Cellulase is a family of at least three groups of enzymes that participate in the sequential hydrolysis of cellulose. Recombinant expression of cellulases might allow reducing their production times and increasing the low protein concentrations obtained with filamentous fungi. In this study, we describe the production of \textit{Trichoderma reesei} cellobiohydrolase II (CBHII) in a native strain of \textit{Wickerhamomyces anomalus}. Recombinant CBHII was expressed in \textit{W. anomalus} 54-A reaching enzyme activity values of up to 14.5 U L$^{-1}$. The enzyme extract showed optimum pH and temperature of 5.0–6.0 and 40$^{\circ}$C, respectively. Enzyme kinetic parameters ($K_M$ of 2.73 mM and $V_{max}$ of 23.1 $\mu$M min$^{-1}$) were between the ranges of values reported for other CBHII enzymes. Finally, the results showed that an enzymatic extract of \textit{W. anomalus} 54-A carrying the recombinant \textit{T. reesei} CBHII allows production of reducing sugars similar to that of a crude extract from cellulolytic fungi. These results show the first report on the use of \textit{W. anomalus} as a host to produce recombinant proteins. In addition, recombinant \textit{T. reesei} CBHII enzyme could potentially be used in the degradation of lignocellulosic residues to produce bioethanol, based on its pH and temperature activity profile.

1. Introduction

Cellulases are enzymes from the glycoside hydrolase family (EC 3.2.1.-) that are expressed by a broad spectrum of bacteria and fungi strains [1, 2]. Among these microorganisms, white- and brown-rot fungi are considered the major producers of cellulases, including \textit{Trichoderma} sp., \textit{Aspergillus} sp., \textit{Schizopyllum commune}, and \textit{Volvariella volvacea} [1, 2]. Cellulase is a family of at least three groups of enzymes: endo-(1,4)-$\beta$-D-glucanase (EC 3.2.1.4, EG), exo-(1,4)-$\beta$-D-glucanase (EC 3.2.1.91, CBH), and $\beta$-glucosidases (EC 3.2.1.21, BG) [3]. These enzymes act sequentially on the cellulose hydrolysis: (1) EG randomly attacks on O-glycosidic bonds resulting in glucan chains of different lengths, (2) CBH acts on glucan chains ends to release $\beta$-cellobiose, and (3) BG hydrolyzes the $\beta$-cellobiose to produce glucose [3].

\textit{Trichoderma reesei} is one of the most important cellulases producing filamentous fungi, since it contains the core enzymes necessary for the complete hydrolysis of lignocellulosic material [4]. Among these enzymes, cellobiohydrolases have been shown to be one of the most important components within this process [4]. Enzymes involved in hydrolysis
of cellulose polymer molecules have been recombinantly expressed [5], which might allow reducing the production times and increasing the low proteins concentrations obtained with filamentous fungi. Recombinant expression might also facilitate the scaling up of the enzyme production, as well as the implementation of Simultaneous Saccharification and Fermentation (SSF) and Separate Hydrolysis and Fermentation (SHF) processes. Saccharomyces cerevisiae has been used for the production of recombinant CBH from Phanerochaete chrysosporium [6] and Talaromyces emersonii [7], while T. reesei CBHII has been expressed in S. cerevisiae [8, 9], P. pastoris [10, 11], and Y. lipolytica [11].

Conventional microbial systems (e.g., E. coli, S. cerevisiae, and P. pastoris) have allowed the production of a long list of recombinant proteins [12]. However, there is a growing interest in the identification of new hosts that allow the production of high-quality or cost-efficient recombinant proteins [13]. Recently, a native strain of Wickerhamomyces anomalous was isolated from sugar cane bagasse at Puerto López, Meta, Colombia (unpublished results). W. anomalous is a yeast that has been isolated from different sources [14], and its reported applications include food biopreservation, bioremediation, and production of phytases and biofuels [14, 15]. However, to the best of our knowledge, there are no reports showing its use as host to produce recombinant proteins.

