KLUYVEROMYCES LACTIS AS AN EXPRESSION HOST FOR ENZYMES THAT DEGRADE LIGNOCELLULOSIC BIOMASS

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Lignocellulose is the most abundant regenerative raw material in the world and is therefore an important substrate for the conversion of biomass into biofuels and other high-value products. The efficient microbial degradation of plant biomass in nature requires a set of different enzymes that act in concert to degrade different parts of the lignocellulose molecule. For example, cellulases are responsible for cellulose hydrolysis, and peroxidases and laccases are the main enzymes for lignin degradation. One strategy for the generation of tailor-made enzyme cocktails is the production of specific combinations of different enzymes for different biomass sources.

Because the best known biomass degraders are fungi, a eukaryotic expression system is preferable for the expression of biomass-degrading enzymes. We therefore chose the yeast *Kluyveromyces lactis* for the over expression of enzymes from fungi such as *Trichoderma reesei* and *Pycnoporus cinnabarinus*. *K. lactis* combines eukaryotic post-translational modifications with easy single-cell fermentation conditions. Furthermore, it can secrete the recombinant enzymes to avoid expensive and time-consuming downstream processing steps.

Here, we demonstrate that *K. lactis* is an appropriate host for the production of recombinant enzymes that degrade lignocellulose, using the endoglucanase *Tr*Cel5A and the laccase *Pc*LCC3-1 as examples. Both enzymes were secreted efficiently into the culture medium, and following affinity purification were active against the model substrates Azo-CMC (*Tr*Cel5A) and ABTS (*Pc*LCC3-1).

Keywords: *Kluyveromyces lactis*, heterologous expression, protein secretion, cellulose, lignin, cellulase, laccase

INTRODUCTION

Plant biomass is a promising alternative to conventional, non-renewable sources of energy. The conversion of lignocellulose into high-value products, such as fuels and chemicals, is an important route for the production of second-generation biofuels. First-generation biofuels are derived from easily-fermentable carbon sources, such as starch and sucrose, but the conversion process for lignocellulosic materials is much more complex. Lignocellulose is a heteropolymer composed of carbohydrates (cellulose and hemicelluloses) and the aromatic, non-carbohydrate component lignin. Lignocellulose is recalcitrant to chemical and biological degradation due to the hydrogen bonds within the crystalline cellulose matrix and the complexity of lignin, which is covalently bound to hemicelluloses. Therefore, many industrial biomass conversion methods require harsh physicochemical conditions to remove lignin from the carbohydrate fraction. Physical pretreatments include milling or extrusion, whereas chemical pretreatments include alkaline or acid digestion, as well as the Organosolv and Organocat processes.[1,2] The biological degradation of lignocellulose is carried out by several fungi, which tend to focus on either cellulose or lignin. Cellulose is used industrially for the manufacture of products like bioethanol.[3] This requires the sequential degradation of cellulose into smaller compounds and ultimately glucose, which is used for the production of ethanol by fermentation. In nature, cellulose is degraded by the hydrolytic activity of three major enzyme classes: endoglucanases, cellobiohydrolases, and β-glucosidases, in concert with auxiliary proteins such as swollenin, which decrystallizes parts of the cellulose (Figure 1A).[4]

The separated lignin is usually burned during the process to generate power for the conversion of the carbohydrate compounds. However, lignin as an energy-rich compound might also be used for the generation of other valuable products, e.g. fuel additives, composites, plastics or other chemicals.[3] In contrast to the cellulases system, little is known about the activity of lignin-degrading enzymes. Three main groups of enzymes have been identified: lignin-degrading peroxidases (heme peroxidases), laccases (copper-containing oxidases) and auxiliary enzymes (e.g. oxidoreductases) (Figure 1B). Whereas manganese peroxidases and lignin peroxidases specifically target either the phenolic or non-phenolic structures, versatile peroxidases include the features of both and have dual specificity. Laccases degrade phenolic structures, but may modify non-phenolic structures in the presence of a mediator. Additional enzymes are thought to regulate lignin degradation by reducing the methoxy radicals generated by peroxidases and laccases or by producing the H_2O_2 required for peroxidase activity.[5]

