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ORIGINAL PAPER

Characterization, optimization, and scale-up of cellulases production by *trichoderma reesei* cbs 836.91 in solid-state fermentation using agro-industrial products

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Abstract The application of cellulases in saccharification processes is restricted by its production cost. Consequently, new fungal strains able to elaborate higher cellulases titers and with special activity profiles are required to make the process economical. The aim of this investigation was to find a promising wild-type *Trichoderma* strain for cellulases production. The *Trichoderma reesei* strain 938 (CBS 836.91) was selected among twenty strains on the basis of cellulase-agar-plate screening. Evaluation of the selected strain on six solid substrates indicated the highest activities to be obtained from wheat bran. Statistical analyses of the experimental design indicated a significant effect of pH and moisture on the generation of endoglucanase (EGA) and filter-paper (FPA) activity. Furthermore, a central-composite design-based optimization revealed that pH values between 6.4 and 6.6 and moisture from 74 to 94 % were optimal for cellulases production. Under these conditions, 8–10 IU gds^{-1} of FPA and 15.6–17.8 IU gds^{-1} of EGA were obtained. In addition, cultivation in a rotating-drum reactor under optimal conditions gave 8.2 IU gds^{-1} FPA and 13.5 IU gds^{-1} EGA. Biochemical characterization of *T. reesei* 938 cellulases indicated a substantially higher resistance to 4 mM Fe^{+2} and a slightly greater tolerance to alkaline pH in comparison to Celluclast®. These results suggest that *T. reesei* 938 could be a promising candidate for improved cellulases production through direct-evolution strategies.

Keywords *Trichoderma reesei* · Cellulases production · Solid-state fermentation · Drum bioreactor · Design of experiments

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Introduction

Cellulose the main structural component of plant cell walls and the most abundant renewable carbon and energy source in the world is a polysaccharide composed of glucose units linked by ß-1,4-glycosidic bonds and is naturally degraded to glucose and other saccharides with different grades of polymerization by cellulolytic microorganisms [1]. Cellulose hydrolysis is required for different industrial fields such as agriculture, detergents, food, and textiles and has currently become of major relevance to bioethanol production [2]. The hydrolysis of cellulose is carried out by the synergistic action of three groups of enzymes: endoglucanases (EG), cellobiohydrolases, and ß-glucosidases [2, 3].

The lignocellulose biomass obtained from agro-industrial waste is the major source of low-cost and renewable feedstock for fueling ethanol production (i.e., bioethanol). In this process, the lignocellulose biomass is hydrolyzed to fermentable sugars by a saccharification reaction; this step defines the process costs and the ethanol yield. Two hydrolysis methods are currently employed, by acids and by hydrolases: the first generates furfural and hydroxymethyl furfural, which products inhibit fermentation [4]. Alternatively, the enzymatic methods, the most widely employed, produce a higher saccharification yield and without the generation of toxic compounds that inhibit fermentation. The enzymatic treatment, however, represents 22.5–43.4 % of the total cost in cellulose-based ethanol production. For this reason, a means of reducing the cost of enzyme production is needed in the bioethanol industry [5].

In order to reduce the production costs of cellulases, several reports have focussed on generating the enzyme by solid-state fermentation (SSF) through the use of different inexpensive lignocellulose substrates such as horticultural waste [6], rice straw [7], apple pomace [8], and wastes from the vinegar industry [9]. To that same end, numerous wild-type and mutant fungal strains of the genus *Aspergillus* and *Trichoderma* have been employed in several reports [10–13]. In particular, *T. reesei* Rut C30 is used in industrial cellulases production because this fungus has the ability to produce large amounts of endoglucanases and cellobiohydrolases [14]. In most of these instances, however, the cellulases production required several days of incubation in order to obtain a maximum productivity [12, 15, 16].

Within this context, the aim of this investigation was to find a promising novel *Trichoderma* sp. strain and define the optimal culture conditions in SSF in order to utilize a more economical agro-industrial substrate for cellulases production.

Materials and methods

Maintenance of microorganisms

Trichoderma reesei strains 155, 158, 159, 160, 179, 282, 283, 665, 938, 1127, 1282, and 3419 and *Trichoderma parareesei* strains 524, 661, 634, and 717 were kindly supplied by Irina S. Druzhinina (Institute of Chemical Engineering, TU Wien) [17] and *Trichoderma reesei* QM6a by the Agricultural Research Service culture collection (NRRL), while the *Trichoderma virens* strains IIB1218, IIB1219, and IIB1220 were isolated from straw bran. The strains were maintained on potato dextrose agar plates at 28 °C until sporulation; at which time the spores were collected in an aqueous 0.08 % (v/v) Tween 80 solution, counted in Neubauer chamber, and stored either at −70 °C in 50 % (w/v) aqueous glycerol for long periods or at 4 °C in water for times no longer than a week.

Strain selection

The preliminary solid-agar-plate screening of cellulolytic activity was carried out according to Florencio et al. [18] in Czapek-Dox agar containing carboxymethylcellulose (CMC) or acid-swollen cellulose (ASC; Walseth cellulose) for estimation of endoglucanase and cellobiohydrolase activity, respectively. As the ASC degradation requires the synergistic action of the endoglucanases and cellobiohydrolases, this substrate has been employed to estimate the cellulolytic capacity of the strains [19]. The enzymatic-productivity index (*EPI*) was calculated using the Eq. (1).

$$\mathrm{EPI} = D_h/(D_c D), \quad (1)$$

where D_h is diameter of hydrolysis zone, D_c is diameter of colony and D is the days of incubation.