In this paper, we describe the production of recombinant cellobiohydrolase II (CBHII, EC 3.2.1.91) from T. reesei using the native strain W. anomalous 54-A. Furthermore, the effects of pH and temperature on enzyme activity were characterized, as well as the kinetic properties of the enzyme and its application on the hydrolysis of a lignocellulose material. Overall, the results showed the potential of W. anomalous 54-A as host to produce recombinant proteins and showed that recombinant T. reesei CBHII has similar characteristics to those reported for the wild-type enzyme.

2. Materials and Methods

2.1. Microorganism, Culture Media, and Vectors. W. anomalous 54-A was previously isolated from sugar cane bagasse at Puerto López, Meta, Colombia. Initially, the microorganism was identified by carbon assimilation profile using the API 20C AUX kit (bioMérieux, Marcy l’Étoile, France). Further microorganism identification was done by amplification of internal transcribed spacer (ITS), using the primers ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 5'-TCCTCCGCTTATGGATATGC-3' [17] and Pfu DNA polymerase (Thermo Fisher Scientific Inc., San Jose, CA, US), under manufacturer instructions. Identity of W. anomalous 54-A was finally confirmed by MALDI-TOF analysis (Unidad de Investigación en Proteómica y Micosis Humana, Pontificia Universidad Javeriana).

Plasmid pKS2-ST (Dualsystems Biotech, Schlieren, Switzerland) was used as expression vector. In this vector, protein expression is regulated by the alcohol dehydrogenase II promoter (EC 1.1.1.1, ADH2), while the secretion signal of the Saccharomyces cerevisiae invertase Suc2 mediates the secretion of the recombinant protein. Escherichia coli DH5 was used for cloning purposes, which was cultured in Luria-Bertani (LB) medium supplemented with 100 mg mL\(^{-1}\) ampicillin. Yeast cultures were performed in YPD supplemented with 500 mg mL\(^{-1}\) geneticin for the selection of clones and 60 mg mL\(^{-1}\) for protein expression cultures.

2.2. Expression Vector and Recombinant Strains. Cellobiohydrolase II gene (cbhII) from T. reesei (GenBank Number GU724763.1) was previously codon-optimized for its expression in S. cerevisiae by GeneArt (Thermo Fisher Scientific Inc.) (unpublished data). The sequence encoding for the CBHII signal peptide was identified by using SignalP [16] and removed from the gene sequence. Comparison of the codon usage tables of S. cerevisiae and W. anomalus showed a similar profile between both microorganisms (Supplementary Figure 1 in Supplementary Material available online at https://doi.org/10.1155/2017/6980565) Two codons (CGC and CGG), which encode Arg (14 residues, 3%, in CBHII) showed significant differences between both microorganisms. Nevertheless, the other two Arg codons have a comparable usage between S. cerevisiae and W. anomalus. In this sense, this codon optimized sequence was used for the CBHII expression in W. anomalus 54-A. Codon optimized cbhII gene was inserted downstream of the Suc2 secretion signal in the vector pKS2-ST, to produce pKS2-ST:CBHII (Supplementary Figure 2). The pKS2-ST:CBHII vector was used to transform competent cells of W. anomalus 54-A by electroporation, using the Gene Pulser electroporation system Xcell (Bio-Rad Laboratories) at 1400 V and 200.Ω. The W. anomalus 54-A clones were selected in YPD medium supplemented with 500 mg mL\(^{-1}\) geneticin. Clones were confirmed by PCR using the primers 5'-GAGGAGGACATAGAAATGGGG-3' and 5'-CAGCAGTAGCCATAGCCA-3', which amplify a fragment from cbhII gene. The PCR-positive clones were evaluated at 55 mL scale, according to vector pKS2-ST manufacturer’s instructions (Dualsystems Biotech). Briefly, each clone was incubated for 10 h in 10 mL YPD, after which 15 mL of fresh YPD medium was added. After 14 h incubation, 30 mL of fresh YPD medium was added to reach a final culture volume of 55 mL. After 6 h of incubation, it was expected that the glucose was exhausted, and this time was considered as the beginning of the induction phase. 1 mL aliquots were taken every 24 h for 96 h to measure extracellular enzyme activity and cell density. All the assays were performed in triplicate at 28°C and 180 rpm. Residual glucose quantitation was carried out by DNS method [17]. Crude extracellular fractions of the clone with the highest enzyme activity were loaded and processed by SDS-PAGE.

