirms *P. rubens* B13 as a fungal strain with promising applications in the degradation of lignocellulose.

The untargeted metabolite analyses performed in the present work constitute a useful initial approach to identify putative bioactive molecules that could hinder the industrial applicability of the enzyme extracts. The verification of the putative annotations is challenging and depends on the availability of standards and MS/MS fragmentation patterns libraries. In this regard, efforts are being made to facilitate the reliability of the annotations, such as the creation of open-access databases including extensive MS/MS information (Creek et al. 2014; Degnes et al. 2017; Wang et al. 2016b; Zhu et al. 2013). The metabolites that were putatively annotated from the analyzed *P. rubens* B13 samples included a mycotoxin and two antibiotics. The putative mycotoxin, aflatoxin B1, was found in both the enzyme samples originating from the M-SBP cultures and in the M-SBP medium. Since *P. rubens* is not among the known aflatoxin B1 producers (*Aspergillus* spp.) (Frisvad et al. 2005; Tola and Kebede 2016), this toxin was most likely produced by other fungal species during storage of the natural plant derivative SBP. Thus, the putative identification of this mycotoxin in the analyzed samples is not necessarily an obstacle to the commercialization of the enzyme extracts. Furthermore, the identification was qualitative, and the levels of this mycotoxin could be compliant with the EU mycotoxins limits and regulations (McEvoy 2016; van Egmond et al. 2007; Vila-Donat et al. 2018). Regarding antibiotics, penicillin G was putatively identified in samples from cultivations on M-SBP and M-OAT media, whereas penicillin V was only found in M-OAT. Although these antibiotics were putatively identified in cultivation broths, they were not found in the enzyme extracts. Given that enzyme extracts are more suitable for commercialization than raw cultivation broths, this finding is favorable toward its industrial applicability. The presence of limited amounts of antibiotics in the mentioned extracts would be problematic, as it could lead to the appearance of allergies and bacterial antibiotic resistance, which is an increasing global concern (Franco et al. 2009; Laich et al. 2002; Lobanovska and Pilla 2017; Ventola 2015). These findings should be taken into consideration, and further analyses are advisable to ensure the safety of the use of the enzyme extracts, concerning mycotoxin levels and bioactivity. The putative presence of undesirable bioactive compounds in the enzyme extracts may also be avoided by means of heterologous enzyme production in GRAS expression platforms, such as other filamentous fungi, yeast, or bacteria (Sewalt et al. 2016). Furthermore, another alternative to prevent the presence of undesirable metabolites would be targeted knockouts in the biosynthesis of the actual compounds by the employment

of molecular engineering techniques such as CRISPR-Cas9-based genome editing, which has been developed and successfully applied to *P. rubens* (Pohl et al. 2016).

The genetic engineering techniques and proteomic approaches carried out in this work increase the knowledge of the renowned penicillin producer *P. rubens* (formerly, *Penicillium chrysogenum*) Wisconsin 54–1255 cultivated in conditions that lead to the production of plant cell wall degrading enzymes. Several enzymatic activities and putative lignocellulolytic enzymes were detected in the enzyme extracts obtained from this fungal strain, including cellulases, hemicellulases, and pectinases. These findings point to *P. rubens* Wisconsin 54–1255 as a novel source of lignocellulolytic enzymes to be taken into consideration for further studies.

The present work identified two sequences in the genome of *P. rubens* Wisconsin 54–1255 encoding putative FAEs, according to their homology to *Aspergillus* sp. FAEs (ORFs Pc20g07010 and Pc12g08300, encoding the putative FAEs PrFaeA and PrFaeB, respectively). The overexpression of PrFaeA and PrFaeB, followed by FAE activity assays, confirmed their FAE activity. To our knowledge, this constitutes the first report of FAE activity in the aforementioned gene products.

Furthermore, the PrFaeB overexpressing strain obtained and studied in this work (*P. rubens* B13) is a promising candidate for further studies on FAEs and potential industrial applications, considering its FAE activity and suitability for scale-up of the cultivations. The FAE activity detected in its enzyme extract, which surpassed that of the analyzed commercial preparations, has potential applications in industries such as food, feed, pulp and paper, cosmetics, pharmaceuticals, food, feed, and biofuels.

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Author contribution LGC, RRC, RVU, SMA, HS, and CB conceived and designed research. LGC, RRC, MFA, CMV, KFD, and CB conducted experiments. LGC, RRC, KFD, and CB analyzed data and contributed to visualization. LGC and RRC wrote the original draft. LGC, RVU, SMA, KFD, HS, and CB reviewed and edited the draft. All authors read and approved the manuscript. RVU, HS, and CB acquired resources and funding.

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Data availability Data is available in the article's Supplementary material. Additional data and biological material are available on request from the authors. The data that support the findings of this study are available from the corresponding authors (Carlos Barreiro and Ricardo V. Ullán), upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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Table 1. List of oligonucleotides used in this work. Their sequence and applications are indicated. Target sequences for relevant restriction sites appear underlined.

Primer	Sequence (5' → 3') and features	Application
faeA-F	GGAAGATCTATGAAAATCTCCGCACCACG (*Bgl*II site)	Construction of p*faeA* plasmid
faeA-R	AAAAGGCCTCTACCAGCTGCAAGCTCCGC (*Stu*I site)	Construction of p*faeA* plasmid
faeB-F	GGAAGATCTATGACTCGTCTTCATGTGTT (*Bgl*II site)	Construction of p*faeB* plasmid
faeB-R	AAAAGGCCTCTAAACACACTGCCAAGCGT (*Stu*I site)	Construction of p*faeB* plasmid
P*gdh*-F	GGACTCCCTAATGGATTCCGA	Creation of specific probes from *faeA*/*faeB* genes, used in RT-qPCR
T*cyc*-R	GAAAAGGGGGACGGATCTCCG	Used together with P*gdh*-F for the same purposes
actA-F	CTGGCCGTGATCTGACCGACTAC	Creation of *actA* specific probe from *actA* constitutive gene, used in RT-qPCR
actA-R	GGGGGAGCGATCTTGACCT	Used together with *actA*-F for the same purposes
1-*faeB*-F	CTTCATGTGTTGCCCTTGTT	Clone sequencing and verification
2-*faeB*-F	TTCCCGAAACCAACAT	Clone sequencing and verification
3-*faeB*-F	CGCTGGTGCCCCTGCTATC	Clone sequencing and verification
4-*faeB*-F	ATCCTCGCTGCACCAATAAT	Clone sequencing and verification
5-*faeB*-F	ATTGCGGTGATGGAGATG	Clone sequencing and verification
1-*faeB*-R	CACACTGCCAAGCGTTCT	Clone sequencing and verification
2-*faeB*-R	CAGCTTGGAGTTGTCG	Clone sequencing and verification
3-*faeB*-R	GACTTGGGCGCTTGTGAGAC	Clone sequencing and verification
4-*faeB*-R	TTGTCGAAGCCCTCCTCA	Clone sequencing and verification
5-*faeB*-R	CGTGATCTCGCTGCTA	Clone sequencing and verification

Table 2. Protein spots identified by mass spectrometry (MALDI TOF/TOF). Mowse score from Mascot search (protein score), protein coverage (%), secretion parameters [classical secretion systems: SignalP and score; nonclassical systems: SecretomeP and score] are indicated. COG functional categories [C: energy production and conversion; E: amino acid transport and metabolism; G: carbohydrate metabolism and transport; I: lipid transport and metabolism; J: translation, ribosomal structure, and biogenesis; M: cell wall/membrane/envelope biogenesis; P: inorganic ion transport and metabolism; Q: secondary metabolites biosynthesis, transport, and catabolism; S: function unknown], EC numbers, CAZy families and enzyme descriptions are also included. [N/A, not applicable].

