Chapter

Recombinant Fungal Cellulases for the Saccharification of Sugarcane Bagasse

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Abstract

Cellulases are important enzymes in cellulose degradation that occurs in nature, this degradation involves a system of extracellular multienzymes and have wide application. The construction of a high-quality system for the production of these enzymes is important for its application in the process of saccharification of biomass involved in the biofuel production process. Several species of fungi are capable of synthesizing and secreting high amounts of cellulase, most studies with fungal species use linearized plasmid, since these are encompassed to chromosomal DNA, improving its stability and expression efficiency. Advances in the production of recombinant enzymes focus on the search for industrially viable microorganisms capable of producing enzymes under various conditions, expressing them in a highly efficient manner, aiming at the synthesis of several copies of genes and a strong promoter. To resay these restrictions, molecular biology combined with recombinant DNA technology is a viable tool in enzymatic production. In subsequent topics, the production of endoglucanases, exoglucanases and β-glucosidase of fungi cloned in *Escherichia coli*, *Pichia pastoris* and other different expression systems will be addressed.

Keywords: recombinants cellulases, fungal cellulases, lignocellulosic biomass, cellulose degradation, heterologous systems, CRISPR/Cas9

1. Introduction

Alternative renewable fuel as bioethanol in the form of biofuel derived from biomass can contribute sources to replace fossil fuel-based conventional energy sources [1].

Cellulases are important enzymes in cellulose degradation that occurs in nature, this degradation involves a system of extracellular multienzymes and have wide application [2, 3].

Cellulase enzymes play an important role in industrial processes, representing about 20% of the global enzyme market worldwide and presenting a wide range of application, from food, feed, textile, pulp and pulp industries. An application that has been growing in recent years is the conversion of biomass into fermentable sugars for the production of biofuels [4–6].

Cellulases act on cellulosic fiber, catalyzing the degradation of β-1,4-glycocydic bonds [7] and includes three different types that act synergistically, based on classification the mode of action and specificities of the substrate, these: endoglucanases (EC 3.2.1.4) that randomly hydrolyze β-1.4 bonds in the cellulose molecule; cellobiohydrolases or exoglucanases (EC 3.2.1.91) which release a cellobiose unit and act procedurally at the end of the chain; and β-glycosidases (EC 3.2.1.21) that hydrolysis cellobiose to glucose [2, 8].

The construction of a high-quality system for the production of these enzymes is important for its application in the process of saccharification of biomass involved in the biofuel production process [9]. Current efforts have focused on fungal cellulases to transform lignocellulosic biomass into fermentable sugars that can be converted into ethanol. This process will allow the production of renewable fuel from cellulosic biomass [10].

Advances in the production of recombinant enzymes focus on the search for industrially viable microorganisms capable of producing enzymes under various conditions, expressing them in a highly efficient manner, aiming at the synthesis of several copies of genes and a strong promoter. Several species of fungi are capable of synthesizing and secreting high amounts of cellulase; most studies with fungal species use linearized plasmid, since these are encompassed to chromosomal DNA, improving its stability and expression efficiency [11].

For genetic engineering, the main expression systems are: *E. coli*, a bacteria classified as belonging to the *Bacteria* Domain*, Proteobacteria philum; Gammaproteobacteria* class, *Enterobacteriales* order and *Enterobacteriaceae* family, which has a high rate of development, easy to manipulate, transform and capture of plasmids, and can grow with high cell density. *E. coli* is generally transformed with self-replicated plasmid that does not integrate with chromosomal DNA and continues to replicate independently of cell divisions [12, 13]; and *Pichia pastoris,* a yeast classified as belonging to the Fungi Kingdom*, Eucomycota* division*; Ascomycota* subdivision*;* class *Hemoascomycetes,* the order *Endomycetales,* family *Sacharomycetaceae* and subfamily *Sacharomycetoideae.* A remarkable physiological characteristic of this yeast is the fact that it is methyltrophic, that is capable of growing in culture medium containing methanol as the only source of carbon and the ability to secrete high amounts of extracellular proteins [14, 15].

Due to the advance in the techniques of recombinant expression, the production systems of recombinant enzymes are promising strategies for the efficient production of industrial cellulase that can increase productivity in several industrial applications, including biomass in the processing of biofuels and thus meet the increasing demands of this enzyme [16].

The cost of obtaining sugars from the biomass of sugarcane bagasse for fermentation is still high, mainly due to the low enzymatic yield of fungal production. Thus, it generates the need for cellulase supplementation to these enzymatic cocktails. To resay these restrictions, molecular biology combined with recombinant DNA technology is a viable tool in enzymatic production. In subsequent topics, the production of endoglucanases, exoglucanases and β-glucosidase of fungi cloned in *E. coli* and *Pichia pastoris* will be addressed.

2. Lignocellulosic biomass

Lignocellulosic biomass is characterized mainly by the presence of two carbohydrate polymers (cellulose and hemicellulose), as well as an aromatic polymer called lignin, in addition to other components found in smaller amounts, such as ash, pectin, proteins, non-structural carbohydrates (glucose, fructose and sucrose)

and lipids. Most of the biomass of lignocellulosic materials is composed of cellulose (40–50%), hemicellulose (20–30%) and lignin (10–25%) and the specific composition of lignocellulosic biomass varies depending on different factors, mainly plant species, age, growth stage and environmental factors, genetic variability, and cultivation conditions of plant material [2, 17, 18].

Lignocellulosic biomass has a complex internal structure and several of its main components also have complex structures. Cellulose and hemicellulose are polysaccharides composed of simple sugars while lignin is a complex network of aromatic alcohols. In general, hemicelluloses and lignin provide an amorphous matrix in which crystalline cellulose microfibrils are dispersed [2, 18].

Corn straw, sugarcane bagasse, rice straw and wheat bran are promising and abundant lignocellulosic raw products from plant residues in the United States, South America, Asia and Europe [19].