Substrate preparation and selection

Substrate screening was performed with wheat bran, cotton husks, alfalfa hay, dandelion, oat straw, and the Argentine green tea, *yerba mate* (*Ilex paraguariensis*). All substrates were dried overnight at 80 °C and milled in a grain blender (Grindomix GM200, Retsch GmbH). For substrate selection, 100 mL Erlenmeyer flasks containing 2 g of each substrate were hydrated with 1.6 mL of water and sterilized by autoclaving. Then 1.6 mL of (2×) sterile modified Mandel's medium containing the following amounts: urea—0.3, $(NH_4)_2SO_4$—1.4, KH_2PO_4—2.0, $CaCl_2{\cdot}2H_2O$—0.4, $MgSO_4.7H_2O$—0.3, proteose peptone—0.75, yeast extract—0.25 in (g L^{-1}), and $FeSO_4{\cdot}7H_2O$—5.0, $MnSO_4{\cdot}7H_2O$—1.6, $ZnSO_4{\cdot}7H_2O$—1.4, $CoCl_2{\cdot}6H_2O$—2.0 in (mg L^{-1}) was added to the sterile solid substrate. The flasks were

inoculated with 10^6 spores per g of dry substrate (gds) and incubated at 28 °C in a moist chamber for 4 to 6 days.

Enzyme extraction

The crude extracts were obtained by soaking the culture in a 1:10 (w/v) ratio of extraction buffer [0.1 M NaCl with 0.1 % (v/v) Tween 80] and shaking in an orbital shaker at 250 rpm, for 1 h at 28 °C. Glass beads (5 mm diameter) were added as extraction adjuvant at 7 g of beads per g of culture. Solids were separated by centrifuging at $3000\times g$ for 20 min. Finally, the clarified extract was fractionated and maintained at −20 °C until use in enzymatic assays.

Enzymatic assays

The filter-paper activity (FPA) and endoglucanase activity (EGA) were assayed in the crude extracts according to the protocols of Xiao et al. [20, 21], with filter paper and CMC as substrate, respectively. The activities were reported in international units per g of dry substrate IU gds^{-1}. One international unit of enzyme was defined as the amount of enzyme required to release 1 μmol of glucose equivalents per minute from filter paper or CMC under standard assay conditions.

Plackett–Burman design (PBD)

A two-level fractional factorial design without interactions between factors (Plackett and Burman) was used for detection of significant parameters. The main effect of each parameter was calculated as the difference between the average of the response values obtained for the high-level (+1) and the low-level (−1) conditions.

The parameters screened for cellulases production were urea, ammonium sulfate, potassium phosphate, calcium chloride, magnesium sulfate, lactose, pH, temperature, inoculum size, and moisture. The experiments were carried out in duplicate for 3.5 days, and the EGA and FPA values taken as responses. The composition of the media, incubation conditions, and inoculum concentrations are shown in Support Information section (Table S1). The experimental designs were formulated through the use of the Essential Experimental Design software (version 2.216) [22]. The data analysis was conducted using the Statgraphics centurion XVI® and the half-normal plot was used to assess which factors are significant important or not significant to make the regression model.

Surface-response methodology

A central-composite design (CCD) type inscribed with start points outside of the minimum and maximum values was employed to study the main effects of the parameters and their interactions. The experiments were performed in duplicate under the conditions shown in Support Information section (Table S2). Essential Experimental Design software (version 2.216) was employed for the experimental design and ANOVA analysis [22]. The desirability analysis was conducted using the Statgraphics centurion XVI®.

Bioreactor for SSF

A rotatory-tank bioreactor with a carbon dioxide and oxygen gas-analyzer module (Terraforce-IS; Infors HT) was used for SSF. The solid media consisted of 1 kg of wheat bran moistened with 1 L of 0.2 M NaOH to give an initial pH of 6.8 and 100 % g gds^{-1} of initial moisture. The medium was in situ vapor-sterilized before inoculation with 5×10^6 spores per gram of moist substrate. The culture was carried out at 26 °C and the composition of the exhaust gas used for growth-phase monitoring. The bioreactor was rotated for 2 min at 2 rpm in each direction (clockwise and counterclockwise) to insure a thorough mixing before sampling.

Enzyme characterization

The crude extract from the *T. reesei* 938 and the commercial Celluclast® (Novozyme) were partially purified by dialysis against distilled water with 10 K MWCO dialysis tubing (Snake Skin™, Thermo). The dialyzed samples, corresponding to 4 mL of the crude extracts, were concentrated to 1 mL in a 10 K MWCO centrifugal-concentrator tube (Vivaspin™, Sartorius) and finally adjusted to 1 IU mL^{-1} of FPA to carry out the determinations. The optimal temperature was ascertained by assaying activities at different reaction temperatures (45.3–64.5 °C) in 50 mM phosphate-citrate buffer (pH 5.0). For optimum pH determination, cellulases activities were assayed at the optimum temperature in 50 mM phosphate-citrate buffer for pH values from 2.6 to 8.0. The EGA and FPA thermal and pH stabilities were determined by incubating the samples under the optimal conditions for different times (0, 30, 90, 150, 210, 270, 330 min) before conducting the assays under optimal conditions.

Because of the complexity of the reaction occurring during inactivation by heat or pH, several equations have been proposed to model these kinetics. In this work, a first-order kinetic model was selected that fits the Eq. (2).

$$A/A_0 = e^{(-kt)}, \quad (2)$$

where A/A_0 represents the residual enzyme activity at time t (min), while k (min^{-1}) is the rate constant of the reaction at a given temperature.