Spot no	Protein name	Description	Protein score	Coverage (%)	SignalP	Likelihood (SignalP)	SecretomeP	NN-score (SecretomeP)	Functional category (COG)
1	Pc21g23210	Pc21g23210 protein (glycoside hydrolase family 16, CRH1, predicted)	428	34	Yes	0.9964	__	__	G, M
2	Pc18g01940	β-glucosidase	311	27	Yes	0.9637	__	__	G
3	Pc18g01940	β-glucosidase	605	35	Yes	0.9637	__	__	G
4	Pc12g11110	β-glucosidase	151	17	Yes	0.9768	__	__	G
5	Pc22g14710	Pc22g14710 protein (O-glucosyl hydrolase; xylan 1,4-β-xylosidase activity; glycoside hydrolase family 3)	181	21	Yes	0.967	__	__	G
6	Pc22g14710	Pc22g14710 protein (O-glucosyl hydrolase; xylan 1,4-β-xylosidase activity; glycoside hydrolase family 3)	128	26	Yes	0.967	__	__	G
6	Pc22g24800	β-N-acetylhexosaminidase	112	17	Yes	0.9954	__	__	G
7	Pc22g24800	β-N-acetylhexosaminidase	127	24	Yes	0.9954	__	__	G
8	Pc22g24800	β-N-acetylhexosaminidase	184	27	Yes	0.9954	__	__	G
9	Pc22g24800	β-N-acetylhexosaminidase	660	36	Yes	0.9954	__	__	G
10	Pc16g03030	Pc+G13:G1416g03030 protein (saponin hydrolase)	296	25	Yes	0.82	__	__	S
11	Pc16g03030	Pc16g03030 protein (saponin hydrolase)	281	38	Yes	0.82	__	__	S
12	Pc20g10170	Pc20g10170 protein (β-glucosidase; glycoside hydrolase family 3)	351	28	Yes	0.9782	__	__	G
13	Pc20g10170	Pc20g10170 protein (β-glucosidase; glycoside hydrolase family 3)	664	28	Yes	0.9782	__	__	G
14	Pc16g11860	Catalase	178	24	Yes	0.9604	__	__	C, P, Q
15	Pc16g11860	Catalase	486	44	Yes	0.9604	__	__	C, P, Q
16	Pc16g11860	Catalase	349	30	Yes	0.9604	__	__	C, P, Q
17	Pc16g11860	Catalase	657	41	Yes	0.9604	__	__	C, P, Q
18	Pc16g11860	Catalase	886	49	Yes	0.9604	__	__	C, P, Q
19	Pc16g11860	Catalase	335	34	Yes	0.9604	__	__	C, P, Q
20	Pc16g11860	Catalase	575	39	Yes	0.9604	__	__	C, P, Q
21	Pc20g02640	Pc20g02640 protein (glutaminase)	265	29	Yes	0.9841	__	__	S
22	Pc16g11860	Catalase	787	46	Yes	0.9604	__	__	C, P, Q
23	Pc20g02640	Pc20g02640 protein (glutaminase)	306	29	Yes	0.9841	__	__	S
24	Pc20g02640	Pc20g02640 protein (glutaminase)	244	26	Yes	0.9841	__	__	S
25	Pc20g02640	Pc20g02640 protein (glutaminase)	202	26	Yes	0.9841	__	__	S
26	EN45_021350	β-galactosidase	230	15	Yes	0.8834	__	__	G
26	Pc20g02640	Pc20g02640 protein (glutaminase)	191	26	Yes	0.9841	__	__	S
27	Pc16g11860	Catalase	1090	51	Yes	0.9604	__	__	C, P, Q
28	Pc22g24800	β-N-acetylhexosaminidase	350	26	Yes	0.9954	__	__	G
29	Pc20g02640	Pc20g02640 protein (glutaminase)	794	42	Yes	0.9841	__	__	S

Table 2 (continued)

Spot no	Protein name	Description	Protein score	Coverage (%)	SignalP	Likelihood (SignalP)	SecretomeP	NN-score (SecretomeP)	Functional category (COG)
30	Pc22g08520	Pc22g08520 protein (β-glucuronidase; glycoside hydrolase family 79)	133	22	Yes	0.9896	__	__	S
31	Pc20g02640	Pc20g02640 protein (glutaminase)	568	32	Yes	0.9841	__	__	S
32	Pc22g08520	Pc22g08520 protein (β-glucuronidase; glycoside hydrolase family 79)	495	29	Yes	0.9896	__	__	S
33	Pc13g15360	Carboxylic ester hydrolase	138	20	Yes	0.9458	__	__	G, I
34	Pc12g08730	Pc12g08730 protein (β-N-acetylglucosaminidase, glycoside hydrolase family 84)	562	32	Yes	0.9976	__	__	J, O
35	Pc16g08980	Endo-1,3(4)-β-glucanase	416	42	Yes	0.9603	__	__	G, M
36	Pc16g11860	Catalase	352	27	Yes	0.9604	__	__	C, P, Q
37	Pc22g01750	Pc22g01750 protein (amidase)	99	27	No	0.0007	No	0.531	J
38	Pc20g01970	Glucanase	226	38	Yes	0.9925	__	__	G
39	Pc12g08300	Carboxylic ester hydrolase	261	24	Yes	0.9953	__	__	G
40	Pc20g01970	Glucanase	479	42	Yes	0.9925	__	__	G
41	Pc12g08300	Carboxylic ester hydrolase	494	30	Yes	0.9953	__	__	G
42	Pc12g08300	Carboxylic ester hydrolase	734	43	Yes	0.9953	__	__	G
43	Pc22g08520	Pc22g08520 protein (β-glucuronidase; glycoside hydrolase family 79)	413	22	Yes	0.9896	__	__	S
43	Pc20g10360	β-N-acetylhexosaminidase hex	260	30	Yes	0.978	__	__	G
44	Pc12g08300	Carboxylic ester hydrolase	744	37	Yes	0.9953	__	__	G
45	Pc20g10360	β-N-acetylhexosaminidase hex	426	46	Yes	0.978	__	__	G
46	Pc20g10360	β-N-acetylhexosaminidase hex	438	42	Yes	0.978	__	__	G
47	Pc20g10360	β-N-acetylhexosaminidase hex	472	35	Yes	0.978	__	__	G
48	Pc20g10360	β-N-acetylhexosaminidase hex	446	48	Yes	0.978	__	__	G
49	Pc20g10360	β-N-acetylhexosaminidase hex	270	30	Yes	0.978	__	__	G
50	Pc20g01970	Glucanase	513	43	Yes	0.9925	__	__	G
51	Pc20g10360	β-N-acetylhexosaminidase hex	278	25	Yes	0.978	__	__	G
52	Pc20g10360	β-N-acetylhexosaminidase hex	279	25	Yes	0.978	__	__	G
53	Pc20g10360	β-N-acetylhexosaminidase hex	242	28	Yes	0.978	__	__	G
54	Pc20g10360	β-N-acetylhexosaminidase hex	412	42	Yes	0.978	__	__	G
55	Pc20g10360	β-N-acetylhexosaminidase hex	520	42	Yes	0.978	__	__	G
56	Pc20g10360	β-N-acetylhexosaminidase hex	463	42	Yes	0.978	__	__	G
57	Pc20g10360	β-N-acetylhexosaminidase hex	361	30	Yes	0.978	__	__	G

Table 2 (continued)

Spot no	Protein name	Description	Protein score	Coverage (%)	SignalP	Likelihood (SignalP)	SecretomeP	NN-score (SecretomeP)	Functional category (COG)
58	Pc20g10360	β-N-acetylhexosaminidase hex	375	37	Yes	0.978	__	__	G
59	Pc20g01970	Glucanase	245	40	Yes	0.9925	__	__	G
60	Pc20g10360	β-N-acetylhexosaminidase hex	759	46	Yes	0.978	__	__	G
61	Pc20g10360	β-N-acetylhexosaminidase hex	706	46	Yes	0.978	__	__	G
62	Pc20g10360	β-N-acetylhexosaminidase hex	957	44	Yes	0.978	__	__	G
63	Pc21g14410	Pc21g14410 protein (endoglucanase; glycoside hydrolase family 5)	271	34	Yes	0.9964	__	__	G
64	Pc22g19620	α-L-arabinofuranosidase	173	35	Yes	0.9955	__	__	G, M
65	Pc14g00370	Rhamnogalacturonate lyase	504	40	Yes	0.9952	__	__	G, M
66	Pc14g00370	Rhamnogalacturonate lyase	217	35	Yes	0.9952	__	__	G, M
67	Pc18g05490	Glucanase	159	33	Yes	0.9873	__	__	G
68	Pc18g05490	Glucanase	167	30	Yes	0.9873	__	__	G
69	Pc18g05490	Glucanase	176	30	Yes	0.9873	__	__	G
70	Pc18g05490	Glucanase	84	27	Yes	0.9873	__	__	G
71	Pc12g14860	Extracellular acid phosphatase PhoA	253	39	Yes	0.96	__	__	O, S
72	Pc22g19620	α-L-arabinofuranosidase	95	28	Yes	0.9955	__	__	G, M
73	Pc21g12590	FAD-linked oxidoreductase chyH	284	36	Yes	0.9255	__	__	C
74	Pc22g19620	α-L-arabinofuranosidase	318	35	Yes	0.9955	__	__	G, M
75	Pc22g19620	α-L-arabinofuranosidase	701	40	Yes	0.9955	__	__	G, M
76	Pc21g12590	FAD-linked oxidoreductase chyH	391	27	Yes	0.9255	__	__	C
77	Pc22g19620	α-L-arabinofuranosidase	102	29	Yes	0.9955	__	__	G, M
78	Pc14g00370	Rhamnogalacturonate lyase	666	50	Yes	0.9952	__	__	G, M
79	EN45_000350	Rhamnogalacturonate lyase	911	54	Yes	0.9952	__	__	G, S
80	Pc22g19620	α-L-arabinofuranosidase	331	35	Yes	0.9955	__	__	G, M
81	Pc22g19620	α-L-arabinofuranosidase	754	42	Yes	0.9955	__	__	G, M
82	Pc20g10360	β-N-acetylhexosaminidase hex	263	29	Yes	0.978	__	__	G
83	Pc21g12590	FAD-linked oxidoreductase chyH	543	37	Yes	0.9255	__	__	C
84	Pc22g01850	1,4-α-D-glucan glucohydrolase	469	53	Yes	0.8674	__	__	G
85	Pc16g14170	α-1,2-mannosidase	260	47	Yes	0.9931	__	__	G
86	Pc22g22810	Pc22g22810 protein (putative thioredoxin reductase)	207	43	Yes	0.9456	__	__	C, O
87	Pc21g19010	Pc21g19010 protein (metal-independent α-mannosidase; glycoside hydrolase family 125)	89	24	Yes	0.9869	__	__	G, S
88	Pc21g23210	Pc21g23210 protein (glycosidase; glycoside hydrolase family 16)	178	29	Yes	0.9964	__	__	G, M
89	Pc21g23210	Pc21g23210 protein (glycosidase; glycoside hydrolase family 16)	542	56	Yes	0.9964	__	__	G, M

Table 2 (continued)