3. Heterologous systems

A potential tool to develop better industrial production of cellulase are techniques of heterologous expression. This technology leads to enzyme yields at an economically viable level, since it allows the creation of microbial strains that express sets of adapted and synergistically active enzymes, within a single cell or combining different strains [20]. There are a variety of protein expression systems available, including bacterial and yeasts expression systems.

For the bacterial expression system, the most used is *Escherichia coli*, whose genetic characteristics are already well described. In addition, it has easy of manipulation, has an abundance of commercially available strains and vectors and has great ability to express recombinant genes with high yields [20–22].

As an alternative to the bacterial system, yeasts are often used, where *Pichia pastoris* yeast has become the most widely used host system for the expression of many heterologous proteins with relative ease of technique and at lower costs than those of most other eukaryotic systems [23–25].

Data from the last 15 years describing the recombinant fungal cellulases candidates for cellulose hydrolysis produced in the expression systems *E. coli*, *P. pastoris* or other different systems are presented below.

4. Recombinant fungal Cellulases produced by different expression systems

Numerous study techniques have been improved in recent years for cloning, heterologous expression and characterization of cellulases (**Table 1**). Several studies show efficient technologies to produce endoglucanases and β-glucosidases cloned in *E. coli* strains.

The Fungus *Trichoderma virens* ZY-01 expressed an endoglucanase cloned in a vector of expression pET-32-EG, being successfully elaborated, and expressed, in a heterologous way, in *Escherichia coli* and the target protein presented a weight of 39 kDa by electrophoresis SDS-PAGE [36].

A. fumigatus gene encoding endo-1,4-β-glucanase (Afu6g01800), studied by Bernardi et al. [46], was cloned in the vector pET-28a (+) and expressed in the strain of *E. coli R*osetta ™ (DE3). The research results showed that the afegl7 enzyme belonged to the GH7 family, in which the Af-egl7 gene encodes the protein comprising 460 amino acids with a CBM1 domain in the 424–460 residues and molecular mass of 52 kDa.

Fungus	Vector	Enzyme	Gene	Molecular mass	Activity Enzyme	Substrate	Author
Lentil edodes	*E. coli*	celobiohydrolase	cel6B	46.4 kDa	0.12 U/min/mg	p-nitrophenyl-β-galactopyranoside	Taipakova et al. [26]
Aspergillus niger	*P. shepherds*	endoglucanase	EglA	~30 kDa	63.83 ± 4.68 U/mg	β-glucan CMC	Quay et al. [10]
Trichoderma harzianum	*Pichia Shepherds*	Endoglucanase III	rThEGIII	24.6 kDa	—	CMC	Generoso et al. [27]
Myceliophthora thermophila	*Pichia Shepherds*	endoglucanase	EG7A	46 kDa	468 U/ml	CMC	Karnaouri et al. [28]
Aspergillus niger	*Pichia Shepherds*	celobiohydrolase	CBH1	60 kDa		CMC	Li et al. [29]
Aspergillus niger	*Pichia Shepherds*	β-glycosidase	Bgl1	121 kD	45 U/mL	Celobiosis.	Zhao et al. [30]
Myceliophthora thermophila	*Pichia Shepherds*	β-glycosidase	Bgl3B	130.0 kDa	15 U ml – 1	CMC	Zhao et al. [31]
Penicillium funiculosum	*Pichia Shepherds*	β-glucosidase	Bgl4P	~130 kDa	1,354.3 U/mg	p-nitrophenyl-β-glucode and celobiosis	Ramani et al. [32]
Aspergillus niduluns	*E. coli TOP 10F*	endoglucanase	XegA	28 kDa	33.3, (U/mg) 75% / 2 h	Sugarcane Bagasse	Lima et al. [33]
Mr Harzianum IOC-3844	*E. coli (pET28a)*	β-glucosidase (GH1 and GH3)	RThBgl	54.72 kDa	relative (> 60%) between pH 5.0 and 7.0	pNPG (Sigma-Aldrich) as substrate	Santos et al. [34]
Aspergillus nidulans AN2227	*pPICZ*	β-glucosidase	—	100 kDa	0.52 μmole / ml / min	nitrophenyl-p-D-glucopiranoid (pNPG)	Auta et al. [35]
Trichoderma Virens ZY-01	*E. coli*	endo-1,4-β-d-glucanase (EG)	Eg	39 kDa 1069 bp	expression was Km = 13.71 mg / mL and Vmax = 0.51 μmol / min · Ml.	CMC	Zeng et al. [36]
Aspergillus niger F321	*E. coli vector pGEM-T*	β-glucosidase	ANRA 2.6 and ANRA12.9	1,190 bp and 1,950 bp,	—	—	Auta et al. [37]