2.3. Evaluation of Carbon Sources. The clone that showed the highest enzyme activity was used to evaluate the effect of carbon source (i.e., glucose and glycerol) on the enzyme production. Cultures were carried out in 2% (w/v) tryptone and 1% (w/v) yeast extract, supplemented with glycerol and glucose in different concentrations: 2% (w/v) glucose, 1% (w/v) glucose, 1% (w/v) glycerol, 2% (w/v) glucose, 1% (w/v) glycerol, and 2% (w/v) glycerol. All cultures were supplemented with 60 mg mL\(^{-1}\) geneticin.
2.4. Culture at 2.4 L Scale. The _W. anomalus_ 54-A clone that showed the highest CBHII activity at 55 mL scale was scaled up to 2.4 L in a 3.7 L Bioengineering KFL2000 bioreactor. Cultures were done in modified YPD medium [2% (w/v) tryptone, 1% (w/v) yeast extract, 1% (w/v) glucose, and 1% (w/v) glycerol] supplemented with 60 mg mL\(^{-1}\) geneticin. Briefly, 2 mL from the cell bank was inoculated into 18 mL of culture medium and incubated for 24 h at 28°C and 180 rpm. The preinoculum was used to inoculate 180 mL of fresh culture medium and incubated for 24 h at 28°C and 180 rpm. Finally, the inoculum was used to inoculate 1000 mL of fresh culture medium at the bioreactor. After 15 h culture, 1200 mL of fresh medium was added to reach a final volume of 2.4 L and cultured during an additional 96 h at 28°C, 400 to 800 rpm, and pH 6.0.

2.5. Cellobiohydrolase Activity Assay. The CBHII activity was evaluated at different pH and temperature conditions, using pNPC. To evaluate the effect of pH and temperature on enzyme activity, the enzyme activity assay was performed at 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 ± 0.2 and at 30, 40, 50, 60, and 70°C for 1 h. The reactions were stopped and read as described above. Apparent kinetic parameters (_K_M_ and _V_max_) were estimated for the crude extract by using pNPC between 0 and 6.5 mM and fitting the experimental data to a Michaelis-Menten model using the software GraphPad PRISM 6.0.

2.6. Characterization of Recombinant CBHII. The CBHII enzyme extract was evaluated at different pH and temperature conditions, using pNPC. To evaluate the effect of pH and temperature on enzyme activity, the enzyme activity assay was performed at 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 ± 0.2 and at 30, 40, 50, 60, and 70°C for 1 h. The reactions were stopped and read as described above. Apparent kinetic parameters (_K_M_ and _V_max_) were estimated for the crude extract by using pNPC between 0 and 6.5 mM and fitting the experimental data to a Michaelis-Menten model using the software GraphPad PRISM 6.0.