The effect of divalent cations on cellulases activities was evaluated through an enzymatic assay at pH 5.0 and

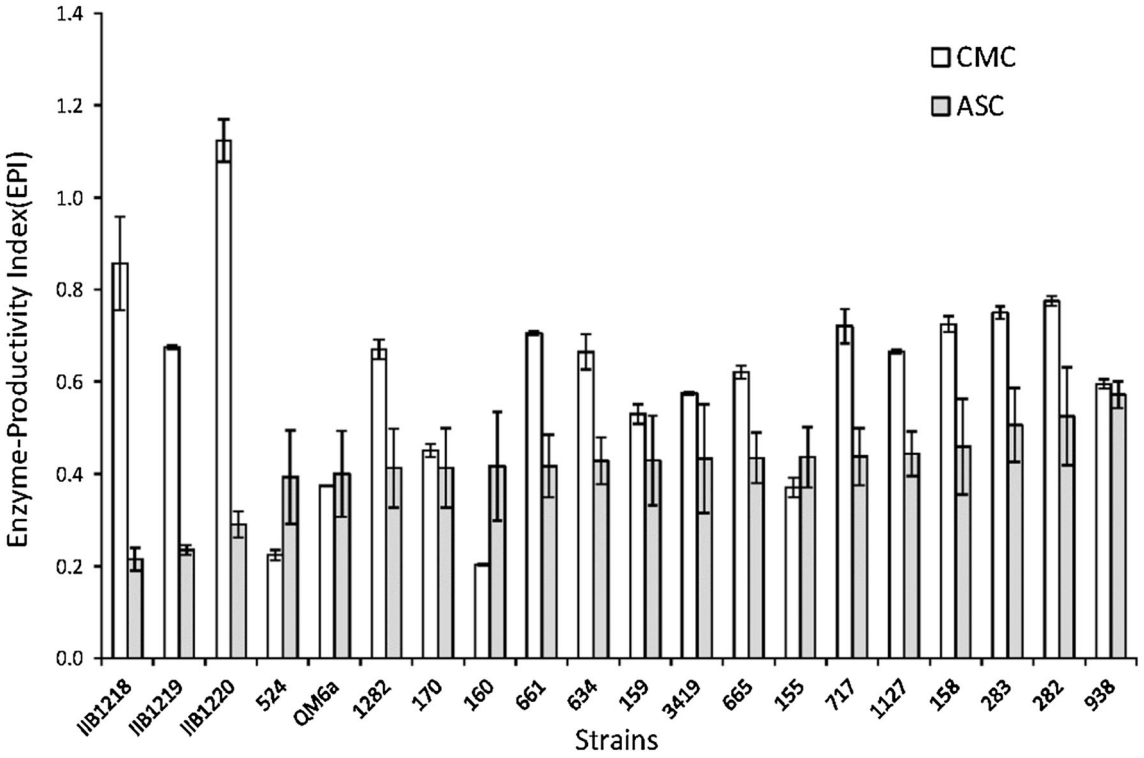


Fig. 1 Enzyme-productivity screening on agar plates. The strains indicated on the *abscissa* were tested in agar media for cellulase activity, expressed as EPI (enzyme-productivity index) on the *ordinate*, with carboxymethyl cellulose (CMC, *white bars*) or acid-swollen cellulose (ASC, *gray bars*) as substrate. For the definition of EPI, *cf*, "Materials and methods." QM6a was used as a reference control. The *error bars* indicate the standard deviation among three independent experiments

56 °C in 50 mM acetate buffer with the inclusion of each ion ($FeCl_2$, $CaCl_2$, $CoCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$) at 4 mM final concentration.

Results and discussion

Strain and substrate selection

The EPI values obtained from agar-plate screening with carboxymethyl and acid-swollen cellulose as substrates indicated that the *T. reesei* 938 displayed the highest cellulases productivity with respect to activity with the latter substrate among the twenty *Trichoderma* strains evaluated (Fig. 1). Furthermore, this strain presented a balanced productivity ratio with respect to the activity on both substrates (0.59 on CMC vs 0.57 on ASC). This balance has been suggested as yielding a higher saccharification of lignocellulosic biomass [23, 24].

The EGA and FPA activities obtained by SSF through the action of *T. reesei* 938 on agriculture feedstocks indicated that although wheat bran, dandelion, and oat straw were the best substrates for the production of EGA and FPA. However, the wheat bran exhibited the highest activity at 4 days of cultivation with values of 5.11 ± 0.33 and 0.82 ± 0.08 IU gds^{-1} for EGA and FPA, respectively (Fig. 2), indicating a potentially higher productivity with that substrate. It should be mentioned that wheat bran has been employed in numerous reports for enzyme production in SSF, because it is a good source of nitrogen and carbon [25]. On the basis of these results, the wheat bran was selected as the indicated substrate for further experiments.

Screening of the main parameters for cellulases production by the PBD

The effect of the medium composition and culture conditions was evaluated by means of the PBD design in 12 experimental runs involving 10 parameters at two levels shown in the Tables 1 or 2. The condition tested in the ninth trial resulted in maximal values for EGA (8.31 IU gds^{-1}) and FPA (3.62 IU gds^{-1}). In contrast, the condition selected in the third trial resulted in the minimal values (Support Information, Table S1).

The activity values were fitted to curves for regression analysis in order to obtain a linear model for EGA (Eq. 3) or FPA (Eq. 4), respectively. The coefficient R^2 was higher than 0.98 in both models which indicated that the model as