Fungus	Vector	Enzyme	Gene	Molecular mass	Activity Enzyme	Substrate	Author
Myceliophthra thermophila	*Pichia Shepherds*	endoglucanase (EG)	MtEG5A	75 kDa	160 h – 53 U / ml	Wheat Straws, Sorb, Birch	Karnaouri et al. [38]
Athermophila Myceliopthor B1J	*pGAPZαA*	endoglucanase	Mt-Egl	47 kDa	70% activity after 3 h of pH exposure 5–12	wheat bran, corn cob, sunflower splints, rice straw and rice bran	Phadtare et al. [39]
Aspergillus fumigatus DBiNU-1	*E. coli DH5 (Kluyveromyces lactis)*	endoglucanase	Cel7	48.19 kDa	0.80 U/ml with a specific activity of 3.08 U/mg protein	*Li et al. 2016*	Rungrattanakasin et al. [40]
Trichoderma Reesei ZU-02	Dh5α *E. coli* (pUC18-PsT	β-glucosidase	Bgl (2.5 kb)	—	112.2 IU/mL after 84 h of fermentation and the FPA reached 89.76 FPU/mL after 96 h.	Corn straw	Xia et al. [41]
T. reesei Rut-C30 A. niger NL02	*pCAMBI*	glucosidase (BGA), endoglucanase	Pcbh1 CBH2	—	BGA = 1.93 ± 0.28 IU/mL and EG =716.11 ± 41.16 U/mL	Steam-blasted corn bagasse	Zhao et al. [42]
Thermophilum chaetomium	*PPIC9K Express Pichia Shepherds*	endoglucanase	ctendo7	48 kDa	3.05 IU/mg in optimal reaction condition of 55°C, pH 5.0	Pretreated Wheat Straw	Hua et al. [43]
Thermoascus aurantiacus	*P. pastoris X-33*	native endoglucanase	Reg	~ 33 kDa	142 IU / mg	CMC in sodium citrate buffer	Jain et al. [44]
Pinophilus talaromyces	*Saccharoys cerevisiae*	β-glucosidase	Bgl3B	92 kDa	0.56 nkat/mg pH 4.0 enzymatic activity: 1 to 60° C.	a wheat-rye hybrid	Trollope et al. [45]
Aspergillus fumigatus	*E. coli* (pET-28a) RosettaTM strain	endo-1,4-β-glucanase	AfEGL7	52 kDa	(51.98 ± 0.0069 U mg^{-1})	Sugarcane bagasse (SEB), Barley	Bernardi et al. [46]
Aspergillus glaucus CCHA	*P. Pastorals GS115*	endoglucanase	AgCM ase,	55.0 kDa	343.81 ± 2.77 μM /mg/min	Rice and corn straws	Li et al. [47]

Fungus	Vector	Enzyme	Gene	Molecular mass	Activity Enzyme	Substrate	Author
Aspergillus fumigatus	*Pichia pastoris X-33*	endo-1,4-β-glucanase	Af-EGL7	70 kDa	(40–45%) after 72 and 48 h	Sugarcane bagasse "in natura"	Bernardi et al. [48]
Penicillium verruculosum (PvBGL)	*P.canescens RN3–11–7).*	β-glucosidase	BGL1	90 kDa	85 and 124 units /mg of protein, respectively	pNPG) p-nitrophenyl-p-Dglucopiranodes hydrolysis of MCC	Volkov et al. [49]
Talaromyces leycettanus	*Pichia Shepherds*	TlCel5A and TlCel6A	TlCel5A and TlCel6A	45 kDa and 65 kDa	3,905.6 U/mg vs. 109.0 U/mg in liquenae and 840.3 and 0.09 U/mg in CMC.	steam-blasted corn straw (SECS), corn cob, soybean meal and wheat bran	Gu et al. [50]
Trichoderma Reesei	*P. pastoris SMD1168*	exoglucanase	Cel6A	50 kDa 750 bp	—	—	Anindyawati et al. [51]
Matsutake Tricholoma (TmEgl5A)	*Pichia pastoris KM71H*	endoglucanase	TmEgl	40 kDa	514 135 U /mg and 104 3 U/mg	grown in barley and vermicuite	Onuma et al. [52]
Hypocrea sp. W63	*Pichia Shepherds*	b-glucosidase (BGL)	EpB-BGL	76.5 kDa	194.25 IU / mg	p-nitrophenyl-b-D-glucoside (pNPG) hydrolysis in sugarcane bagasse	Liang et al. [53]
Trichoderma Reesei	*Pichia Shepherds*	endoglucanase	ReEG I	~ 45 kDa	34.3 U/mg	Pulp, carboxymethylated cellulose, oat xylan, birch xylan, corn straw	Tao et al. [54]
Trichoderma Harzianum	*E. coli* JM109	β glucosidase	cel7a	—	2.24 ± 0.05 IU / mL - 46.66 IU. L^{-1}. H	pretreated sugarcane bagasse	Delabona et al. [55]
Aspergillus fresenii.	*E. coli* (by *K. phaffii X33)*	β-1, 4-glucosidases	Bgl T2	35 kDa	bgl T2 under pH 5.0 per 1 h provides up to 60% activity.	pNPG, CMC-Na, pNPC and Avicel	Yang et al. [56]

Table 1.
Recombinant fungal Cellulases used in the Saccharification of sugarcane bagasse.

RNA-Seq of β-glucosidase and genomic data are instruments that can be used to express *T. harzianum* genes for the deterioration of lignocellulosic biomass. The target gene of the recombinant protein (rThBgl) cloned and expressed heterological in *Escherichia coli* Rosetta was purified with high yields. The results showed a significant increase in the activity of β-glicosidase and in the filter paper cellulase (FPA). The cellulase produced by the transformers reached higher hydrolysis yield, with less enzymatic load during the saccharification of pretreated corn straw [41].

Delabona et al. [55] reported that *Trichoderma harzianum* overexpressed the methyltransferase of the global regulator - LAE1, in order to improve the production of cellulases, considering that the evaluation of the impact of LAE1 to induce cellulases made use of soluble carbon sources and lignocellulose and low cost in an agitated bioreactor. Using sugarcane bagasse with sucrose, the overexpression of lae1 culminated in a significant increase in the expression of the gh61b (31x), cel7a (25x), bgl1 (20x) and xyn3 (20x) genes. As a result, reduction of sugar released from the pretreated sugarcane bagasse, hydrolyzed by the recombinant crude enzymatic cocktail, obtained 41% cloned improvement through plasmid in *Escherichia coli*.