2.7. _Chrysanthemum_ Wastes Degradation Assay. The effect of recombinant CBHII on cellulose hydrolysis was evaluated by using _Chrysanthemum_ wastes, as previously described [18, 19], with modifications. _Chrysanthemum_ wastes were autoclaved at 121°C for 15 min. Experiments were performed in substrate submerged cultures carried out in 100 mL flasks in a rotary shaker at 150 rpm and 30°C, for 20 days. Flasks contained 1% _Chrysanthemum_ wastes in 40 mL of the following enzyme crude extracts: (1) cellulolytic and hemicellulolytic extract from a _Penicillium_ sp. culture (hereafter Ce-Hem extract), (2) concentrated recombinant CBHII extract from _W. anomalus_ 54-A (hereafter rCBHII), and (3) a 1:1 Ce-Hem:rCBHII extracts mixture. Control culture was carried out by using 40 mL of distilled water. All cultures were carried out with 10 mM sodium azide, 0.1% tween 80, and pH 5.0. The response variable was the concentration of reducing sugars as measured by DNS method [17]. Results are reported as g L\(^{-1}\) of reducing sugars after subtraction of the results obtained with the control cultures, and normalized by the initial units of CBHII present in each extract. Production of Ce-Hem extract was carried out by using a _Penicillium_ sp. strain (Collection of Microorganisms of Pontificia Universidad Javeriana). For this purpose, 20 mL of a 10⁶ conidia mL\(^{-1}\) suspension was inoculated in 200 mL of a rice straw medium without nitrogen source and incubated for 8 days at 28°C and 120 rpm. The crude extract was obtained by centrifugation and sequential filtrations through Whatman paper Number 42 and 0.45 and 0.22 μm polyether sulphone membranes (Pall Corp, Port Washington, NY, USA). Concentrated recombinant CBHII extract from _W. anomalus_ 54-A was produced by using a 2.4 L culture of the _W. anomalus_ 54-A Celo-3.2 clone, as described above. Culture medium was centrifuged at 8000 g and filtered sequentially through Whatman paper Number 42 and 0.45 and 0.22 μm polyether sulphone membranes (Pall Corp). Permeate was ultrafiltered through a 30 kDa cut-off membrane (Millipore, Billerica, MA, USA), until reaching a final volume of 20 mL. CBHII activity in both extracts was assayed as described above. The β-glucosidase (BG) activity in both extracts was assayed using 5 mM 4-nitrophenyl β-D-glucuronide (Sigma-Aldrich) as previously described [18, 19]. One BG unit was defined as the amount of enzyme releasing 1 μmol of p-nitrophenol per min, and the activity was expressed as U L\(^{-1}\).

2.8. Statistical Analysis. Differences between groups were tested for statistical significance by using one-way ANOVA. An error level of 5% (p < 0.05) was considered significant. All analyses were performed using the software GraphPad PRISM 6.0.

3. Results and Discussion

3.1. Production of Recombinant CBHII in _W. anomalus_ 54-A. In this study, we evaluated the production of the recombinant CBHII in the native yeast strain 54-A, previously isolated from sugar cane bagasse from Puerto López, Meta, Colombia. This strain showed a carbon assimilation profile (API 20C AUX, bioMérieux) different from that observed for _S. cerevisiae_ strains, and it was identified as _Pichia anomala_ (recently renamed as _Wickerhamomyces anomalus_ [21]) with a 94.5% ID and 0.25 T value. Identity of this strain was further confirmed as _W. anomalus_ through ITS amplification (GenBank accession number KX676490, Supplementary Figure 3) and MALDI-TOF analysis (Supplementary Figure 4). _W. anomalus_ is a yeast that have been isolated from different sources, such as food- and feed-related systems, insects, and human clinical samples. This yeast has a wide robustness to environmental stresses conditions like extreme pH or low water activity [14]. Biotechnological applications of _W. anomalus_ strains include biopreservation agent due to antimicrobial activity against variety of microorganisms in fruits and cereals and production of enzymes (e.g., phytases), biofuels, and bioremediation [14, 15]. However, to the best of our knowledge, there are no reports of the use of this yeast as a host to produce recombinant proteins.
Figure 1: Recombinant T. reesei CBHII produced in W. anomalus 54-A. (a) PCR-positive clones of W. anomalus 54-A were evaluated at 55 mL scale and the activity of recombinant T. reesei CBHII was assayed in the extracellular fraction. Among the four PCR-positive clones, only W. anomalus 54-A Celo 3.2 clone showed CBHII activity. No activity was observed in W. anomalus 54-A transfected with the empty vector. Each clone was evaluated in triplicate. (b) Crude extracellular extracts from W. anomalus 54-A Celo 3.2 at 0 and 92 h were evaluated by SDS-PAGE, followed by silver staining. The arrow indicates the recombinant CBHII.