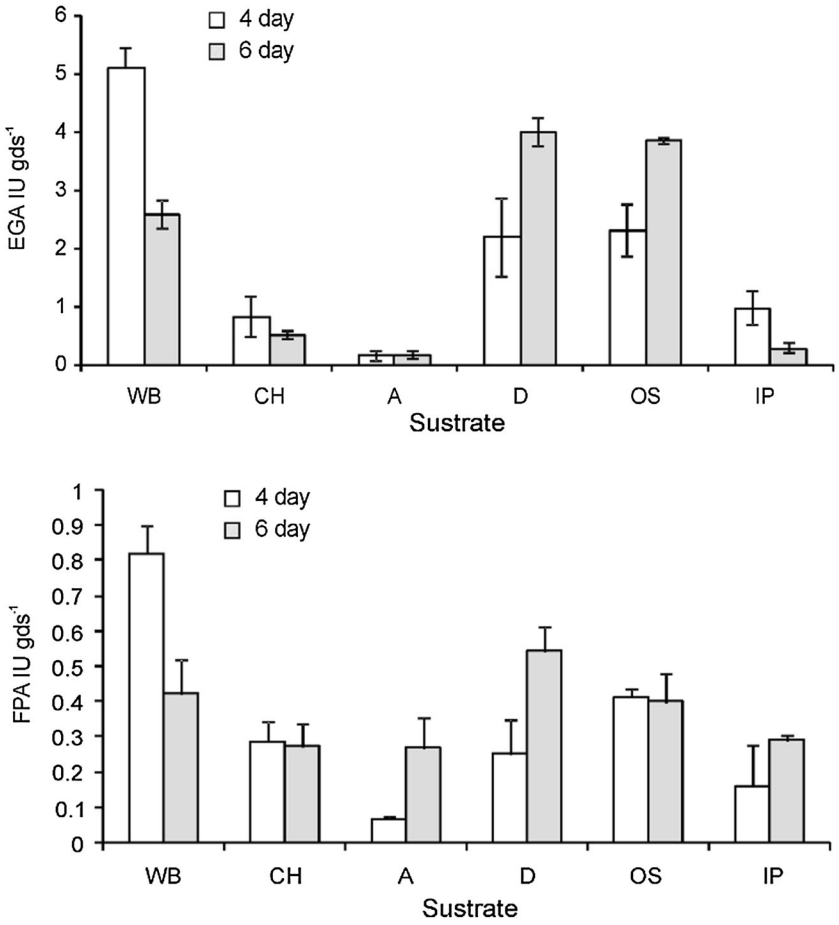


Fig. 2 Substrate selection for enzyme production. Cellulases production by *T. reesei* 938 was assayed after growth on the following solid substrates: wheat bran (*WB*), cotton husks (*CH*), alfalfa hay (*A*), dandelion (*D*), oat straw (*OS*), and *Ilex paraguariensis* (*IP*). EGA (*upper panel*) or FPA (*lower panel*) is plotted on the *ordinate*. The *error bars* indicate the standard deviation among three independent experiments

Table 1 The levels of variables tested with the Plackett–Burman design and their effect on EGA production

Code	Parameter name	Low level (−1)	High level (+1)	Effect estimate	*p* value
X_0	–	–	–	−24.35	0.00174
X_1	Urea	0.05	0.3	23.33	–*
X_2	$(NH_4)_2SO_4$	0.2	1.5	−12.43	–*
X_3	KH_2PO_4	0.3	2.2	−55.70	0.039
X_4	$CaCl_2{\cdot}2H_2O$	0.6	0.3	−104.86	–*
X_5	$MgSO_4{\cdot}7H_2O$	0.05	0.3	306.00	0.096
X_6	pH	4.7	5.7	5.71	8.315E-05
X_7	Inoculum	10	70	−0.01	0.071
X_8	Moisture	70	100	0.031	0.056
X_9	Lactose	0.16	0.4	278.47	0.129
X_{10}	Temperature	26	30	−0.17	0.124

Urea, $(NH_4)_2SO_4$, KH_2PO_4, $CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, and lactose in % (w gds^{-1}); temperature (°C); inoculum (million gds^{-1}), and moisture in % (v gds^{-1})

* Terms not included in the model

fitted explains 98 % of the variability of the responses, EGA or FPA, respectively. The adequacy of the model was significant when analyzed by Fisher's statistical test on the ANOVA results for both activities, indicating that the models can be used for prediction of the responses. Accordingly, the main effects of the individual parameters on enzyme production were calculated as described in "Materials and methods" and the significance levels (*p* values) identified by means of Fisher's test on the ANOVA obtained for each variable are shown in the Tables 1 and 2, respectively. The estimated effect obtained for each variable, illustrated by pareto chart (Fig. 3),

Table 2 The levels of variables tested with the Plackett–Burman design and their effect on FPA production

Code	Parameter name	Low level (−1)	High level (+1)	Effect estimate	*p* value
X_0	–	–	–	−9.65	0.005
X_1	Urea	0.05	0.3	173.98	0.053
X_2	$(NH_4)_2SO_4$	0.2	1.5	−34.70	0.048
X_3	KH_2PO_4	0.3	2.2	−10.60	0.240
X_4	$CaCl_2{\cdot}2H_2O$	0.6	0.3		–*
X_5	$MgSO_4{\cdot}7H_2O$	0.05	0.3	209.48	0.033
X_6	pH	4.7	5.7	1.73	0.001
X_7	Inoculum	10	70	−0.01	0.013
X_8	Moisture	70	100	0.014	0.062
X_9	Lactose	0.16	0.4		–*
X_{10}	Temperature	26	30	0.07	0.139

Urea, $(NH_4)_2SO_4$, KH_2PO_4, $CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, and lactose in % (w gds^{-1}); temperature (°C); inoculum (million gds^{-1}), and moisture in % (v gds^{-1})