Spot no	Protein name	Description	Protein score	Coverage (%)	SignalP	Likelihood (SignalP)	SecretomeP	NN-score (SecretomeP)	Functional category (COG)
90	Pc21g23210	Pc21g23210 protein (glycosidase; glycoside hydrolase family 16)	147	23	Yes	0.9964	__	__	G, M
91	Pc18g05530	Pc18g05530 protein (FAD-linked oxidoreductase)	184	22	Yes	0.896	__	__	C
92	Pc21g19010	Pc21g19010 protein (metal-independent α-mannosidase; glycoside hydrolase family 125)	349	28	Yes	0.9869	__	__	G, S
93	Pc13g09520	Chitinase	86	33	No	0.0039	No	0.586	G
94	Pc13g09520	Chitinase	237	48	No	0.0039	No	0.586	G
95	Pc22g00820	Beta-xylanase	117	20	Yes	0.9637	__	__	G
96	Pc18g01880	Exo-arabinanase abnx	190	24	Yes	0.7453	__	__	G, S, O
97	Pc18g01880	Exo-arabinanase abnx	228	24	Yes	0.7453	__	__	G, S, O
98	Pc18g01880	Exo-arabinanase abnx	225	24	Yes	0.7453	__	__	G, S, O
99	Pc21g19010	Pc21g19010 protein (metal-independent α-mannosidase; glycoside hydrolase family 125)	278	24	Yes	0.9869	__	__	G, S
100	Pc18g01880	Exo-arabinanase abnx	658	40	Yes	0.7453	__	__	G, S, O
101	Pc18g01880	Exo-arabinanase abnx	148	24	Yes	0.7453	__	__	G, S, O
102	Pc18g01880	Exo-arabinanase abnx	260	28	Yes	0.7453	__	__	G, S, O
103	Pc18g01880	Exo-arabinanase abnx	643	41	Yes	0.7453	__	__	G, S, O
104	EN45_103640	Chitinase	150	31	Yes	0.9956	__	__	G
105	EN45_103640	Chitinase	405	62	Yes	0.9956	__	__	G
106	EN45_103640	Chitinase	554	80	Yes	0.9956	__	__	G
107	Pc18g03120	Pc18g03120 protein (β-1,3-glucanase; glycoside hydrolase family 64)	283	45	No	0.0024	Yes	0.72	S
108	Pc22g15910	Probable Xaa-Pro aminopeptidase P	105	15	No	0.0017	No	0.434	E
109	Pc13g09680	Penicillopepsin (aspartic-type endopeptidase activity)	140	32	Yes	0.975	__	__	M, O
110	Pc21g09850	Pc21g09850 protein (pectin lyase; polysaccharide lyase 1)	363	47	Yes	0.9005	__	__	G
111	Pc22g24890	Pc22g24890 protein (pectate lyase; polysaccharide lyase family 1)	219	49	Yes	0.989	__	__	G
112	Pc22g24890	Pc22g24890 protein (pectate lyase; polysaccharide lyase family 1)	573	50	Yes	0.989	__	__	G
113	Pc22g24890	Pc22g24890 protein (pectate lyase; polysaccharide lyase family 1)	876	52	Yes	0.989	__	__	G
114	Pc13g08730	Endo-1,3-beta-glucanase eglC	140	46	Yes	0.9941	__	__	G
115	Pc21g14160	Alkaline serine protease AAM33821	182	43	Yes	0.924	__	__	O
116	Pc13g08730	Endo-1,3-beta-glucanase eglC	333	73	Yes	0.9941	__	__	G
117	Pc21g23210	Pc21g23210 protein (glycosidase; glycoside hydrolase family 16)	146	44	Yes	0.9964	__	__	G, M

Table 2 (continued)

Spot no	Protein name	Description	Protein score	Coverage (%)	SignalP	Likelihood (SignalP)	SecretomeP	NN-score (SecretomeP)	Functional category (COG)
118	Pc21g23210	Pc21g23210 protein (glycosidase; glycoside hydrolase family 16)	142	33	Yes	0.9964	__	__	G, M
119	Pc21g23210	Pc21g23210 protein (glycosidase; glycoside hydrolase family 16)	184	40	Yes	0.9964	__	__	G, M
120	Pc20g09400	Pc20g09400 protein (serine-type peptidase activity; peptidase family S9)	174	17	No	0.0041	Yes	0.682	E, O
120	Pc22g13950	Pc22g13950 protein (aminopeptidase; peptidase family M18)	168	25	No	0,0063	No	0.532	E

Table 3. Cellulase and xylanolytic activities of *P. rubens* B13. Reactions performed at pH 5.0 and 50 °C. U, unit, defined as the amount of enzyme that releases one μmole of product (4-nitrophenol for cellobiohydrolase, β-glucosidase, and β-xylosidase; glucose for endoglucanase; xylose for endoxylanase) per min at the given conditions. Values given as means, including standard deviations.

Lignocellulolytic activity	Specific activity (U/mg total protein)
β-glucosidase	2.65 ± 0.27
Cellobiohydrolase	0.52 ± 0.03
Endoglucanase	0.33 ± 0.039
β-xylosidase	0.33 ± 0.02
Endoxylanase	0.3 ± 0.03

Table 4. Simple sugars released after treatment of sugar beet pulp (SBP) with enzyme extracts recovered from *P. rubens* Wisconsin 54–1255 (WIS) and transformant B13 (B13) fermentations on M-SBP. Reactions performed adding 15 mg extract/g SBP, 24 h at pH 5.0, 50 °C and 175 rpm. Values given as means from three replicates, including the standard deviations.

Monosaccharide/ disaccharide	Saccharification power (g/L released) by WIS	Saccharification power (g/L released) by B13
Glucose	2.72 ± 0.41	3.35 ± 0.99
Galactose	N/D [a]	N/D [a]
Fructose	1.88 ± 0.20	2.08 ± 0.67
Arabinose	3.62 ± 0.82	3.83 ± 0.68
Sucrose	N/D [a]	N/D [a]

[a] N/D, non-detected

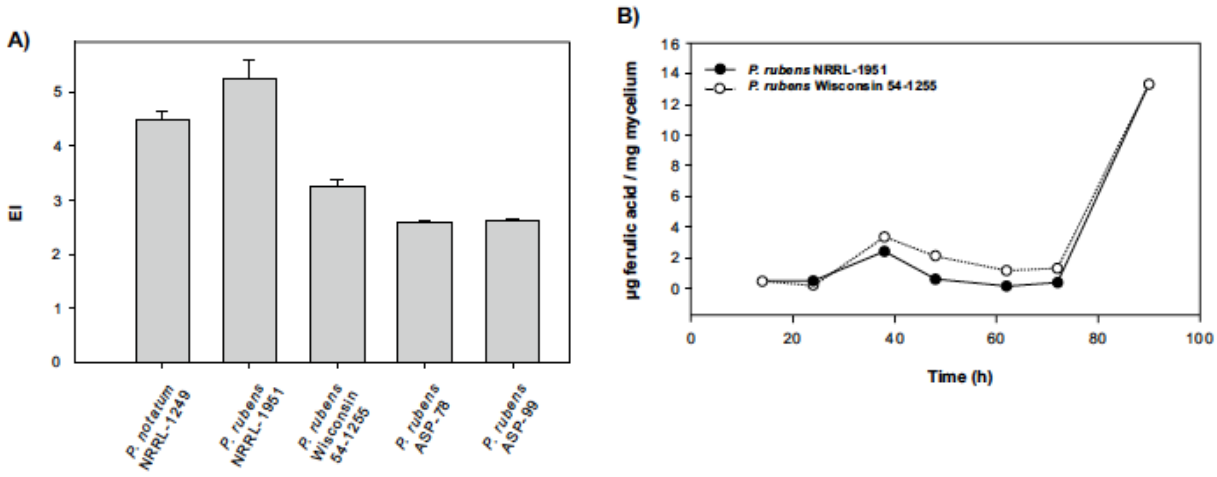

Fig. 1. A Enzymatic indices (EI) for each analyzed strain (indirect measurement of FAE activity through the ability to hydrolyze ethyl ferulate on a plate assay; [EI = (diameter of the colony + diameter of the degradation halo) / diameter of the colony]). Strain names are indicated in the figure. **B** FAE activity of *P. rubens* Wisconsin 54–1255 and *P. rubens* NRRL-1951, determined by the release of ferulic acid from sugar beet pulp in liquid medium at 28 °C and 250 rpm.

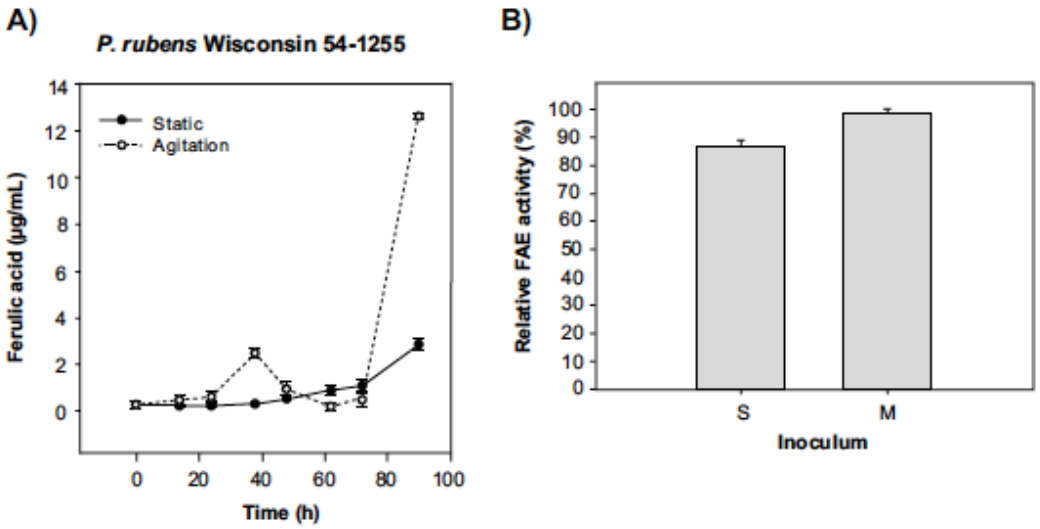

Fig. 2. A Comparative analysis of *P. rubens* Wisconsin 54–1255 FAE activity in submerged culture at 28 °C, with (250 rpm) or without constant agitation, through the release of ferulic acid from sugar beet pulp. **B** FAE activity of enzyme extracts obtained from *P. rubens* Wisconsin 54–1255 grown in M-SBP medium, after being inoculated with a spore suspension (S) or 10% (v/v) of a seed culture grown for 24 h in PMMY medium, at 28 °C and 250 rpm (M). Data expressed as relative FAE activity values compared to the maximum (100%).