Two new genes of the β-glucosidases of *Aspergillus niger* 321 were successfully cloned in the pGEM-T vector [37]. *Aspergillus fresenii* (JCM 01963) *Escherichia coli* TOP 10 and *K. phaffii* X-33 (Invitrogen, USA) were used as host strains. The bgl T2-opt gene was synthesized according to the *K. phaffii* codon trend and constructed in the (pPICZαA vector Invitrogen, USA) with the sites of the restriction enzymes EcoRI and XbaI. This article effectively discovered a new β-1,4-glucosidase bgl T2 and its *Aspergillus fresenii* ORF, under the help of high-yield sequencing of the mRNA technique. Such a method is more convenient than the traditional of obtaining a new enzyme and its genetic information, as deluded in the discussion section. The bgl T2 gene was expressed by *K. phaffii* X33. The properties of bgl T2 were tested, including pH and temperature optimums for catalysis, pH tolerance, thermostability, effects of unusual chemicals and kinetic properties against pNPG [56].

The gene of *M. thermophila* coding for endoglucanase (EG) was isolated from fungal genomic DNA and then cloned and amplified in *E. coli* strains and, finally, expressed heterologic in *P. pastoris* and two basic strategies were followed for the production of EG. These strategies include controlling proteolysis through low temperature and adding numerous amino acid supplements to the culture medium. The enzyme presented high thermostability and was able to hydrolyze several natural substrates, cellobiose as the main product, characteristics that reflect its potential use in different biotechnological applications [38].

The study by Bernardi et al. [48] used the vector *Pichia pastoris* X-33 on to improve the characterization of an endo-1,4-β-glucanase, thermostable GH7 of *Aspergillus fumigatus* (Af EGL7). The kinetic parameters Km and Vmax were estimated and evidenced a robust enzyme which provided an improved hydrolysis of sugarcane bagasse "in natura", exploded sugarcane bagasse, corn cob, rice straw and bean straw [48]. A recombinant thermoalkalin endoglucanase of *Myceliopthor thermophila* BJA (rMt-egl) was used in the application and enzymatic saccharine of agro residues. The gene of this codon-optimized endoglucanase (Mt-egl) was expressed, constitutively in *Pichia pastoris* under the regulation of the GAP promoter. It was confirmed that recombinant endoglucanase (rMt-egl), efficiently hydrolyzed industrial agro residues, which were tested, and wheat bran. The effort aims to improve the production of rMt-egl by various approaches to molecular biology and cultivation [39].

Zhao et al. [42] investigated the fungi *T. reesei* and *Aspergillus niger* with the intention of further improving cellulase production and performance in enzymatic

hydrolysis. For this study, *Escherichia coli* DH5a was used for plasmid propagation. *Agrobacterium tumefaciens* AGL-1 was used for the transformation of recombinant *T. reesei*, constructed, and transformed into a pCAMBIA1300-PsCT vector. The vector *Pichia pastoris* was used for the production of recombinant CBH II by Zhao et al. [42] using a cloning vector of pUCm-T (Sangon, Shanghai, China) to obtain the *T. reesei* Rut-C30 and *A. niger* NL02. A vector pUC18-PsT containing fragments of 1.6 kb of Pcbh1-ss and 1.4 kb of Tcbh1 was used as vector structure to construct the set of DNA sequences with the expression information. As a result, a binary vector pCAMBIA1300-hph, in which it was added to this hygromycin gene, culminating in a final expression vector pCAMBIA1300-hph-PsCT. The results confirmed that the BG and CBHII genes provide a good performance in the hydrolysis of steam exploded corn pomace.

The recombinant endoglucanase (EG I) gene of *Trichoderma reesei* was successfully expressed in *Pichia pastoris*, with the objective of producing oligosaccharides from various biomass-derived substrates. Recombinant endoglucanase I (ReEG I) showed catalytic activity in relation to cellulose and xylan hydrolysis. Among several glucan and xylan substrates (paper pulp, carboxymethylated cellulose, oat xylan, birch xylan), birch xylan exhibited higher yield of xylooligosaccharides (XOS) [54].

An endoglucanase gene (ctendo7) of the fungus *Chaetomium thermophilum* was expressed in *Pichia pastoris*. The recombinant enzyme was purified by affinity chromatography with Ni^{2+} and subsequently characterized, through this analysis it was possible to conclude that the enzyme belongs to the family of glycosides hydrolase 7 and exhibited considerable activity against carboxytyl sodium cellulose (CMC-Na) and xylan of 1.91 IU / mg and 3.05 IU/mg in the ideal reaction condition of 55°C, pH 5.0, respectively, showed high hydrolytic efficiency in multiple lignocellulosic substrates at high temperatures [43].

The fungus *T. aurantiacus* RCKK was cloned in *P. pastoris* X-33 for overexpression. After the expression of recombinant endoglucanase (rEG), of molecular size of ~33 kDa confirmed by SDS-PAGE and western blotting, followed by determination of gel activity by zymogram analysis, the recombinant was successfully expressed in *P. pastoris* X-3 and bioreactor tests demonstrated that the enzyme is suitable for industrial applications [44].

An endoglucanase (TmEgl) was isolated from the solid-state culture of the ectomycorrhizal fungus *Tricholoma matsutake* (TmE-gl5A) cultivated in barley and vermiculite, which purified by fractionation of ammonium sulfate, ionic exchange, hydrophobic and gel filtration. TmEgl5A showed a molecular mass of approximately 40 kDa, as determined by SDS-PAGE. The gene encoding TmEgl was cloned and expressed in *Pichia pastoris* KM71H. These results suggested that *T. matsutake* produces a typical endoglucanase in solid state culture. *T. matsutake* presents itself as a strong candidate for the production of enzymes that degrade the cell wall of plants [52].

The unique candidate for GH5 cellulase of *A. glaucus* produced an endoglucanase called AgCMCase, which was cloned and expressed in the *Pichia pastoris* system [47]. The purified AgCMCase degraded the CMC-Na and was also able to hydrolyze the corn straw and rice to release sugar. The study showed that AgCMCase activity was retained by more than 95% after 4 h of incubation in the presence of NaCl 4 M, suggesting that it is a halotolerant enzyme. Thus, the interesting properties of AgCMCase can make it a potential candidate for industrial applications.