* Terms not included in the model

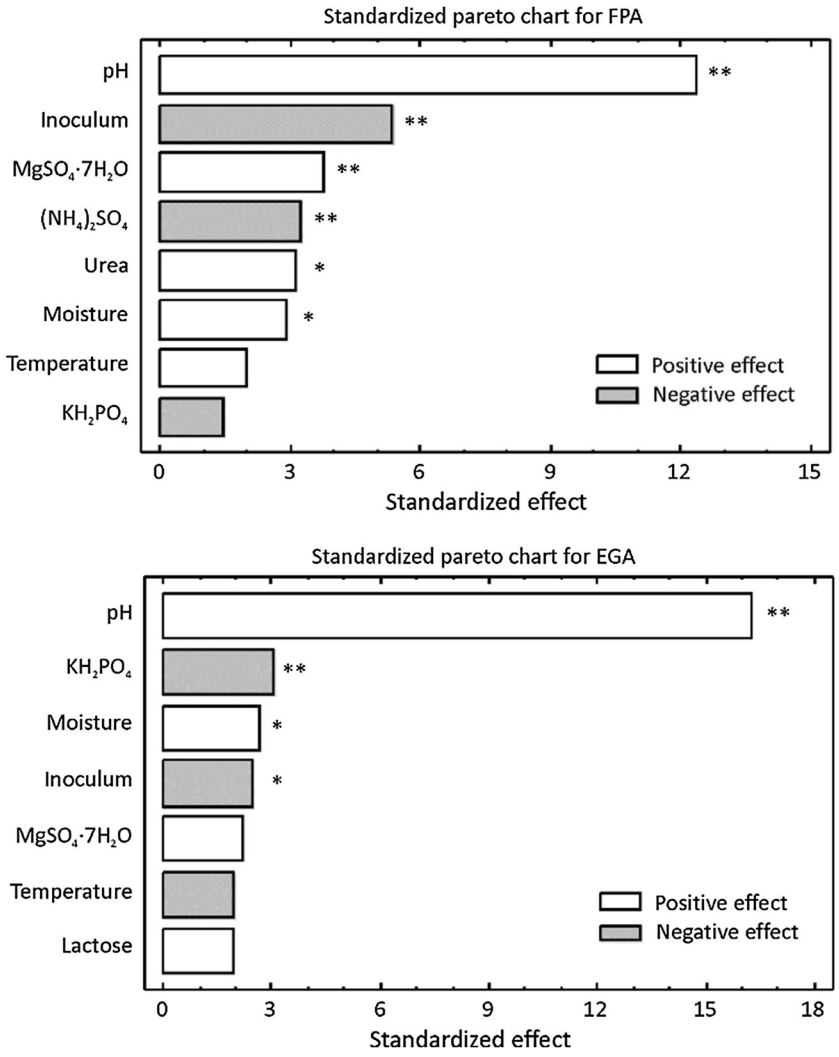


Fig. 3 Effect of the main parameters on cellulases production. The magnitude and effect of each parameter and the values of statistical significance (*$p < 0.07$; **$p < 0.05$) obtained by Fisher's test (ANOVA) are indicated above or below *each bar*

indicated that an increase in the initial pH and moisture content resulted in an enhancement of both activities. Based on these results, the initial moisture content and the pH were selected for further optimization to improve cellulases production.

$$EGA\left[\text{IU gds}^{-1}\right] = -24.35 - 55.70\,X_3 + 306.00\,X_5 + 5.71\,X_6 - 0.01\,X_7 + 0.038\,X_8 + 278.47\,X_9 - 0.17\,X_{10} \quad (3)$$

$$FPA\left[\text{IU gds}^{-1}\right] = -9.65 + 173.98\,X_1 - 34.70\,X_2 - 10.60\,X_3 + 209.48\,X_5 + 1.73\,X_6 - 0.01\,X_7 + 0.014\,X_8 + 0.07\,X_{10}, \quad (4)$$

where X_i are the independent variables, see Tables 1 or 2 for references.

Optimization of cellulases production by a central-composite design

On the basis of the PBD results, the conditions of pH and moisture were optimized by means of a central-composite design involving 12 experimental runs (Support Information, Table S2). The FPA and EGA activities (responses) were fitted to a second-order polynomial equation Eq. 5 and 6. The F values for Fisher's test on the ANOVA results were 10.44 and 29.56 for FPA and EGA, respectively; thus suggesting that both models are relevant. The correlation coefficient (R^2) for the observed and predicted values was 0.897 for FPA and 0.961 for EGA, indicating the adequate correlation for both activities Tables 3 and 4, respectively.

$$EGA\left[\text{IU gds}^{-1}\right] = -118.88 + 51.75\,X_1 - 0.743\,X_2 - 5.094\,X_1^2 - 0.002\,X_2^2 + 0.165\,X_1\,X_2 \quad (5)$$

$$FPA\left[\text{IU gds}^{-1}\right] = -161.50 + 55.20\,X_1 - 0.345\,X_2 - 4.666\,X_1^2 - 0.001\,X_2^2 + 0.081\,X_1\,X_2, \quad (6)$$

Table 3 ANOVA test for the selected quadratic model for EGA

Reference	Parameter	Value	p value
X_0	–	−118.88	0.0410
X_1	pH	51.75	0.0071
X_2	Moisture	−0.743	0.0023
X_1^2	pH pH	−5.094	0.0016
X_2^2	Moisture Moisture	−0.002	0.0003
X_1. X_2	pH Moisture	0.165	0.0002
Lack of fit	–	–	0.8600

Coefficient of variation (CV) = 6.82 %, coefficient of determination (R^2) = 0.961, correlation coefficient (R) = 0.980, and adjusted R^2 = 0.723

Table 4 ANOVA test for the selected quadratic model for FPA

Reference	Parameter	Value	p value
X_0	–	−161.50	0.0111
X_1	pH	55.20	0.0046
X_2	Moisture	−0.345	0.0522
X_1^2	pH pH	−4.666	0.0022
X_2^2	Moisture moisture	−0.001	0.0075
X_1. X_2	pH moisture	0.081	0.0060
Lack of fit	–	–	0.8150

Coefficient of variation (CV) = 12.80 %, coefficient of determination (R^2) = 0.897, correlation coefficient (R) = 0.947, and adjusted R^2 = 0.811

where X_i are the independent variables, see Tables 3 or 4 for references.

The contour plot for each model was used for determining the visualization and interaction of the parameter for obtaining a maximal cellulases yield (Fig. 4). According to the elliptical profile observed in these contour plots for pH and moisture, a strong interaction between those two parameters was observed for both models [26]. The highest FPA was obtained at an initial pH and moisture content of 6.4–7.1 and 74.4–123.4 %, respectively, while the maximum EGA occurred at an initial pH and moisture content of 5.94–6.61 and 50–90 %, respectively. The desirability function was used for the simultaneous optimization of the EGA and FPA responses, based on the predicted responses from the fitted model the optimal values of variables (pH and moisture) to give the maximal response correspond to second trial (Support Information, Table S3). Finally, the adequacy of the EGA and FPA models corresponding to Eqs. (4) and (5) was validated by performing a total of four verification experiments around the optimal values (Support information, Table S4). The data from the validation trials were statistically analyzed by Pearson correlation coefficient and the R values obtained for EGA and FPA were 0.96 and 0.85, respectively. This good correlation between experimental and predicted values confirms the accuracy of the models.