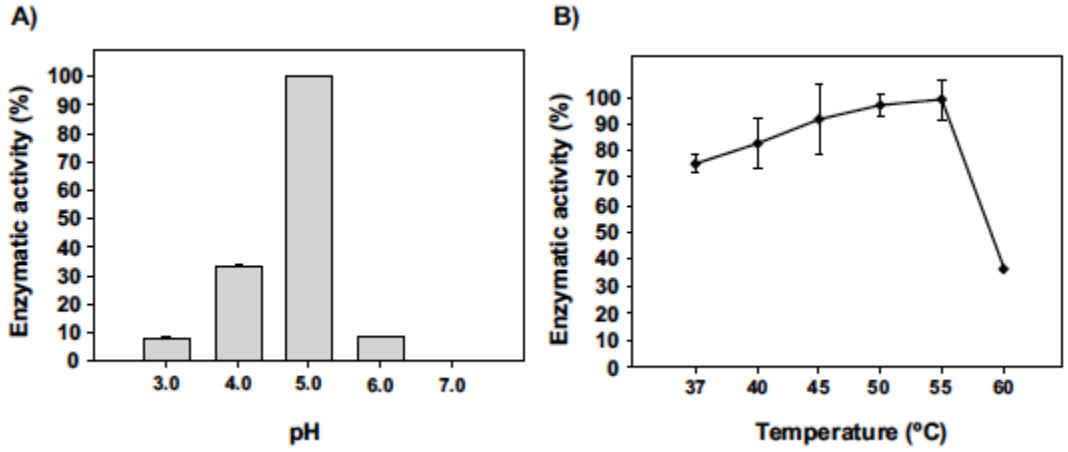

Fig. 3. Effect of pH and temperature on FAE activity of *P. rubens* Wisconsin 54–1255 enzyme extract against methyl ferulate. FAE activity is expressed as relative values compared to the maximum (100%). **A** Influence of pH on FAE activity at 37 °C. **B** Influence of temperature.

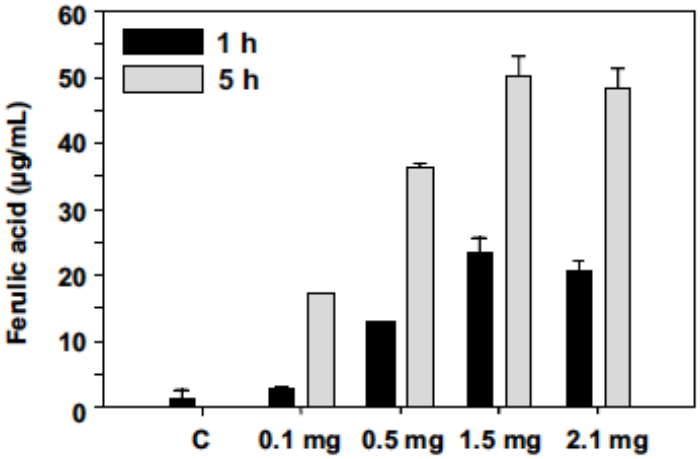

Fig. 4. Release of ferulic acid from sugar beet pulp at pH 5.0, 50 °C, and 175 rpm, by different concentrations of *P. rubens* Wisconsin 54–1255 enzyme extract and at two incubation times (1 h and 5 h). C, control without enzyme extract.

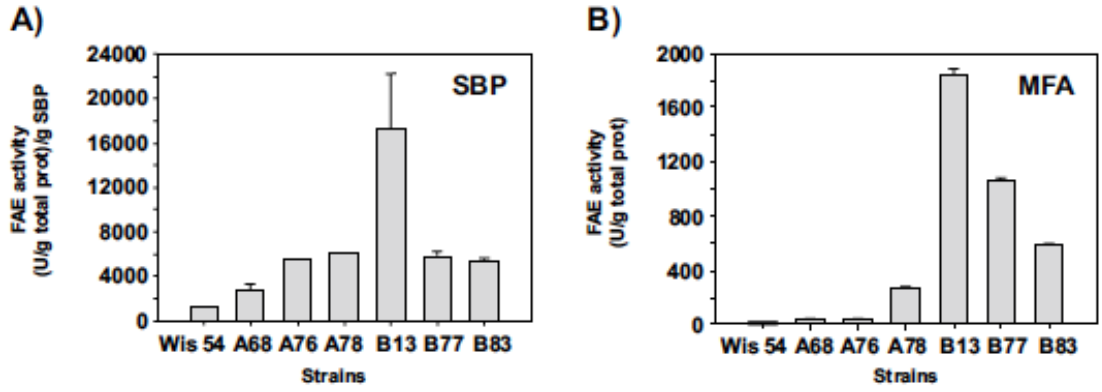

Fig. 5. FAE activity of *P. rubens* Wisconsin 54–1255 and its transformants A68, A76, A78, B13, B77, and B83 enzyme extracts against the substrates sugar beet pulp (SBP) **(A)** and methyl ferulate **(B)**. One unit (U) corresponds to the amount of enzyme that releases one μmol of ferulic acid per min under the assay conditions (pH 5.0 and 50 °C).

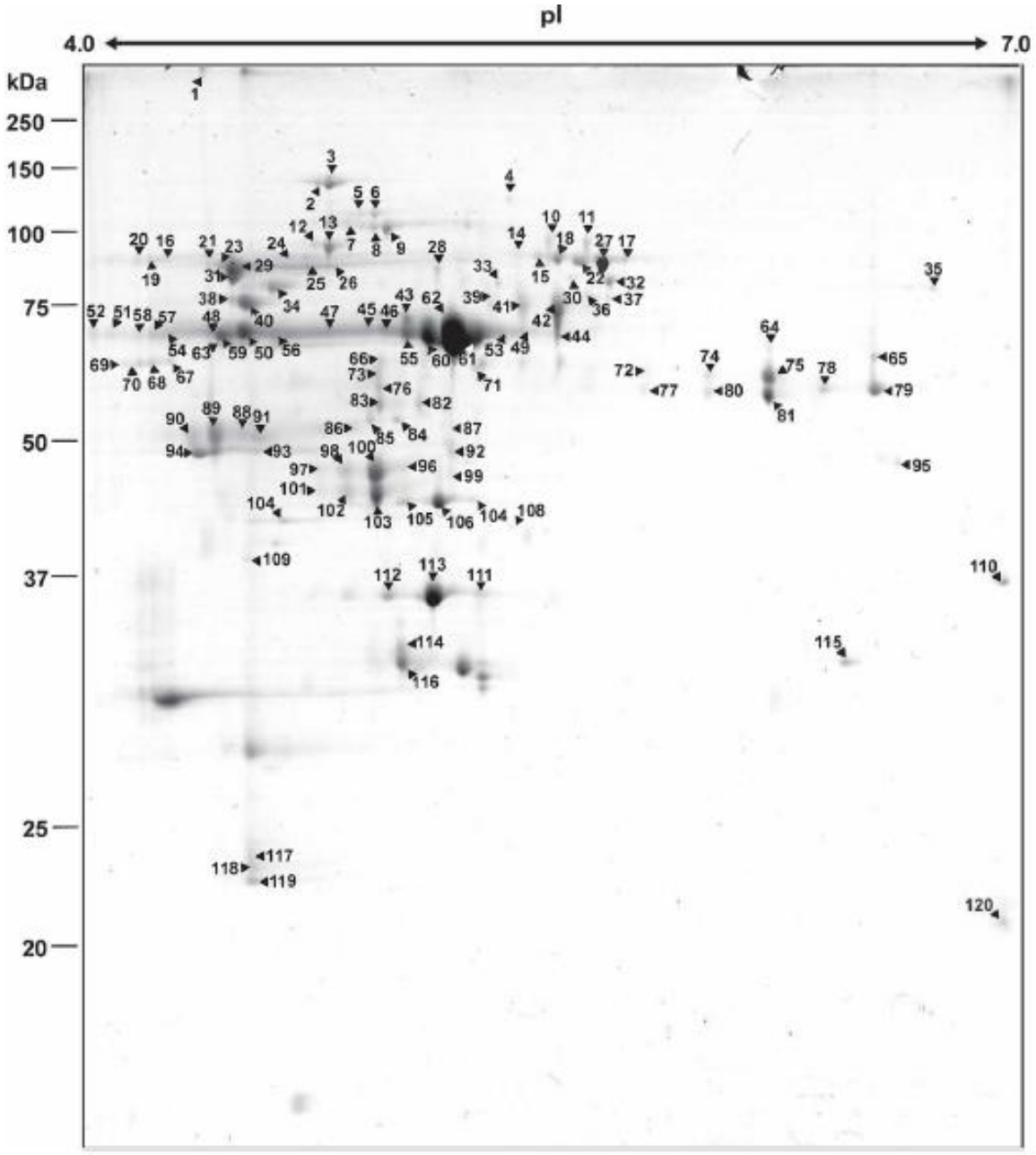

Fig. 6. Reference map of the *P. rubens* B13 grown in M-SBP partial extracellular proteome; 205 protein spots were visualized in a 4.0–7.0 pH range, and 120 were successfully identified (numbered in the gel image. Numbers correspond to those in Table 2 and Supplementary File S7). The pH range is marked above the gel and molecular weights (kDa), on the left.