Recombinant β-glucosidase (EC 3.2.1.21) of *Aspergillus nidulans* AN2227 was expressed using buffered methanol complex medium (BMMY). Purification was performed using precipitation with ammonium sulfate and anionic exchange chromatography in the DEAE-Sephadex A-50 column. The enzyme was purified 2.58

times from the crude extract. The β-glucosidase was purified for electrophoretic homogeneity, containing a relative molecular weight of 100 kDa, as determined by electrophoresis in polyacrylamide gel, with sodium dodecile sulfate (SDS-PAGE). In the study, β-glucosidase was purified for electrophoretic homogeneity from the crude extract. The characteristics expressed from *P. pastoris* X33, with high-level expression, were described. The study suggests that the protein may be present in the monomeric form, with the enzyme having a good pH and temperature stability, making it an excellent candidate for cellulose hydrolysis [35].

A new bgl1 gene, which encodes a GH3 family of β-glucosidase of *Penicillium verruculosum* (PvBGL) was cloned and expressed heterological in the strain of *P. canescens* RN3–11–7 (niaD-) under the control of the xylAgene promoter. After the construction of the rPvBGL vector its properties were studied and compared with those of rAnBGL of *Aspergillus niger*, previously expressed in the same fungal host. It was observed that rPvBGL had an observed molecular mass of 90 kDa (SDS-PAGE data). It was possible to verify that rPvBGL converted polymeric substrates into glucose much faster than the recombinant BGL of *A. niger* (rAnBGL). Thus, this study showed the possibility of using rPvBGL for the construction of complex and balanced enzymatic preparations of cellulase based on the fungus *P. verruculosum* [49].

A β-glucosidase (BGL) of *Hypocrea sp.* W63 was cloned and expressed in *Pichia pastoris* and recombinant enzyme after purification presented a specific activity of 194.25 IU/mg. This study used *C. autoethanogenum* and *A. succinogenes* for the co-production of ethanol and succinic acid, using sugarcane bagasse as a source of fermentable sugars. The good conversion of epB-BGL suggests a great potential for the biorefining of cellulosic material [53].

Other study produced an exoglucanase (Cel6A) cloned in *Pichia pastoris*. The Cel6A gene was derived from *Trichoderma reesei* was produced synthetically, and the codon optimized for better expression in yeast *P. pastoris*. The gene was placed under the regulation of the GAP promoter, and the recombinant plasmid, called pLIPI-TrCel6A, inserted with the *T. reesei* Cel6A gene (TrCel6A), integrated into the genome of *P. pastoris* SMD1168H. The recombinant enzyme was successfully expressed by *P. pastoris*, with a main product that shows a molecular size of about 50 kDa. The recombinant Plasmid Cel6A selected was linearized with the enzyme BamHIO recombinant plasmid, pLIPI-TrCel6A, carrier of the *T. reesei* Cel6A gene (TrCel6A) integrated into the genome of *P. pastoris* SMD1168H [50].

4.1 Recombinant endoglucanases

Generoso et al. [27] using the expression system in *Pichia pastoris*, obtained a β-1,4-endoglucacase belonging to Glycosil hidrolases 12 (cel12a), cloned in the vector pPICZαA, isolated from the filamentous fungus *Trichoderma harzianum* IOCzianum-3844. The recombinant enzyme rThEGIII presented a molecular mass of 25 kDa, which is similar to the predicted mass, which is 24.6 kDa, as demonstrated by the authors. A large amount of rThEGIII was produced after 24 h of methanol induction, where in approximately 48 h, 300 mg of the purified enzyme was obtained from 1 L of medium. The optimum pH and temperature for rThEGIII activity were 5.5 and 48.2°C, respectively, similar to other EGIII already described. These characteristics indicate that rThEGIII is promising for simultaneous saccharification and fermentation, since the authors showed that the enzyme presented stability at temperatures close to ideal, lasting several days with acceptable activity.

Another recombinant endoglucanase was reported by Quay et al. [10], from the fungus *Aspergillus niger* ATCC 10574. The coding gene for the enzyme (EglA) was cloned in a pPICZαC vector and expressed in recombinant form in *P. pastoris* X-33.

After purification, the recombinant protein obtained presented a mass of ~30 kDa. Based on biochemical characterization, EglA had excellent activity at 50°C and ideal pH of 4.0, with a high stability at temperatures between 30 and 50°C and pH between 2.0 and 7.0. EglA showed greater affinity in the presence of β-glucan followed by carboxymethylcellulose (CMC) with a specific activity of 63.83 and 9.47 U/mg, respectively. Significant increase in activity was also observed with the presence of metal ions (Mn^2, Co^{2+}, Zn^{2+}, Mg^{2+}, Ba^{2+}, Fe^{2+}, Ca^{2+} and K^{+}). Based on these attributes, this enzyme can be signaled in order to be explored for enzymatic hydrolysis of agro-industrial residues.

A recombinant endoglucanase (MtEG7a), belonging to the family of glycosides hydrolase 7, was obtained by Karnaouri et al. [28], isolated from the fungus *Myceliophthora thermophila*; cloned in a pPICZαC vector and functionally expressed in the yeast *Pichia pastoris*. The purified recombinant enzyme (MtEG7a) was tested for its activity in relation to different substrates; where the enzyme showed high activity for β-glucan of barley (298 U/mg) and carboxymethylcelllulase (177 U/mg), also presenting activity for xylan-containing substrates, such as wheat arabinoxylan (5 U/mg). The highest activity levels were verified at pH 5.0 and the ideal activity temperature was 60°C, rapidly losing its activity at temperatures above 65°C. This study shows that the primary enzymatic activity of MtEG7 a hydrolysis the β-1.4 bonds of substrates because the activity of MtEG7a in β-1,3-glucan bonds was completely inhibited. In addition, the characteristics in terms of catalytic efficiency and thermostability of MtEG7a, makes it a good candidate for industrial applications, including the saccharification of lignocellulosic materials [28].