Four clones were obtained after transformation of W. anomalus 54-A with the vector pKS2-ST::CBHII (Celo-2.2, Celo-3.2, Celo-4.2, and Celo-5.1), which were confirmed by PCR. The ADH2 gene promoter is repressed in the presence of glucose, since this metabolite inhibits the expression of the alcohol dehydrogenase regulator protein (Adr1) that is an activator of the ADH2 gene. On the other hand, under glucose depletion conditions, the expression of Adr1 is increased leading to the induction of the ADH2 gene [22]. In this sense, we first determined the residual glucose during the culture of W. anomalus 54-A pKS2-ST::CBHII clones, to establish the beginning of the induction phase. We observed, for all the evaluated clones, that, at the final culture step, the glucose was consumed within the first 6 h of incubation (Supplementary Figure 5). In this sense, this point was considered as the beginning of the induction phase, which agrees with previous reports of the production of recombinant proteins in yeast under the control of the ADH2 gene promoter [22, 23]. Under these culture conditions, extracellular CBHII activity was only detected in W. anomalus 54-A Celo-3.2 clone, reaching a final enzyme activity of 5.7 U L⁻¹ after 96 h of induction (Figure 1(a)). As expected, CBHII activity was not detected in W. anomalus 54-A transfected with the empty vector.

As was mentioned above, T. reesei CBHII has been expressed in S. cerevisiae [8, 9], P. pastoris [10, 11], and Y. lipolytica [11]. Recombinant CBHII produced in S. cerevisiae showed an activity of 25 S.I. U mL⁻¹ using barely β-glucan as substrate [8], while recombinant CBHII produced in P. pastoris GSH15 showed an enzyme activity of 5.84 U mL⁻¹ at 96 h using microcrystalline cellulose PH101 as substrate [10]. In P. pastoris X-33 and Y. lipolytica POld, extracellular recombinant CBHII activities were 0.25 and 0.36 U mL⁻¹, respectively, using phosphoric acid swollen cellulose (PASC) prepared from Avicel PH-101 as substrate [11]. The differences in promoters, secretion signals, hosts, and enzyme activity substrates limit the comparison between these results and those obtained for recombinant CBHII produced in W. anomalus 54-A. However, a crude culture broth from Penicillium sp. containing wild-type cellulase and hemicellulase enzymes (see Chrysanthemum Wastes Degradation Assay) showed a CBHII activity of 8.5 U L⁻¹ using the pNPC substrate, which was similar to that observed for the recombinant CBHII produced in W. anomalus 54-A. Nevertheless, these results are lower than those observed for erlenmeyer flask cultures of Pleurotus ostreatus, which showed an activity of 445.3 ± 27.6 U L⁻¹ using the pNPC substrate [18, 19].

SDS-PAGE analysis of extracellular fraction of W. anomalus 54-A Celo-3.2 showed that recombinant CBHII has an apparent molecular weight of about 66 kDa (Figure 1(b)). This recombinant CBHII is higher than the wild-type enzyme produced by T. reesei (50–58 kDa) [24]. However, recombinant CBHII produced in W. anomalus 54-A has a molecular weight similar to that reported for a CBHII produced in P. pastoris X33 and Y. lipolytica POld (~63–65 kDa) [11] but higher than that reported for the enzyme produced in P. pastoris GSH15 (~58 kDa) [10]. Differences in the molecular weight between wild-type and the recombinant counterpart could be associated with the hypermannosylation observed
in yeast, while the differences in the molecular weight among the recombinant CBHII could be associated with several factors of the culture conditions that affect the expression of glycosyltransferases [25].

3.2. Evaluation of Carbon Sources. To improve the production of recombinant CBHII, two glucose and glycerol concentrations were evaluated. First, 1% (w/v) glucose was evaluated, which is lower than the concentration suggested by the vector manufacturer (2% w/v). We hypothesize that 1% (w/v) glucose would be depleted faster than 2% w/v, allowing a reduction in the induction time and a longer and higher enzyme production [22]. In addition, glycerol, alone or in combination with glucose, was also evaluated as a carbon source, since it was expected that glycerol would be used for yeast growth after depletion of glucose, increasing the production of the recombinant enzyme.