A cocktail of several enzymes that degrade cellulose and lignin may be necessary for the successful and efficient conversion of lignocellulose into valuable products. The enzyme cocktail must be tailored to the composition of the biomass on a case-by-case basis. One approach is the expression of individual recombinant enzymes that can be formulated into a defined enzyme mixture. A eukaryotic expression host is preferable because the most active and best characterized lignocellulose-degrading enzymes are of fungal origin and thus require posttranslational modifications, such as disulfide bonds and glycosylation, to ensure stability and activity. We chose to work with the yeast *Kluyveromyces lactis*, for which a commercial vector system is available to facilitate cloning and selection. We discuss the challenges of using *K. lactis* as an expression host and some successful examples of recombinant protein expression.

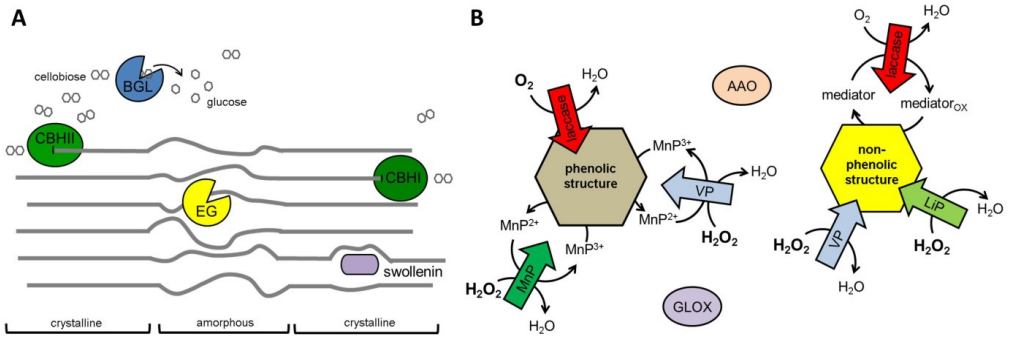

Figure 1: Enzymatic degradation of lignocellulose. A: The cellulolytic system. Endoglucanases (EG) cleave mainly within the amorphous part of the cellulose. Cellobiohydrolases (CBH) attack either the reducing or non-reducing ends of the crystalline cellulose structure, releasing cellobiose, which is digested by β-glucosidases (BGL) to produce glucose. Swollenin decrystallizes parts of the cellulose (adapted from[4]). B: The lignin-degrading system. Three main groups of enzymes are involved in the degradation of lignin. Peroxidases (MnP, VP, LiP) and laccases degrade the phenolic and non-phenolic structures using H_2O_2 or oxygen as an electron acceptor and, if applicable, mediators or manganese as intermediates. Accessory enzymes (e.g. AAO, GLOX) enhance enzyme activity, e.g. by producing H_2O_2. MnP: manganese peroxidase, VP: versatile peroxidase, LiP: lignin peroxidase, AAO: aryl alcohol oxidase, GLOX: glyoxal oxidase[5]

EXPERIMENTAL

Cloning and transformation

Genes encoding the enzymes of interest were synthesized and ligated into the commercial vector pKLAC1, which had been modified by the introduction of a C-terminal His_6 tag sequence into the expression cassette. Transformation and selection were carried out according to the manufacturer's protocol (*K. lactis* Protein Expression Kit, New England Biolabs).

Protein expression and purification

Cultivation and protein expression were carried out as previously described.[6] The proteins were recovered from the supernatant by immobilized metal-ion affinity chromatography (IMAC).