According to the levels curves, it is possible to conclude that the optimal range for producing both activities in SSF with WB as the substrate is given by a pH of 6.40–6.61 and a humidity of 74.4–90.0 %. Under these conditions, the model predicted an FPA of 8–10 IU gds^{-1} and an EGA of 15.6–17.8 IU gds^{-1} after 2.5 days of culture (Fig. 4). The corresponding productivities actually attained were 6.7 EGA (IU gds^{-1} day^{-1}) and 3.8 FPA (IU gds^{-1} day^{-1}) for the second trial (Support Information, Table S2). This FPA value was considerably higher than the productivities obtained for other wild-type strains: For example, *Trichoderma* sp. growing on apple pomace produce 2.3 FPU

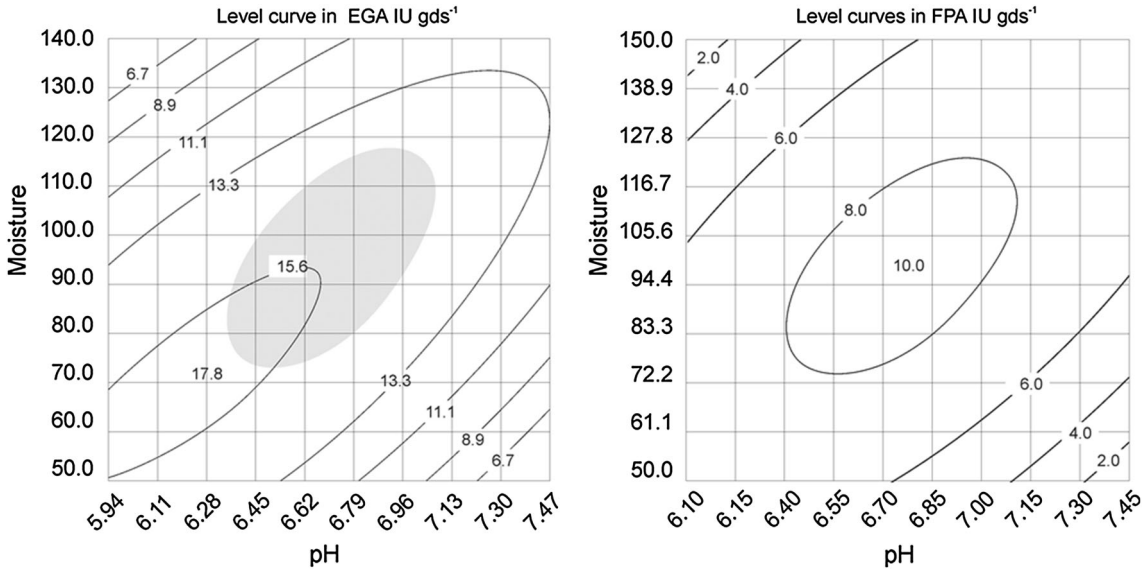


Fig. 4 Optimization of cellulase-activity-production parameters through the use of central-composite design. Contour plot obtained for the effects of initial pH and moisture content on cellulases yield (*left graph*, EGA; *right graph*, FPA, activities shown on the *curves*). *Gray shadow* in the EGA *contour plot* indicates the 8–10 FPA contour area

gds^{-1} at 120 h [8], *T. reesei* QM 9414 growing on rice bran elaborates 2.8 FPU gds^{-1} at 55 h [27], and *T. reesei* HY07 growing on corn stalk and wheat bran attains 3.0. FPU gds^{-1} at 120 h [28]. Likewise, the *T. reesei* 938 displayed a higher EPI than QM6a, the parental strain of the hyper-producer mutant *T. reesei* Rut-C30 (Fig. 1). On the basis of these observations and comparisons, the *T. reesei* 938 would likely be a promising parental strain for improving cellulases productivity by protocols employing mutagenesis and/or directed evolution.

Cellulases production in a rotating-drum bioreactor

The scaling-up effect on cellulases production was evaluated in a rotating-drum bioreactor under the optimal conditions for FPA and EGA production (pH 6.8 and 100 % moisture, previously selected in CCD analysis). The impact of the aeration rate on cellulases production was evaluated at 0.5 and 2 L min^{-1}. At the lower aeration rate, the respective FPA and EGA produced at 72 h were 5 and 8.5 IU gds^{-1}. In contrast, when the aeration rate was 2 L min^{-1}, 6.5 IU gds^{-1} of FPA and 11.5 IU gds^{-1} of EGA were obtained at 72 h. Under both conditions, however, the activity obtained was lower than in the incubations conducted in Erlenmeyer flasks during the optimization experiments. Nevertheless, at 96 h, the activities obtained in the rotating-drum bioreactor at 2 L min^{-1} were equal to those predicted by the model, as illustrated in Figs. 4 and 5, respectively. These results demonstrate that the aeration rate affects the culture productivity (Fig. 5), so that the optimization of this parameter will be required in order to improve the SSF yield of enzyme in a rotating-drum bioreactor. In SSF, the porosity of the medium, the aeration, and the moisture content are the main parameters altered in scaling up for industrial production [29].

Biochemical characterization of the crude enzyme extract

In order to establish whether the crude extract from *T. reesei* 938 can be useful as an alternative in saccharification processes, some of the preparation's biochemical properties were determined and then compared with those of a well-known commercial cellulases-containing extract from *T. reesei* (i.e., Celluclast® by Novozyme). The temperature-optimization experiment indicated an activity maximum at around 56.1 °C for both activities (FPA and EGA) along with similar temperature profiles for the crude extracts and the commercial preparation (Fig. 6, Panel A).

A similar behavior was likewise found for the pH, with both preparations having essentially the same pH optimum with either substrate (Fig. 6b). The *T. reesei* 938 extract, however, retained a minimal level of residual EGA at a pH of 6.8 or higher (Fig. 6, Panel B, left graph). This differential tolerance of alkalinity confers a slight advantage on the *T. reesei* 938 extract over the commercial preparation because that degree of resilience extends the pH range through which the enzyme retains its usefulness.