Figure 2 shows the results of volumetric activity (U L\(^{-1}\)) and enzyme activity normalized by biomass (U g\(^{-1}\) dry weight of cells). A similar activity profile was observed between volumetric and normalized enzyme activities, suggesting that the production of recombinant CBHII was associated with yeast growth. In fact, the profile of recombinant CBHII production was similar to that previously reported for the ADH2 promoter, with a complete induction in the late stationary phase (>36 h culture) [22]. The results showed that in 2% (w/v) glycerol cultures no CBHII activity was detected, suggesting that the glycerol was used for cell growth or that the ADH2 promoter needs to sense the glucose depletion to induce the gene expression. On the other hand, lower CBHII activity was observed at 2% (w/v) glucose, 1% (w/v) glycerol, cultures compared to that obtained with 2% (w/v) glucose (\(p < 0.001\)). Finally, the highest CBHII activity was observed at 1% (w/v) glucose, 1% (w/v) glycerol, although this was not statistically different regarding the levels obtained with 2% (w/v) glucose (\(p > 0.05\)). However, 1% (w/v) glucose, 1% (w/v) glycerol, allowed an earlier detection of the enzyme activity than that observed with 2% (w/v) glucose. In this sense, 1% (w/v) glucose, 1% (w/v) glycerol, was selected to produce recombinant CBHII at 2.4 L scale. The reasons of the effect of glycerol on CBHII activity are unknown, and further investigations should be carried out to clarify this aspect. Nevertheless, it is important to mention that there is a wide variation among yeasts, and even strains, regarding glycerol metabolism (i.e., uptake and catabolism) [26]. Although the metabolic pathways and the uptake and regulation (e.g., catabolic repression) process have been widely described for S. cerevisiae, limited information is available for other yeasts [26]. In this sense, the effect of glycerol on the changes of enzyme activity levels might be associated with the specific glycerol metabolism of W. anomalus 54-A.

3.3. Production of Recombinant CBHII at 2.4 L Scale. The production of recombinant CBHII enzyme at 2.4 L scale was carried out for 117 h after the final feeding step (see Materials and Methods). Figure 3 shows the behavior of dissolved oxygen (DO) during the production of recombinant CBHII at bioreactor scale, which could be associated with the consumption of the carbon sources. The CBHII activity was detected from the first 2 h of culture (0.12 ± 0.08 U L\(^{-1}\)). This activity significantly increased after 10 h (2.6 ± 0.09 U L\(^{-1}\)) and continuously kept increasing until reaching a final enzyme activity of 14.5 ± 0.86 U L\(^{-1}\), which was 2.7-fold higher than that observed at 55 mL scale. These results might suggest that, under controlled culture conditions, the strict repression of the ADH2 promoter might be affected...
allowing the expression of the recombinant protein even in the presence of residual glucose.

3.4. Characterization of CBH Recombinant Extract. The effect of temperature and pH on the enzyme activity of the recombinant CBHII produced in W. anomalus 54-A was evaluated. In this work, crude extract rather than the purified recombinant CBHII was used for characterization, since this crude extract would be used in the production of bioethanol by the degradation of Chrysanthemum residues [19]. As observed in Figure 4, optimum reaction temperature for recombinant CBHII crude extract was 40°C (p < 0.001), while the highest enzyme activities were observed at pH 5.0 and 6.0 ± 0.2 at all evaluated temperatures (p < 0.001). On the other hand, at pH 3.0 and 8.0 ± 0.2, a marked decrease in the enzymatic activity was observed regardless of the reaction temperature.

Using microcrystalline cellulose PH101 as substrate, recombinant CBHII produced in P. pastoris showed an optimum reaction pH and temperature of 5.0 ± 0.2 and 50°C, respectively. In addition, this recombinant enzyme showed a significant loss of activity at pH of 3.0, 70, and 8.0 and at 30°C and temperatures above 65°C [10]. Recombinant CBHII enzyme produced in P. pastoris and Y. lipolytica showed an optimum pH between 5.0 and 6.0 and at a temperature of 60°C. Recombinant CBHII produced in P. pastoris and Y. lipolytica also showed rapid inactivation at pH and temperatures above 70 and 60°C, respectively, while at temperatures below 40°C, an 80% loss in the enzyme activity was observed [11]. In this sense, recombinant T. reesei CBHII produced in W. anomalus 54-A has a reaction pH profile similar to that reported for recombinant CBHII enzymes expressed in other yeast. Nevertheless, it seems that the optimum temperature of reaction differs among the recombinant CBHII enzymes, which might be associated with the substrate used for the enzyme activity assay or the posttranslational modifications carried out for each host. It is noteworthy that recombinant CBHII produced in W. anomalus 54-A only showed 30% reduction in enzyme activity at 30°C. Functionality at 30°C might allow the use of this enzyme in SSF processes for the production of bioethanol, since this temperature is also the optimum growth temperature for S. cerevisiae that is the preferable microorganism used during the fermentation stage of bioethanol production [27].