Rubini et al. [57], reported the isolation and cloning of the first cDNA of *P. echinulatum* (Pe-egl1) that encodes a supposed endoglucanase. This cDNA was expressed in a system of heterologous expression based on the methyl yeast trophic *Pichia pastoris*. *P. echinulatum* EGL1 secreted in the culture supernatant of a recombinant strain of *Picchia pastoris* revealed several characteristics of industrial interest, such as an optimal activity at 60°C and in a wide pH range. Recombinant *P. echinulatum* EGL1 is also interesting for its high thermostability.

Lahjouji et al. [58] described a cDNA of celobiohydrolase Tvcel7a de *Trametes versicolor* cloned and expressed in *Aspergillus niger*. The biochemical properties of purified TvCel7a obtained from both peaks were studied in detail. The optimum pH and temperature were 5.0 and 40°C, respectively. The enzyme is stable in a pH range extending from 3.0 to 9.0 and at temperatures below 50°C. Kinetic parameters with the p-nitrophenyl substrate β-D-cellobioside (pNPC) were 0.58 mM and 1.0 μmol/ min/mg of protein for purified TvCel7a found in peaks 1 and 2. TvCel7a catalyzes the hydrolysis of pNPC, filter paper, β-glucan and avicel in several degrees, but no detectable hydrolysis was observed when the substrates carboxymethylcellulose, laminarin and pNPG were used.

Nakazawa et al. [59] attempted to increase the specific activity of *T. reesei* EG III in *E. coli* by random gene mutagenesis using error-prone PCR followed by plate-assay activity screening. They reported that the yield in the active form of EG III was improved in transforming and the specific activity of their mutant (2R4) was increased. In addition, the stability in the pH and heat of these mutants increased unexpectedly.

Koseki et al. [60] produced an endoglucanase of the glycosyl hydrolase family 61 of *Aspergillus kawachii* (AkCel61) and a truncated enzyme only with the catalytic domain (rAkCel61ΔCBM) in *Pichia pastoris* and analyzed its biochemical properties. The proteins rAkCel61 and rAkCel61ΔCBM produced small amounts of oligosaccharides from soluble carboxymethylcellulose. They also exhibited a slight hydrolytic activity in relation to laminarin. However, they showed no detectable activity in relation to microcrystalline cellulose, arabinoxylan and pectin. Both

recombinant enzymes also showed no detectable activity for p-nitrophenyl-β-D-glucosides, p-nitrophenyl-β-D-cellobiosides and p-nitrophenyl-β-D-celotriosides.

Igarashi et al. [61] report the identification of the gene encoding the endoglucanase (EG) of the family 45 (GH) of *Phanerochaete chrysosporium*, cloning the cDNA, determining its heterologous expression in the methylotrophic yeast *Pichia pastoris* and characterizing the recombinant protein. The recombinant protein showed hydrolytic activity in relation to amorphous cellulose, carboxymethylcellulose, liquena, barley-glucan and glucomannan, but not xylan. In addition, a synergistic effect was observed with cellobiohydrolase of the recombinant GH 6 family of the same fungus for amorphous cellulose as substrate, indicating that the enzyme can act together with other cellulolytic enzymes to hydrolyze cellulosic biomass in nature.

A new β-1,3-1,4-glucanase gene (designated as PtLic16A) of *Paecilomyces thermophila* was successfully cloned and expressed in *Pichia pastoris* as β-1,3-1,4-active extracellular glucanase. The purified enzyme had a molecular mass of 38.5 kDa in SDS-PAGE. It was optimally active at pH 7.0 and at a temperature of 70°C. In addition, the enzyme exhibited strict specificity for β-1,3-1,4-D-glucans. This was the first report on cloning and expression of a β-1,3-1,4-glucanase gene of *Paecilomyces sp* [62].

The gene encoding an endoglucanase of the glycosyl hydrolase (GH) family 45 (Cel45A) was cloned from *P. decumbens* and expressed in *Pichia pastoris* [63]. As far as we know, this is the first report of characterization of a protein of the GH 45 family in *Penicillium* species. The purified recombinant enzyme showed higher activity on glucomannan konjac (KGM) than on sodium carboxymethylcellulose (CMC-Na) or phosphoric acid cellulose (PASC). The highest hydrolytic activity was detected at pH 5.0 in KGM and pH 3.5 in CMC-Na, indicating that the mode of action in both substrates may be different for Cel45A. The optimum temperatures in both substrates were 60°C and about 90% of the relative activities were retained at 70° C. Products released from PASC and CMC-Na were mainly cellobiose, cellotriose. The protein with the highest glucomannanase activity can aid in the efficient degradation of lignocellulose by *P. decumbens* in the natural state.

4.2 Recombinant Exoglucanases

In a study conducted by Li et al. [29], a gene (cbh1) encoding a cellobiohydrolase (CBH) was isolated from the fungus *Aspergillus niger* NL-1. The cellobiohydrolase gene (cbh1) was successfully expressed in *Pichia pastoris* KM71H, presenting molecular mass of approximately 60 kDa. The amino acid sequence encoded by cbh1 shows high homology with the glycoside hydrolase sequence family 7. The recombinant cbh1 exhibited ideal activity at 60°C and pH 4.0 with Km and Vmax for CMC-Na of 13.81 mM and 0.269 μmol/min, respectively. When submitted to 2 h of incubation at 90°C, the enzyme retained more than 80% of its activity and was stable in the pH range 1.0 ± 10.0; due to moderate to high temperature stability and a wide pH range, the authors point out that this enzyme has potential in several industrial applications.