The thermal stability measured at 56 °C, the optimum temperature cited above, fit a first-order model (Fig. 6, Panel C). The half-lives for FPA and EGA were estimated from the curves. The FPA half-life values obtained were 324 min for the enzyme extract and 457 min for

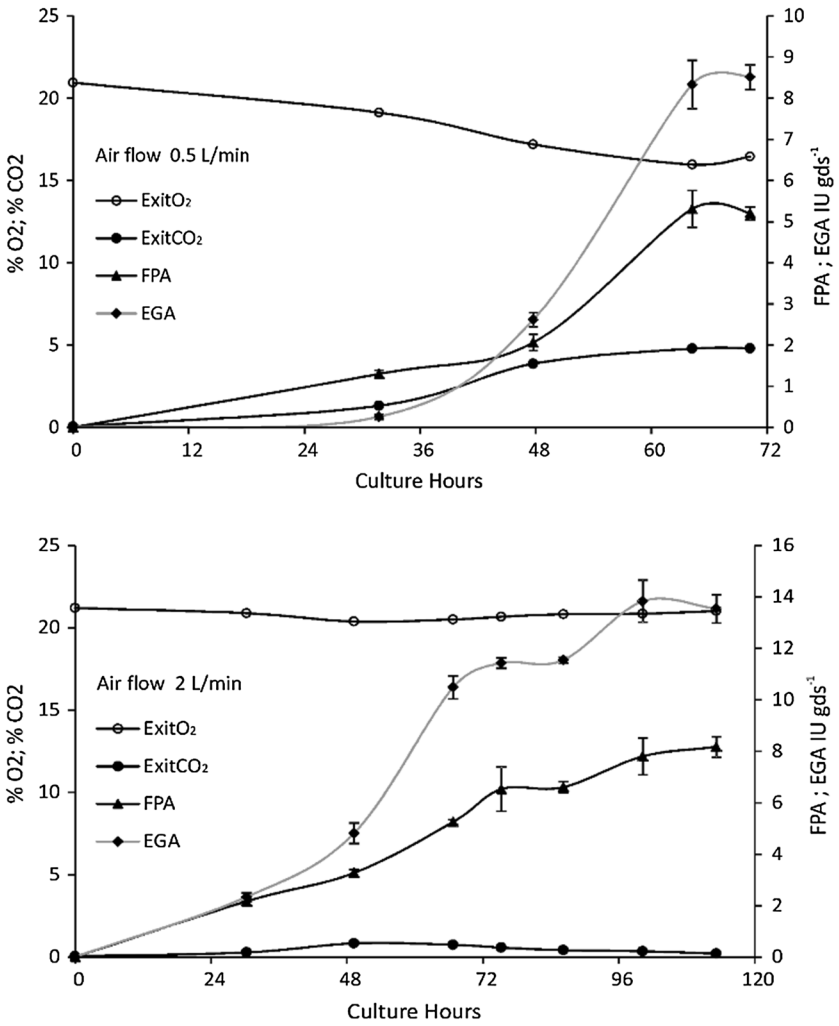


Fig. 5 Bioreactor enzyme generation. Cellulases-production rate at two air-flow rates, 0.5 and 2 L min^{-1} (*upper* and *lower panels*, respectively). The FPA (*solid triangles*) and EGA (*solid diamonds*) values were the average of 4 samples taken from different spots within the culture. The growth phase was monitored online by analysis of the oxygen consumption (*solid circles*), and carbon dioxide production (*open circles*). The *error bars* represent the standard deviation of each experimental point ($n = 4$)

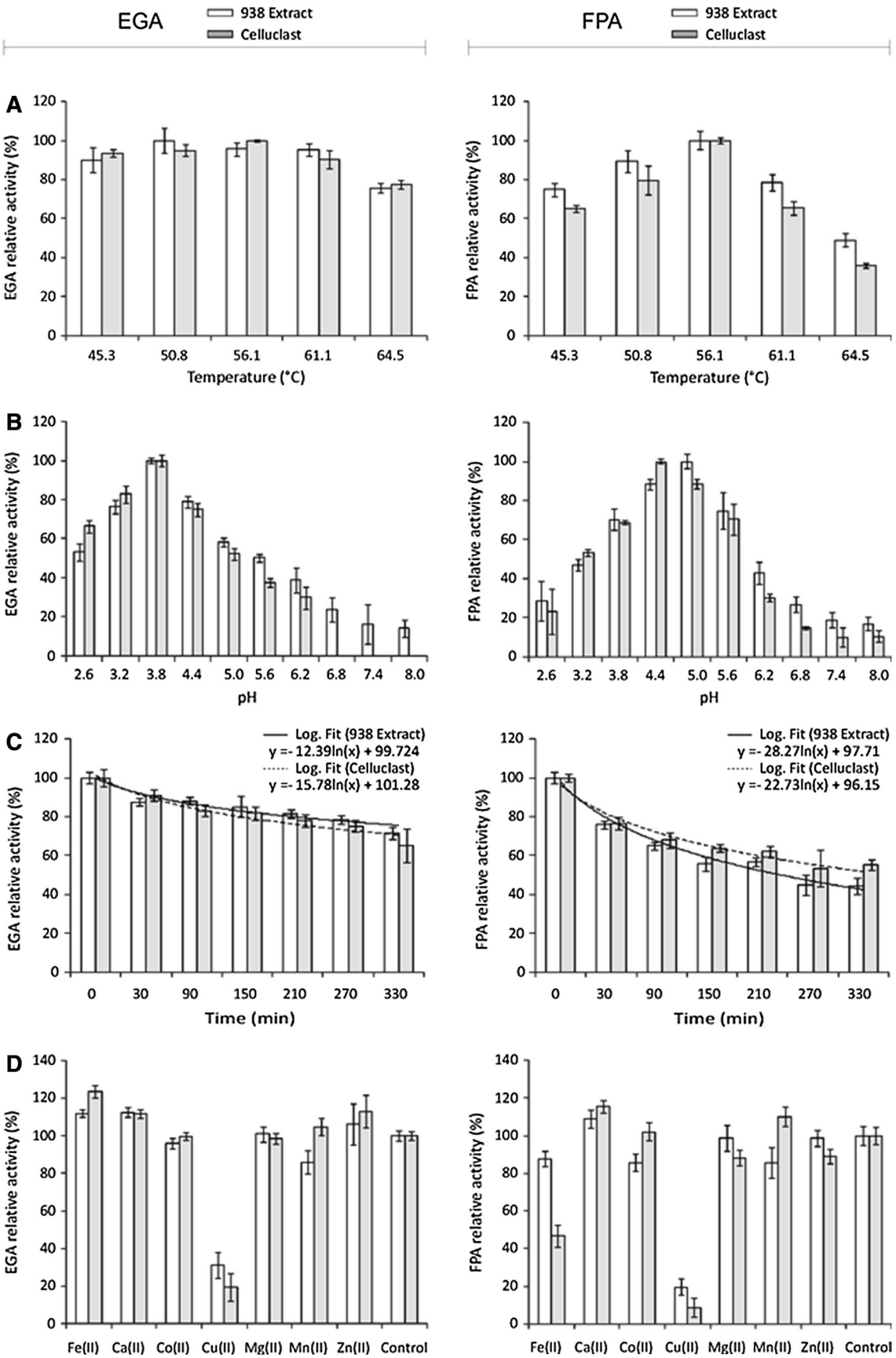
commercial mixture; whereas the EGA half-lives of the extracts and Celluclast® exhibited no differences during the times assayed, thus indicating that the EGA extracted from *T. reesei* 938 is more stable than the FPA. No significant differences in catalytic stability with respect to the EGA were, therefore, observed between the extracts and the commercial product during the time assayed (Fig. 6, Panel C).