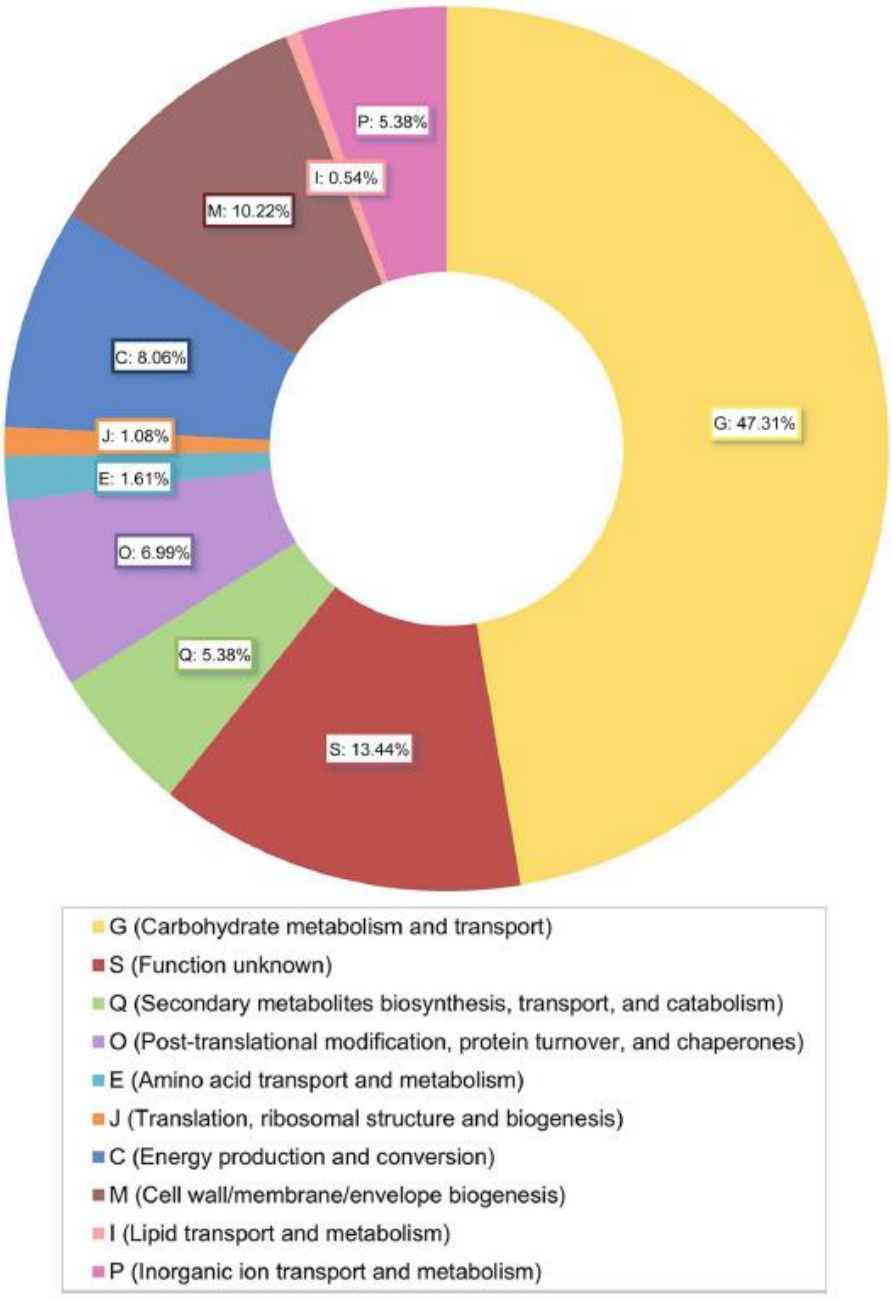

Fig. 7. Functional classification of all the protein species identified from *P. rubens* B13 partial extracellular proteomes when grown on the lignocellulosic natural substrate sugar beet pulp. The classification was based on a functional annotation according to the COG (Clusters of Orthologous Groups of proteins) system, which includes eukaryote organisms (KOG), via the eggNOG 5.0 online orthology resource (Huerta- Cepas et al. 2019; Tatusov et al. 2003).