Kinetic parameters for the crude extract were estimated using pNPC as substrate (Supplementary Figure 6). Recombinant T. reesei CBHII produced in W. anomalus 54-A showed K_M and V_max of 2.73 mM (est. error 0.26) and 23.1 μM min⁻¹ (est. error 0.89), respectively. This K_M value agrees with those reported for wild-type CBHII enzymes (EC 3.2.1.91) isolated from different microorganisms, which vary between 0.1 and 3.1 mM, depending on the source of the enzyme [28]. In the case of T. reesei CBHII enzymes there are no reports of kinetic parameters using pNPC substrate. Nevertheless, a wide variation in K_M values is observed (i.e., between 0.041 and 380 mM), depending on the substrate used for this estimation [28].

3.5. Chrysanthemum Wastes Degradation Assay. To evaluate the effect of recombinant CBHII on cellulose hydrolysis, Chrysanthemum wastes degradation assay was performed using three enzyme extracts: (1) a crude culture broth from Penicillium sp. containing wild-type cellulase and hemi-cellulase enzymes (Ce-Hem extract), (2) a concentrated recombinant CBHII extract from W. anomalus 54-A (rCBHII extract), and (3) a 1:1 Ce-Hem: rCBHII extracts mixture. After treatment, 0.2 ± 0.02, 0.4 ± 0.01, and 1.25 ± 0.1 g L⁻¹ of reducing sugars were observed for Ce-Hem, rCBHII, and 1:1 Ce-Hem: rCBHII extracts, respectively. However, when reducing sugar concentration was normalized by the CBHII units present in each extract at the beginning of the experiment (Figure 5), it was observed that rCBHII extract had production of reducing sugars similar to that obtained with the Ce-Hem extract (p > 0.05). Noteworthy, the use of a 1:1 Ce-Hem: rCBHII extracts mixture allowed an increase of about 5-fold, in the reducing sugars per CBHII unit, in comparison with the results obtained by using the Ce-Hem or concentrated recombinant CBHII extracts alone, suggesting a synergistic effect of the combination of these two enzymatic extracts. To understand the reasons of this synergistic effect, we measured the activity of β-glucosidase (BG) and endoglucanase (EG) in the extracts (Figure 5). Whereas BG was detected in the three evaluated extracts, EG was only detected in Ce-Hem and Ce-Hem: rCBHII extracts. Nevertheless, there was no correlation between BG and EG activity and the increase in reducing sugars observed with the Ce-Hem: rCBHII extract. In this sense, we hypothesize that the synergistic effect observed in the Ce-Hem: rCBHII treatment could be associated with the presence of other enzymes, such as lignin peroxidase, xylanase, laccase, and manganese peroxidase [29].

Chrysanthemum wastes degradation using enzymatic extracts with native or recombinant cellulases has not been
The results showed production of recombinant CBHII in W. anomalus 54-A carrying the recombinant T. reesei CBHII allows production of reducing sugars similar to that of a crude extract from cellulolytic fungi, showing the potential of W. anomalus 54-A as a host to produce recombinant cellulases. Noteworthily, this is the first report on the use of W. anomalus as a host to produce heterologous proteins. Further studies should be focused on the consolidation of W. anomalus as a platform to produce recombinant proteins such as the design of specific expression vectors and the generation of auxotrophic strains.

Additional Points

One-Sentence Summary. This work represents the first report on the use of the yeast Wickerhamomyces anomalus as a host to produce heterologous proteins.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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References