Taipakova et al. [26], obtained the cellobiohydrolase coding gene (Cel6B), belonging to the glycosyl hydrolase 6B family, lentinula edodes isolate cloned in vector pET11d and transformed into *E. coli* (Rosetta DE3). The recombinant protein obtained presented a mass of 46.4 kDa. However, there was the formation of an insoluble inclusion body, preventing enzymatic activity. Such a feature has been observed before, according to Chiang et al. [64], overexpressed proteins in *E. coli* can lead to the formation of the inclusion body. To obtain the recombinant protein in the active form, Taipakova et al. [26], denaturated with 6 M guanidine chloride.

After this stage, the enzyme showed activity of 0.12 U/min, being considered much lower when compared to other celobiohidolases, however, an optimization of this expression system in *E. coli* has a great possibility of obtaining that of active cellulases.

The genome of the basidiomycete *Phanerochaete chrysosporium* contains sequences encoding at least 166 putative hydrolase glycosides, many of which are predicted to β-1,3-glucanases [65]. Kawai et al. [66], cultivated *P. chrysosporium* with laminarin as the only carbon source and found that several β-1,3-glucanases were secreted in the medium. The cDNA encoding a new β-1,3-glucanase with molecular mass of 36 kDa was cloned and expressed in a heterologous way in the methylotrophic yeast *Pichia pastoris*. Based on the catalytic activity of the recombinant enzyme in relation to various substrates β-1, 3-glucan, the recognition pattern for the branched structure of β-1,3/16-glucan is discussed: Lam16A generates non-branched oligosaccharide from branched β-1,3/1,6-glucan.

Voutilainen et al. [67] characterized three new cellobiohydrolases originated from thermophilic ascomycetes fungi. The properties of these three cellobiohydrolases were compared to one of the best characterized celobiohydrolases, *T. reesei* Cel7A. *C. thermophilum* Cel7A showed the highest specific activity and optimum temperature in soluble substrates and these properties also correlate well with its high activity in polymeric substrates.

A gene(cel4) encoding for a cellobiohydrolase II (Ex-4) Ex-4 has been isolated from the basidiomycete of the white rot strain *Irpex lacteus* MC-2 and successfully expressed in yeast *Pichia pastoris*. The recombinant Ex-4 showed endo-processive degradation activity for cellulosic substrates and a synergistic effect on Avicel degradation was observed when the enzyme acted together with cellobiohydrolase I (Ex-1) or endoglucanase (En-1) produced by *I. lacteus* MC-2 [68].

4.3 β-Glycosidase recombinant

Ramani et al. [32] obtained a β-glucosidase (rBgl4) of *Penicillium funiculosum* successfully expressed in the expression system of *Pichia pastoris* KM71H. The recombinant protein rBgl4, after purified presented a weight of ~130 kDa. The rBgl4 activity test at different pH showed ideal activity at pH 5.0 and temperature of 60°C. The enzyme exhibited a high substrate conversion rate for p-nitrophenyl-β-glucosidase and cellobiose, being 3,332 and 2,083 μmol/min/mg, respectively. In addition, rBgl4 demonstrated glucose concentration tolerance of up to 400 mM.

A two-fold increase in glucose yield was observed when supplemented with crude cellulase of *Trichoderma reesei* Rut-C30 in cellulose hydrolysis, suggesting that the recombinant enzyme is a term β-glucosidase and glucose tolerant, and maybe a potential complement to commercial cellulases in cellulose hydrolysis, ensuring profitability in bioethanol production [32].

The gene of a β-glycosidase (bglI) of *Aspergillus niger* NL-1, expressed in *Pichia pastoris,* was obtained by Zhao et al. [30]. The recombinant enzyme showed high activity at pH 4.0 and temperature 60°C and was stable in a pH range of 3.0 to 7.0 and held more than 85% of activity after incubation at 60°C for 30 minutes. The β recombinant glucosidase presented molecular mass of 121 kDa. The authors determined glucose production from avicel compared to recombinant β-glycosidase, where, without the addition of recombinant β-glucosidase, glucose yield was only 49.3%, while with the addition of recombinant β-glucosidase, glucose yield was 63.4%, 70.5% and 78.6%, corresponding to 0.5, 0.75 and 1.0 U/mL, respectively. The results also indicate that BGLI was high glucose tolerant and organic solvent, presenting higher efficiency in the hydrolysis of cellobiose than β-glucosidases.

This study points to the use of β-glycosidase to improve the enzymatic conversion of cellulose to glucose through synergistic action.

Zhao et al. [31] expressed in *Pichia pastoris* a thermostable beta glycosidase of the thermophilic fungus *Myceliophthora thermophila*. The molecular mass of the enzyme after purification was 130.0 kDa the recombinant enzyme (MtBgl3b) MtBgl3b presented pH 5.0 as the ideal for activity at 60°C, and excellent thermostability at 60 or 65°C. The authors also determined the effects of some metal ions and chemical reagents on the activities of MtBgl3b, where Ca^{2+}, Pb^{2+}, K^{+}, Mn^{2+}, EDTA, β-ME and Triton X-100 improved the activity by 6.4–29.9%, while Fe^{3+} completely suppressed the enzymatic activity. In addition, the activities of MtBgl3b were determined in relation to different substrates, for which the enzyme had higher activity against pNPG (258.7 U mg – 1), followed by pNPC (164.5 U mg-1), celotetraosis (125.7 U mg-1), celotriosis (118.0 U mg-1), celobiosis (62.2 U mg-1) and gentilebiosis (63.9 mg U-1). These results indicate that the enzyme presented desirable industrial properties, in addition to thermostability, wide spectrum of substrates and the capacity resistant to ethanol, which makes this protein a great candidate for industrial applications [30].

A β-glucosidase from *A. niger* was successfully expressed in *P. pastoris* and recombinant produced gentileoligosaccharides from glucose. In addition, the main operating parameters of this enzymatic conversion were optimized. At 80% glucose, 60°C, pH 4.5, 1 mmol/ L K +, 60 U of beta-glucosidase per gram of substrate and reaction time of 48 h, the gentiooligosaccharides produced reached 50 g/L [69].