Applied Microbiology and Biotechnology

Penicillium chrysogenum as a profitable fungal factory for feruloyl esterases

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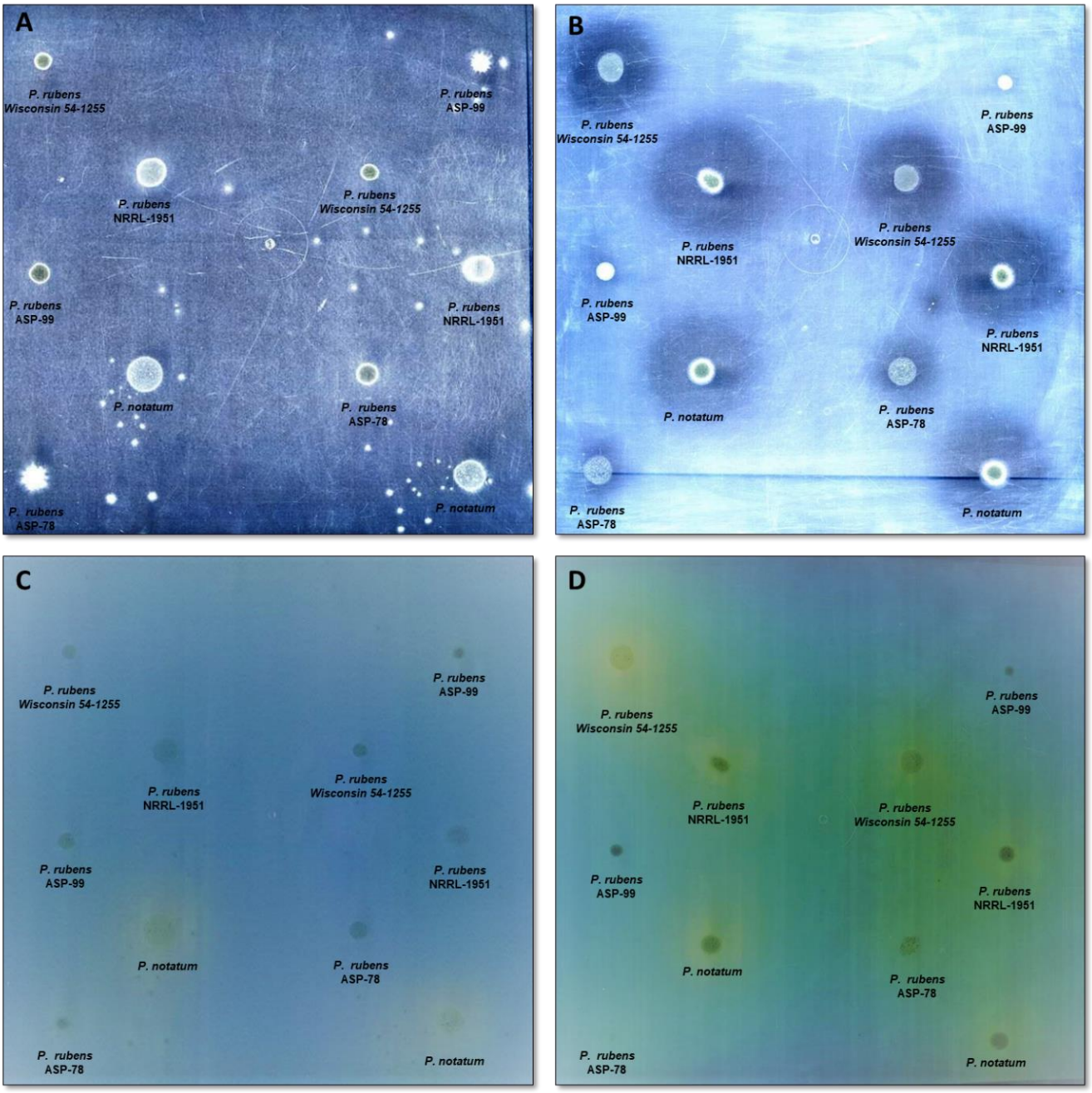
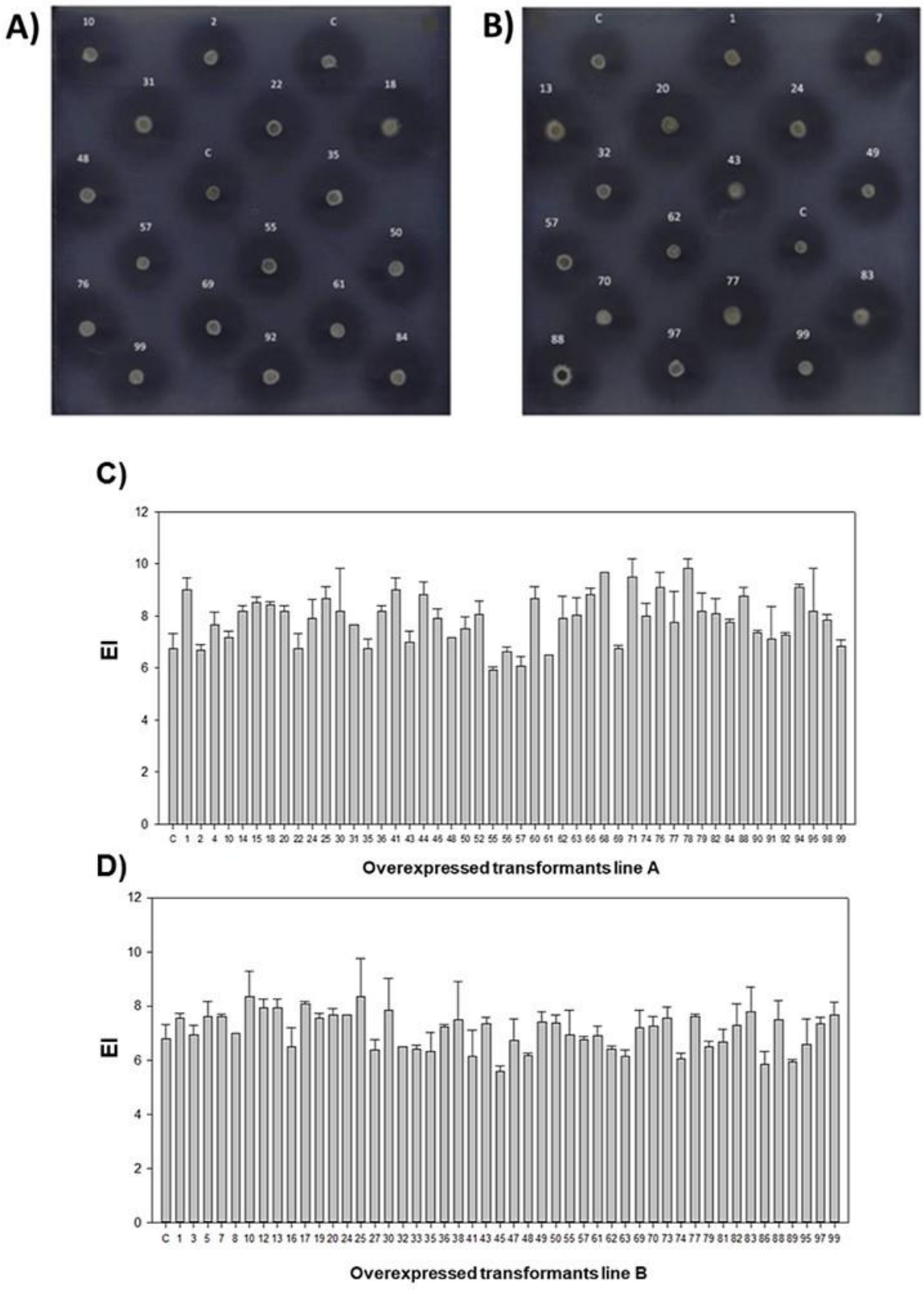
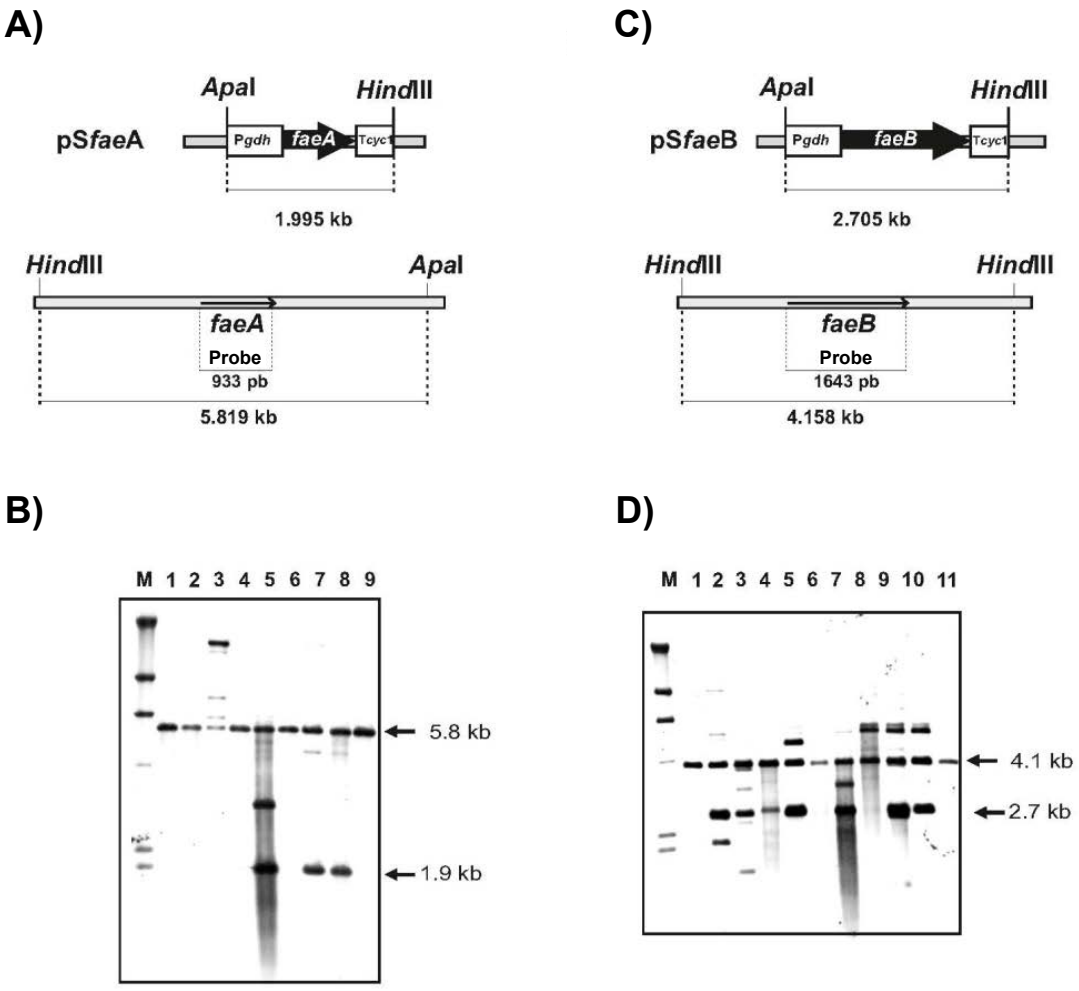


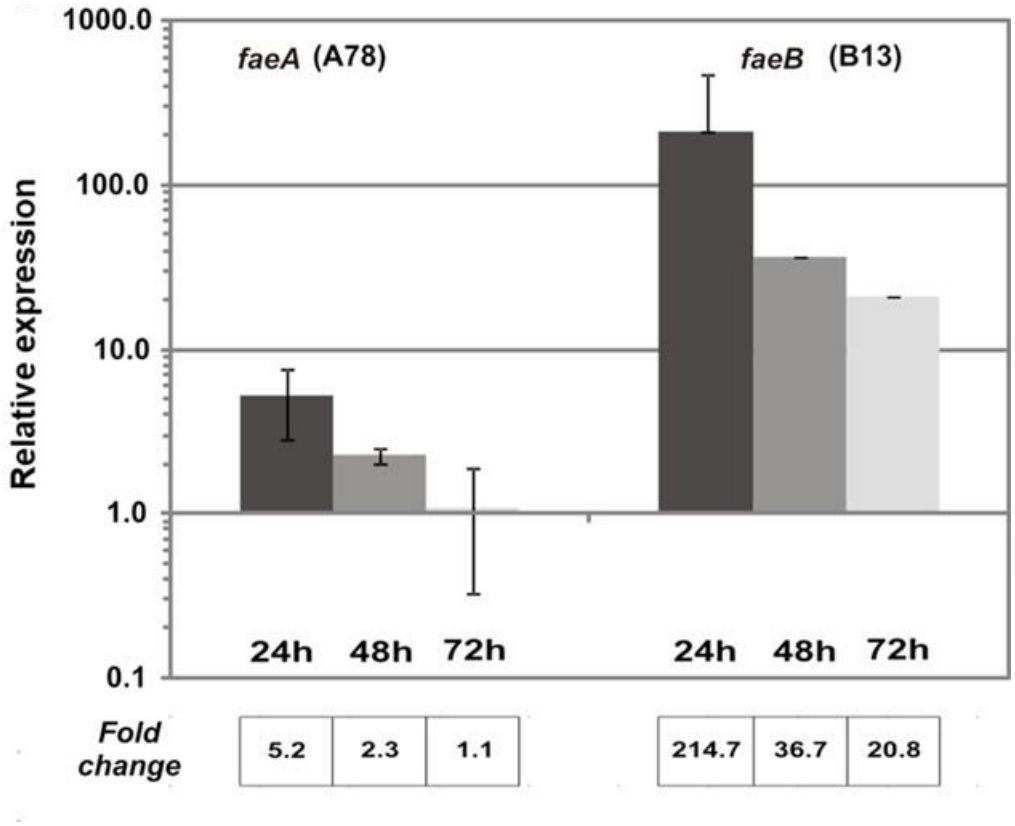
Supplementary Figure S1. FAE activity of several *Penicillium* strains on plate assay. FAE activity is displayed as an EF hydrolysis halo surrounding the colonies. **A)** Control plate without EF. **B)** Plate with EF. **C)** Control plate without EF, stained with bromocresol green. **D)** Plate with EF, stained with bromocresol green.