Divalent cations have been shown to affect cellulases activity [30–32]. In order to evaluate this possibility, several cations were assayed at a final concentration of 4 mM. The FPA from *T. reesei* 938 exhibited a slight enhancement in the presence of Ca^{+2} and strong inhibition in the presence of Cu^{+2}; while Co^{+2}, Mn^{+2}, and Fe^{+2} exerted a moderate inhibition. In contrast, the FPA from the commercial extract retained about half the activity of the *T. reesei* 938 extract in the presence of Fe^{+2} (Fig. 6, Panel D; right graph). The Fe^{+2} and Cu^{+2} ions could be acting as inhibitors of the cellulolytic activity through the redox mechanism suggested by Tejirian et al. [32]. The EGA from both *T. reesei* 938 and the commercial extracts were strongly inhibited by Cu^{+2} but somewhat stimulated by Fe^{+2} and Ca^{+2} (Fig. 6, Panel D; left graph).

We conclude that the enzyme extract from *T. reesei* 938 has similar properties to those of the commercial extract except for a greater resistance to iron and a residual EGA at pHs >6.8. Thus, the enzyme extract from *T. reesei* 938 could be considered a good candidate for use in biotechnological processes.

Conclusions

In the present work, twenty *Trichoderma* strains isolated from around the world were screened to select a new potential strain for cellulases production. Strain *T. reesei* 938 was selected, based on the balanced EPI ratio between the CMC and ASC activities and the highest EPI for ASC among the strains. This last property was observed in the strains 155, 170, and QM6a the latter the parental strain of


EGA
938 Extract
Celluclast
FPA
938 Extract
Celluclast
A
EGA relative activity (%)
FPA relative activity (%)
Temperature (°C)
45.3
50.8
56.1
61.1
64.5
B
pH
2.6
3.2
3.8
4.4
5.0
5.6
6.2
6.8
7.4
8.0
C
Log. Fit (938 Extract)
y = -12.39ln(x) + 99.724
Log. Fit (Celluclast)
y = -15.78ln(x) + 101.28
Log. Fit (938 Extract)
y = -28.27ln(x) + 97.71
Log. Fit (Celluclast)
y = -22.73ln(x) + 96.15
Time (min)
0
30
90
150
210
270
330
D
Fe(II)
Ca(II)
Co(II)
Cu(II)
Mg(II)
Mn(II)
Zn(II)
Control

◀**Fig. 6** Characterization of the crude enzyme extract. For all points, the residual activity is expressed as a percent of the original activities for FPA (*right graphs*) and EGA (*left graphs*). The *white bars* represent the enzymatic extract from *T. reesei* 938 and the *gray bars* the commercial cellulases blend Celluclast®. *Panel A*, the effect of temperature on cellulase activity; *Panel B*, the effect of pH on cellulase activity measured at 56 °C; *Panel C*, the thermal stability at 56 °C and optimum pH (FPA, pH 5.0; EGA, pH 3.8) plotted as cellulase activity (*ordinate*) versus time (*abscissa*); *Panel D*, the effect of different divalent ions on cellulase activity. The *error bars* represent the standard deviation ($n = 3$)

hyperproducer strain Rut-C30. The FPA-productivity values obtained for *T. reesei* 938 proved to be higher than those of other wild-type strains. According to the results obtained in the Plackett–Burman design, the main parameters to enhance the EGA and FPA production were moisture and pH, pH being the most important. In this regard, the optimization of these variables by central-composite design led to an improvement in the cellulases productivity, reaching values from 1.28 to 6.7 and 0.20 to 3.8 IU gds^{-1} day^{-1} for EGA and FPA, respectively. In addition, the comparative biochemical analysis of enzyme produced in the extracts indicated comparable characteristics to those of the commercial preparation Cellucast® in addition to a substantially greater resistance to 4 mM Fe^{+2} and a slightly greater tolerance to alkaline pH for EGA and FPA, respectively. On the basis of these results, *T. reesei* 938 could be regarded as a promising candidate for direct-evolution strategies aimed at improving enzyme production.

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