5. One-time expression with the new CRISPR/Cas9 system technology

In recent years, several genetic tools have been elaborated and applied in various fungi and widely shared in different sectors of the economy. However, there is still a certain limitation in the studies of functional genomics for the production of recombinant cellulases in fungi. For this logic, emerging tools stand out, including CRISPR-Cas9-based genome editing (Clustered Regularly Interspaced Short Palindromic Repeats), i.e., Grouped and Regularly Interspaced Palindromic Repeats, as an agile tool for genome-specific gene edits [70]. The CRISPR-Cas9 system contains two components: the effector protein, which is Cas9 endonuclease, and a single chimeric guide RNA (sgRNA). This tool was involved to allow rapid editing of the genome of several organisms, among them, some varieties of filamentous fungi [71–75].

However, such approaches are not as useful as those available for yeasts and bacteria, considering the complexity of fungi, such as multicellular morphology, cell differentiation, thick chitinous cell walls and lack of adequate plasmids [76]. Composing the need to establish a genome editing system that can be used to develop a hyper cell factory for preparations of lignocellulolytic enzymes and other heterologous proteins, as well as to characterize the mechanisms that regulate protein induction, synthesis, and secretion [77].

In studies with *Myceliophthora thermophile* Li et al. [78] used the CRISPR/Cas9 technique in five highly expressed genes encoding extracellular proteases, degrade extracellular proteins and reduce cellulase yield. The results attest that Mtalp1 is a gene that degrades protease that inhibits cellulase production. To perform this study, five genes were selected and constructed using the CRISPR-Cas9 technique, resulting in a Mutant DMtalp1 that demonstrated protease activity substantially lower than 58.4%, and may be a good initial strain for additional metabolic engineering in order to produce cellulases and other proteins.

Liu et al. [74] used the CRISPR/Cas9 system for effective multiplexed genome engineering, successfully developed in thermophilic species *M. thermophila* and *M. heterothallica*. CRISPR/Cas9 can efficiently modify the imported a mdS gene into the genome through non-homologous nhej end-mediated events. As evidence of principle, the genes of the cellulase production pathway including cre-1, res-1, gh1–1 and alp-1, were chosen as editing targets. Simultaneous multigenic fissures of up to four of these different loci were prepared with the integration of the neomycin selection marker by means of a single transformation, using the CRISPR/Cas9 system.

This genome engineering tool gave rise to several strains that exhibit marked production of hypercellulase, among which extracellular secret activities of protein and lignocellulase increased substantially (up to 5 and 13 times, respectively), in analogy with the parent lineage. In their research, Salazar-Cerezo et al. [79], used *Penicillium subrubescens*, which is an ascomycete fungus with a robust content of families of active enzymes specific to carbohydrates involved in the degradation of ligonocellulosoic biomass. First, a method was developed for the engendering and transformation of protoplasts, using hygromycin as a selection marker. Subsequently, the CRISPR/Cas9 system was established in *P. subrubescens* by successfully excluding the KU70 gene, which was directly involved in the non-homologous end of the DNA repair mechanism. According to Salazar-Cerezo et al. [79], it was possible to consider the implementation of the CRISPR/Cas9 system in the filamentous fungus *P. subrubescens* and the effective protocols for generating and transforming protoplasts were optimized. In this way the MUTATED KU70 gene showed no discrepant phenotypic differences with the wild-type strains reported in the study, enabling the use of these mutants as parental strains for subsequent transformation events.

Rantasalo et al. [80] made use of CRISPR/Cas9 multiplexed in combination with System (S), classified as synthetic expression producing large amounts of the highly pure calB gene; this combination allowed the production of strains in a shorter time. Rantasalo et al. [80], when using the SES tool, it was used by the calB gene indices in an inducing medium, with highly constitutive expression provided by the SES, being possible to produce approximately 4 grams of glucose per liter of calB, in a cellulase inducing medium.

Zheng et al. [81], was able to create a CRISPR/Cas9 system in *Aspergillus niger* for sgRNA expression based on an endogenous U6 promoter and two heterologous promoters of U6. The three u6 promoters tested made feasible the transcription of sgRNA and the interruption of the gene of polyketide synthase albA gene in *A. niger*. In addition, this system allowed the insertion of highly efficient genes in the target genomic locus in *A. niger*, using DNAs from donors with homologous arms of up to 40 bp.

One of the alternatives for bioprospecting enzymes potentially for industry and the use of CRISPR/Cas9 with non-model species, such as the fungus *Huntiella omanensis*, a filamentous fungus ascomycete belonging to the family Ceratocystidaceae, will allow cutting in metabolic and genetic pathways that have not yet been studied in model species [82].

However, filamentous fungi are considered the largest producers of cellulases so far, since for genome editing systems using CRISPR-Cas9, it was stipulated in more than 40 different species of filamentous fungi and oomycetes, being therefore an important strategy regarding the production of potential strains for applications in the industry [83]. Cellulases and heterologous of fungi, with great industrial potential in the manufacture of bioethanol, using one of the most efficient techniques of recent times for genetic engineering, CRISPR/Cas9. It can be concluded that this technique, combined with biotechnological advances, will result in the improvement of fungal cells capable of producing biofuels economically and on an industrial scale, resulting in higher yield and quality of products.

6. Conclusions

Cellulases are important enzymes in cellulose degradation that occurs in nature, this degradation involves a system of extracellular multienzymes and have wide application. Molecular biology combined with recombinant DNA technology is a viable tool in enzymatic production with high activity, what makes recombinant fungal cellulases good candidates for industrial applications, including the saccharification of lignocellulosic materials.

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Conflict of interest

The authors declare no conflict of interest.

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