Supplementary Figure S2. Hydrolysis halos observed in the EF plate assay for some of the 48 transformants chosen from line A (overexpressing *faeA*) **(A)** and from line B (overexpressing *faeB*) **(B)**. The parent strain *P. rubens* Wisconsin 54-1255 was used as control (c). **C)** EI of the analyzed transformants from line A. The figure shows averages and standard deviations from three biological replicates. **D)** EI of the analyzed transformants from line B. The figure shows averages and standard deviations from three biological replicates.

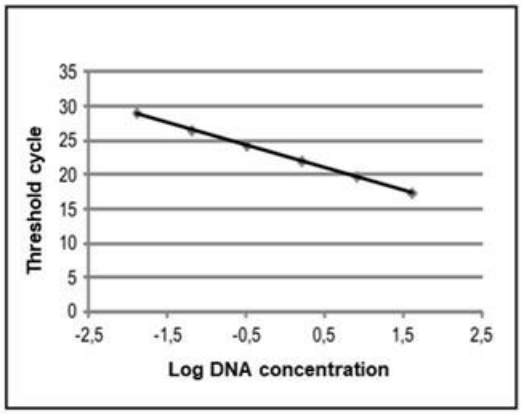


Supplementary Figure S3. Overexpression of *faeA* and *faeB* genes in *P. rubens* Wisconsin 54-1255. **A)** Physical map of the pS*faeA* plasmid for the overexpression of *faeA* and schematic representation of the *P. rubens* genome region containing gene *faeA*. The size of the hybridization band of the P*gdh-fae*A-Tcyc1 expression cassette is indicated. **B)** Southern hybridization of total DNA of 8 transformants and the parent strain *P. rubens* Wisconsin 54-1255 with a 933 bp probe corresponding to the sequence of *faeA*. 1: Wisconsin 54-1255; 2: A1; 3: A18; 4: A31; 5: A68; 6: A71; 7: A76; 8: A78; 9: A84. **C)** Schematic representation of the pS*faeB* plasmid, containing the expression cassette P*gdh-faeB*-Tcyc1, and the region of the *P. rubens* genome containing *faeB*. **D)** Southern hybridization of total DNA of 10 transformants and the parental strain *P. rubens* Wisconsin 54-1255 with a 1643 bp probe corresponding to the *faeB* gene sequence: 1: Wisconsin 54-1255; 2: B1; 3: B7; 4: B10; 5: B13; 6: B17; 7: B20; 8: B27; 9: B77; 10: B83; 11: B88. M: λ DNA/*Hind*III molecular weight marker labeled with digoxigenin.

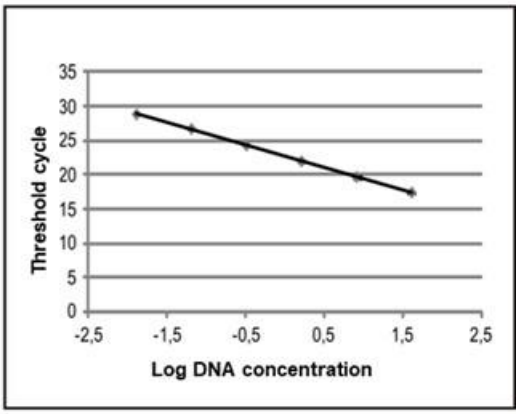


Supplementary Figure S4. Analysis of the expression of *faeA* and *faeB* genes by RT-qPCR in *faeA* overexpressing (A78) and *faeB* overexpressing (B13) *P. rubens* transformants. Relative values refer to the expression values for each *fae* gene in the parent strain *P. rubens* Wisconsin 54-1255. The expression of *actA* (encoding Y-actin) was used as a control. Error bars indicate the standard deviation of the ratio value between three biological replicates and three technical replicates of each sample. Fold change values are shown.