RESULTS AND DISCUSSION

We selected a range of fungal lignocellulose-degrading enzymes for expression in *K. lactis*,

because these enzymes naturally exhibit high specific activities against their target substrates. A set of lignin degrading peroxidases, comprising a lignin peroxidase (LP1), a manganese-dependent peroxidase (LP2) and a versatile peroxidase (LP3), as well as a *Pycnoporus cinnabarinus* laccase (*Pc*LCC3-1) and a *Trichoderma reesei* cellulase (*Tr*Cel5A), were evaluated. The corresponding genes were successfully integrated into the *K. lactis* genome by homologous recombination and transformants were identified using the selectable marker gene *amd*S (Figure 2).

Heterologous protein expression and secretion were confirmed by SDS-PAGE and western blot analysis using an anti-His_6 antibody (Figure 3). As expected, strong western blot signals were observed for all cultures expressing a recombinant His_6-tagged protein, but no signals were visible in the control samples, the wild-type *K. lactis* strain (WT) and a *K. lactis* strain carrying an empty pKLAC1 vector (EV). The western blot signals were stronger than the corresponding band in the stained gel for two independent clones producing *Pc*LCC3-1 (L1, L2) and also the clones producing Cel5A and one lignin-degrading peroxidase (LP2). This indicated that the proteins were secreted efficiently. In contrast, the recombinant lignin-degrading enzymes LP1 and LP3 were not secreted efficiently. Most of the enzyme accumulated within the cell, as shown by the relatively weak western blot signal compared to the stained gel, confirming that most of the protein was retained in the pellet (Figure 3). The smear observed in each lane reflects the hyperglycosylation of proteins in *K. lactis*.

Figure 2: Schematic illustration showing the integration of expression cassettes into the *K. lactis* genome. A modified version of the commercial vector pKLAC1 was used to clone different lignocellulose-degrading enzymes, and *K. lactis* was transformed by homologous recombination following plasmid linearization. Transcription was controlled by the LAC4 promoter (P_{LAC}) and protein secretion was promoted by an α-mating factor (α-MF) peptide sequence located upstream of the gene of interest (GOI). Selection was achieved by expressing an acetamidase selectable marker gene (*amd*S) under the control of the yeast ADH2 promoter (P_{ADH2}:); TT = terminator

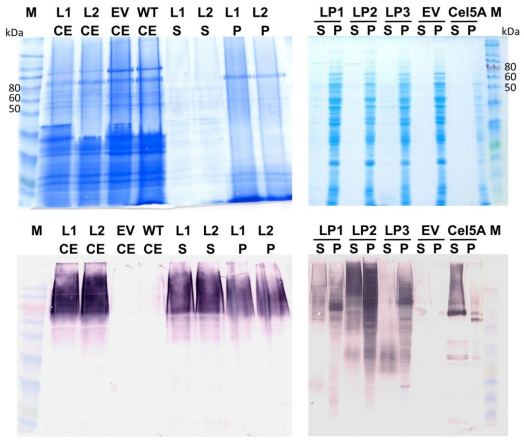

Figure 3: SDS-PAGE (upper panels) and western blot analysis (lower panels) of lignocellulose-degrading enzymes expressed in *K. lactis*. An aliquot of each culture was separated into pellet (P) and supernatant (S) fractions. For laccase, wild-type (WT) and empty vector samples, a crude cell extract (CE) was also loaded onto the gel. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue or blotted and incubated with an anti-His_6 antibody. The smear presumably reflects hyperglycosylation. CE: crude cell extract; EV: empty vector carrying no gene of interest; L1, L2: two different transformants expressing the same laccase; LP1-LP3: three different lignin-degrading peroxidases. Size of the native enzymes: L = 55 kDa; LP = 35-40 kDa; Cel5A = 44 kDa

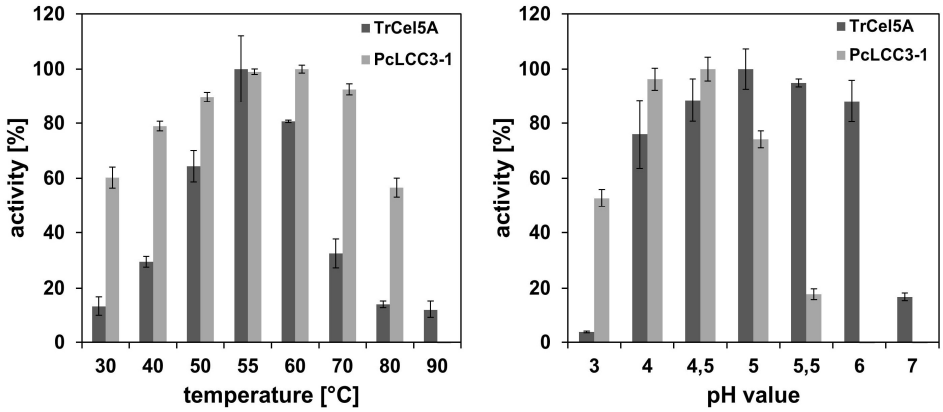

Figure 4: Temperature and pH profiles of the recombinant laccase (*Pc*LCC3-1) and cellulase (*Tr*Cel5A). A: *Tr*Cel5A samples were incubated at pH 5 in the presence of 0.5% AzoCMC for 20 min at the indicated temperatures. Activity was measured as an increase in absorption at 590 nm compared to a buffer control baseline (50 mM sodium acteate pH 5). *Pc*LCC3-1 samples were incubated at pH 4.5 in the presence of 0.8 mM ABTS at different temperatures. The oxidation of ABTS was confirmed by measuring the absorbance at 420 nm for 15 min. B: *Tr*Cel5A and *Pc*LCC3-1 samples were incubated in the presence of 0.5% AzoCMC for 20 min at 55 °C and in the presence of ABTS for 15 min at 30 °C at the pH values shown. *Tr*Cel5A: n = 3; *Pc*LCC3-1: n = 2; bars indicate standard deviations.

The successful secretion of heme peroxidases (such as horseradish peroxidase) and even a manganese peroxidase has been achieved using *Pichia pastoris* as the production host.[7] *K. lactis* also possesses a heme-synthesis metabolism[8] and the expression of a mature protein is therefore likely. However, additives such as $FeSO_4$ and the heme precursor 5-aminolevulinic acid can increase the yield of active protein.[9] Furthermore, the signal peptide sequence also determines the secretion efficiency of recombinant enzymes and minor changes in this sequence can have a strong impact.[10,11] Alternative signal peptides, including native sequences and tailored synthetic peptides, may therefore facilitate the secretion of the lignin-degrading peroxidases LP1 and LP3.

*Tr*Cel5A and *Pc*LCC3-1 were purified by immobilized metal ion affinity chromatography with protein yields of 20 mg/L (*Tr*Cel5A) and 5 mg/L (*Pc*LCC3-1) and specific activities of 6.2 U/mg on Azo-CMC (*Tr*Cel5A) and 0.72 U/mg on ABTS (*Pc*LCC3-1). Furthermore, the pH and temperature optima of the purified secreted laccase (*Pc*LCC3-1) and cellulase (*Tr*Cel5A) were found to be similar (Figure 4). *Pc*LCC3-1 had a pH optimum of pH 4.5 and a temperature optimum of ~60 °C, which is comparable with the native *P. cinnabarinus* laccase.[12] Similarly, *Tr*Cel5A had a pH optimum of pH 5.0 and a temperature optimum of 55 °C, which is comparable to the native enzyme from *T. reseei*.[13]

CONCLUSION

K. lactis can be used as an expression host for recombinant enzymes, but the signal peptide for secretion must be adapted and optimized for each recombinant enzyme. We showed by western blot analysis that three enzymes were expressed and secreted successfully, whereas two peroxidases remained inside the cell. The pH and temperature optima of the successfully expressed and secreted *P. cinnabarinus* laccase and *T. reesei* endoglucanase were similar to the native enzymes.[12,13] This clearly demonstrates the suitability of *K. lactis* for the production of recombinant fungal enzymes. Purified enzymes fused to a His_6 tag sequence could facilitate various downstream applications, such as immobilization procedures using defined amounts of enzyme or the recovery and reuse of enzymes to minimize production costs.

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