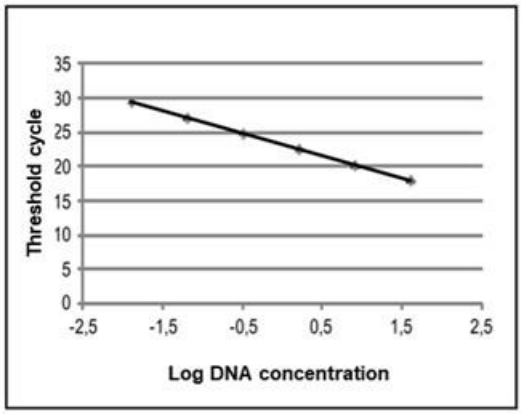
A) *faeA*



B) *faeB*



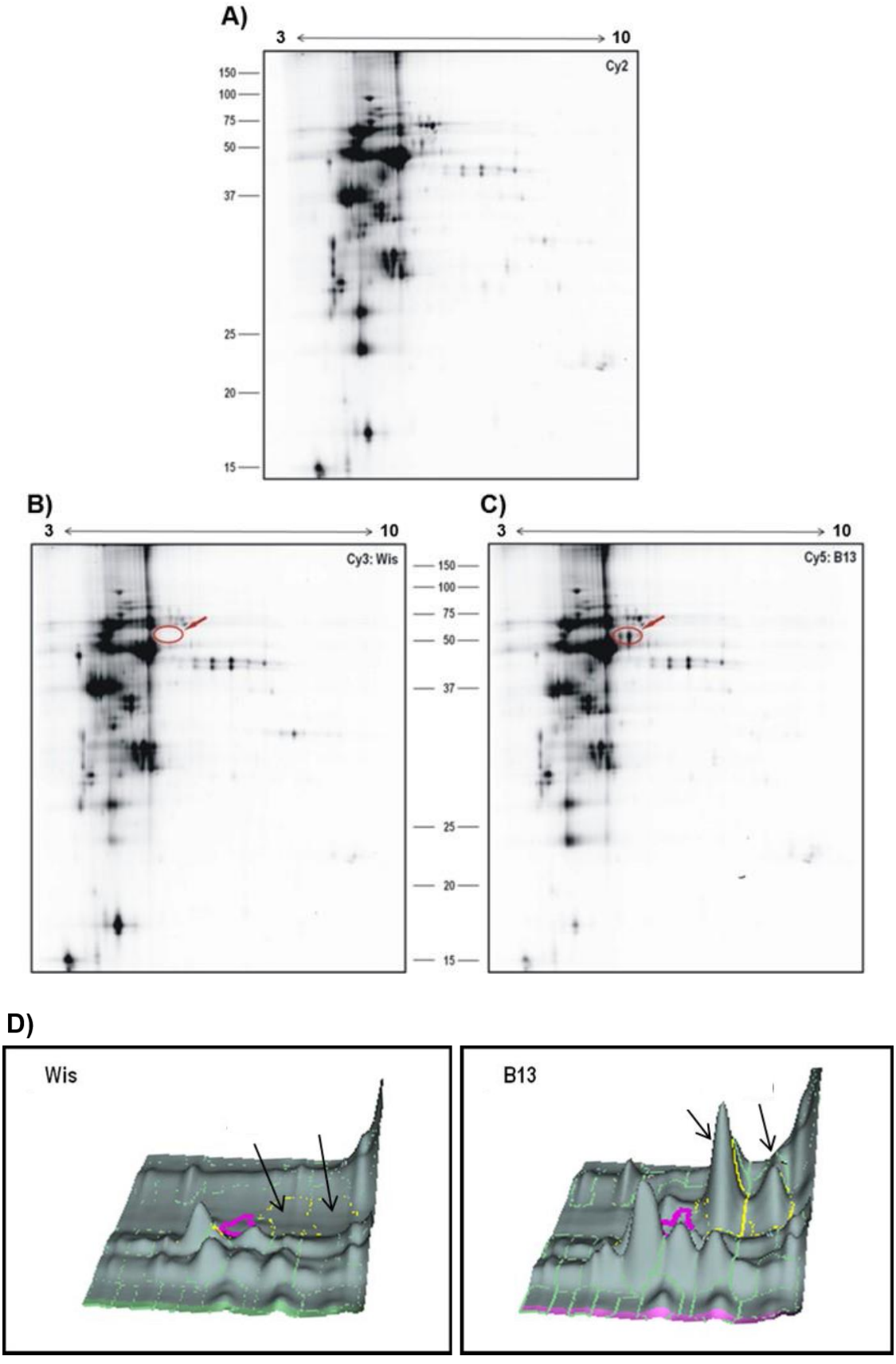
C) *act*



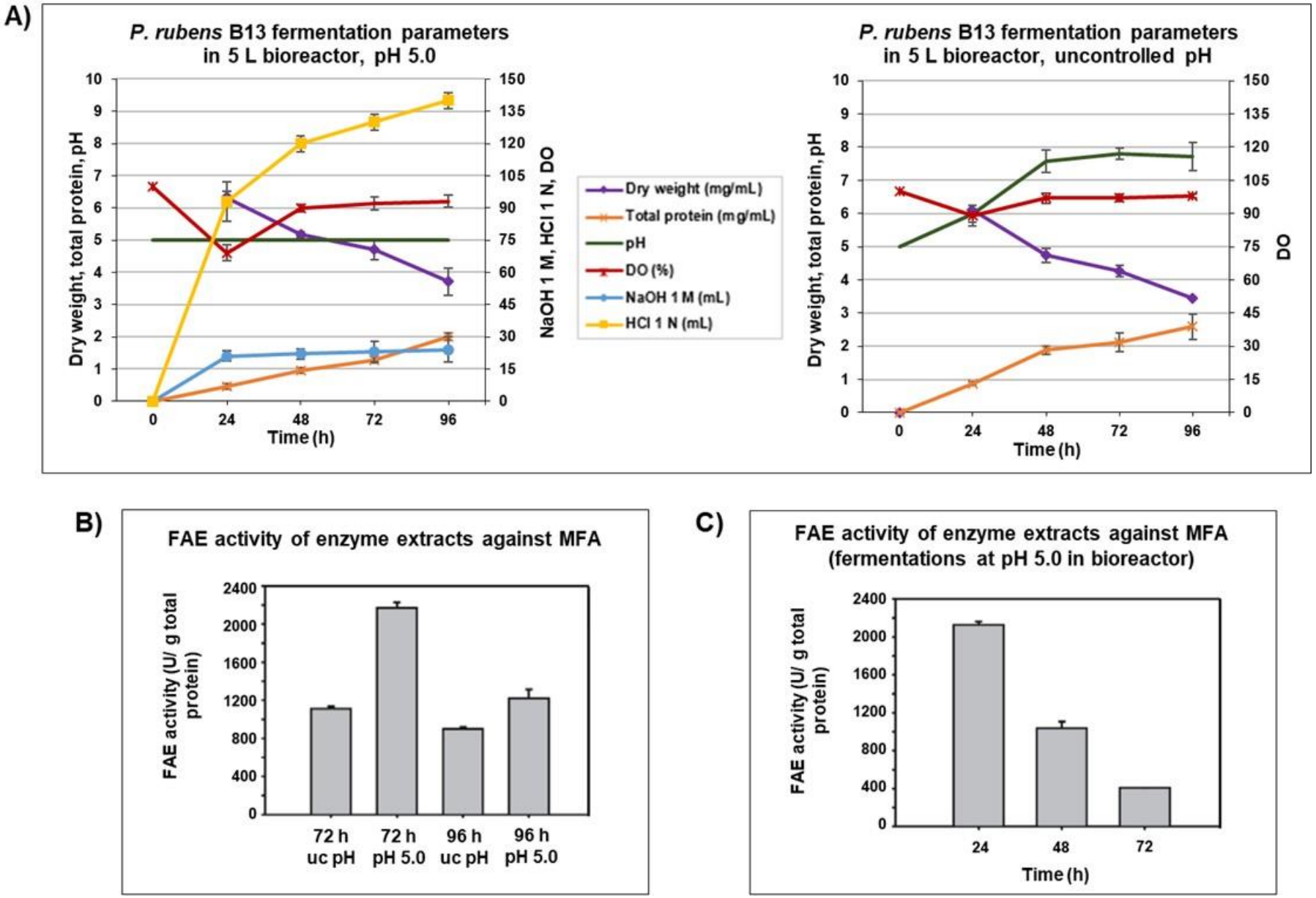
D)

	Slope	R^2	Efficiency
LfaeA/RfaeA	-3.3104	0.999	1.00
LfaeB/RfaeB	-3.2856	0.999	1.01
Lact/Ract	-3.2855	0.999	1.01

Supplementary Figure S5. Efficiency of the RT-qPCR amplification. Representation of the standard concentrations of DNA vs. the threshold cycle, to calculate the efficiency of amplification for the different analyzed genes: *faeA* **(A)**, *faeB* **(B)**, and *actA* **(C)**. **D)** Slope, R2, and efficiency of the amplification data for each of the amplified cDNA fragments of the analyzed genes.

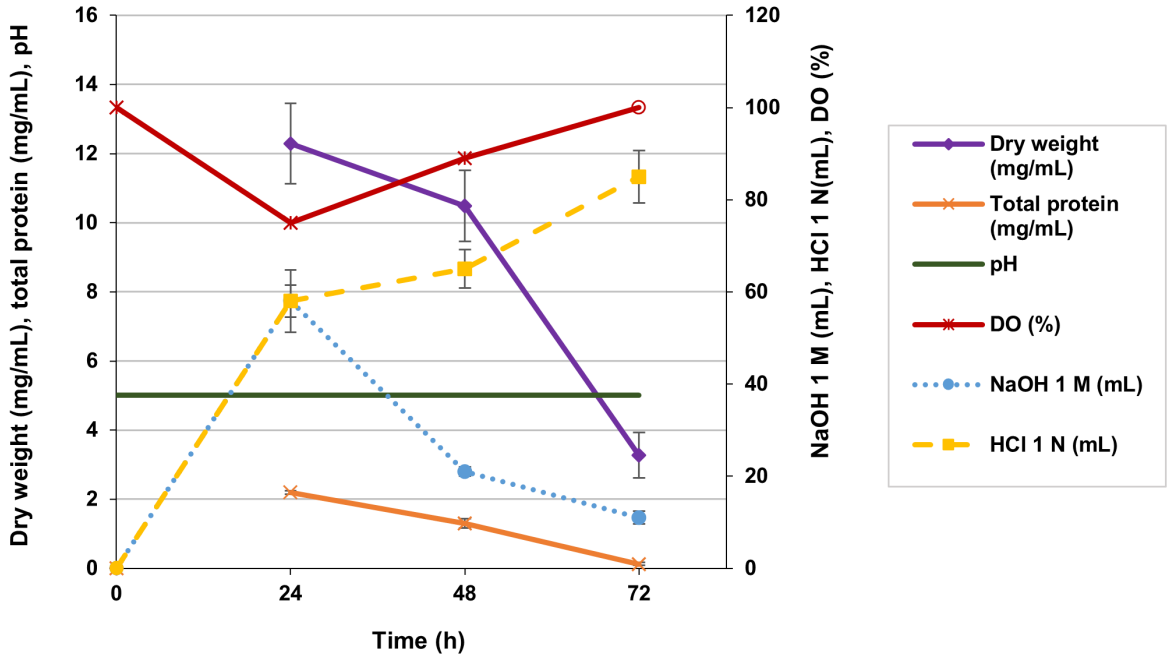


Supplementary Figure S6. 2D-DIGE analysis of differences in the extracellular protein production by *P. rubens* Wisconsin 54-1255 (Wis) and B13 strains, grown on M-SBP medium for FAE production. A) Internal standard (Cy2). B) *P. rubens* Wisconsin 54-1255 strain (Cy3). C) *P. rubens* B13 strain (Cy5). D) 3D representation on DeCyder software (GE Healthcare) of the abundance of the two protein spots identified as PrFaeB (Pc12g08300) in both strains. Arrows indicate the two protein spots identified as PrFaeB.



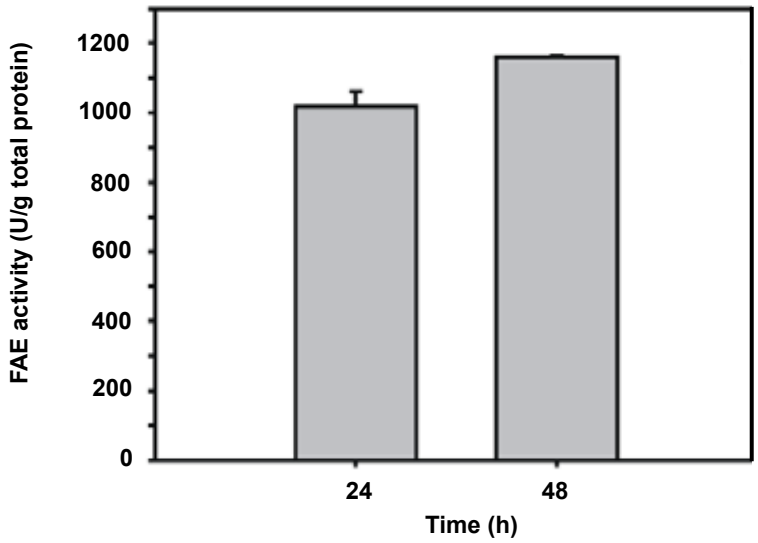
Supplementary Figure S8. Fermentation parameters and FAE activity of *P. rubens* B13 in 5 L bioreactors with M-SBP medium [U= unit; amount of enzyme that releases one µmol of ferulic acid per min under the tested conditions (20 min reactions at pH 5.0 and 50 ºC)]. **A)** Fermentation parameters in 5 L bioreactors at pH 5.0 (left) and uncontrolled pH (right). **B)** FAE activity against MFA of enzyme extracts recovered at 72 and 96 h from fermentations in a bioreactor carried out at 28 ºC, at pH 5.0 and uncontrolled pH (uc pH). **C)** FAE activity against MFA of enzyme extracts recovered at 24, 48, and 72 h from fermentations in a bioreactor carried out at 28 ºC and pH 5.0.

A) ***P. rubens*** **B13 fermentation parameters in 20 L bioreactor, pH 5.0**



B)

FAE activity of enzyme extracts against MFA (20 L bioreactor)



Supplementary Figure S9. Fermentation parameters and FAE activity of *P. rubens* B13 in 20 L bioreactors with M-SBP medium. **A)** Fermentation parameters in 20 L bioreactors at 28 ºC and pH 5.0. The figure shows values for dry weight (mg/mL), total protein (mg/mL), pH, DO (%), NaOH 1 M (mL added since the previous time-point), HCl 1 M (mL added since previous time-point) **B)** FAE activity against MFA of enzyme extracts recovered at 24 and 48 h from fermentations in 20 L bioreactors, containing M-SBP medium, at 28 ºC and pH 5.0. FAE activity is expressed in U/g protein from enzyme extract [U= unit; amount of enzyme that releases one µmol of ferulic acid per min under the tested conditions (20 min reactions at pH 5.0 and 50 ºC)].