Expression and Characterization of Chimeric CBHI in Yeasts and the Effects of CBHI Fusion to EGII

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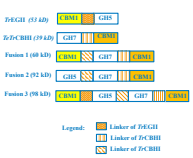
Expression of an Endoglucanase-Cellobiohydrolase Fusion Protein in *S. cerevisiae, Y. lipolytica, and L. starkeyi*

The low secretion levels of cellobiohydrolase I (CBHI) in yeasts is one of the key barriers preventing yeast from directly degrading and utilizing lignocellulose. To overcome this barrier, we have explored the approach of genetically linking an easily secreted protein to CBHI, with CBHI being the last to be folded. The *Trichoderma reesei* eg2 (*Tr*EGII) gene was selected as the leading gene due to its previously demonstrated outstanding secretion in yeast. To comprehensively characterize the effects of this fusion protein, we tested this hypothesis in three industrially relevant yeasts: *Saccharomyces cerevisiae, Yarrowia lipolytica,* and *Lipomyces starkeyi*.

Our initial assays with the *L. starkeyi* secretome expressing differing *Tr*EGII domains fused to a chimeric *Talaromyces emersonii – T. reesei* CBHI (*TeTr*CBHI) showed that the complete *Tr*EGII enzyme, including the glycoside hydrolase (GH) 5 domain is required for increased expression level of the fusion protein when linked to CBHI (Figure 1). We found that this new construct (*Tr*EGII-*TeTr*CBHI, Fusion 3) had an increased secretion level of at least three-fold in *L. starkeyi* compared to the expression level of the chimeric *TeTr*CBHI (results not shown) and significantly improved secretome level activity against Avicel (Figure 2). However, digestion of pretreated corn stover (PCS) with the secretomes of *Y. lipolytica* and *L. starkeyi* showed that conversion was much better using *Y. lipolytica* secretomes (50% vs. 29%, respectively) and that in *Y. lipolytica*, *TeTr*CBHI performed better than the fusion construct (Figure 3). Furthermore, *S. cerevisiae* expression of the *Tr*EGII-*TeTr*CBHI fusion construct was poor and only minimal activity was observed on the substrate pNP-cellobiose; and no activity was observed for the pNP-lactose substrate (results not shown).



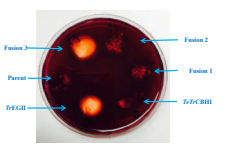


Figure 1. L. starkeyi secretome endoglucanase activity with Congo-Red staining.

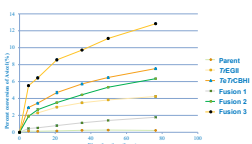


Figure 2. L. starkeyi secretome activity on Avicel.

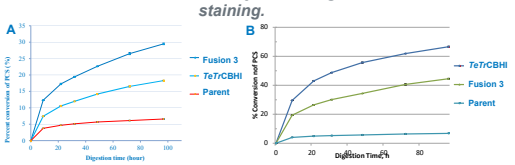


Figure 3. L. starkeyi and Y. lipolytica secretome activity on PCS.

Conclusions

- The Fusion 3 construct (*Tr*EGII-*TeTr*CBHI) has an increased secretion level of at least three-fold compared to that of individual chimeric *TeTr*CBHI in *L. starkeyi*.
  - ➢ The same benefits do not extend to *Y. lipolytica* or *S. cerevisiae*, indicating that this approach is not universally applicable.
- Expression of the *Tr*EGII-*TeTr*CBHI fusion construct in *S. cerevisiae* was poor and only minimal activity was observed with pNP-cellobiose substrate and it has no activity for pNP-lactose indicating that *Tr*EGII may have folded correctly but there were problems with the CBHI domain of the fusion in *S. cerevisiae*.
- Our results indicate that fusion proteins could be used as an engineering strategy in yeasts and possibly other organisms to increase secretion levels and specific activity of not only CBP-biofuels relevant pursuits, but more broadly in the context of general secretion of enzymes from yeast.

Characterization of Chimeric CBHI Expressed in *Y. lipolytica, L. starkeyi* and *S. cerevisiae* and the Effects of Glycosylation

Yeasts are known to have problems with properly folding all the expressed CBHI protein. To understand the properties of chimeric *TeTr*CBHI expressed in yeasts we compared the yields of purified active protein. Table I shows the yield of purified *TeTr*CBHI from *Y. lipolytica, L. starkeyi*, and *S. cerevisiae*. *L. starkeyi* has the lowest level of active purified *TeTr*CBHI at 0.08 mg/L followed by *Y. Lipolytica* with a yield of 1.09 gm/L – a more than 10-fold increase. *S. cerevisiae* has a production level of almost 3-fold higher than *Y. Lipolytica* but with the final recovery of three distinct active *TeTr*CBHI isoforms.

To assess the purity and extent of glycosylation we did SDS-PAGE with Coomassie blue, Western blot and glycosylation staining (Figure 1). All chimeric CBHI samples had multiple bands in Western blot and glycosylation staining indicating degradation and/or variable glycosylation. To thoroughly understand what was happening we analyzed all samples with HPLC size exclusion chromatography and compared with *Tr*CBHI (Table I).

The chimeric *TeTr*CBHI purified from *L. starkeyi* and *Y. lipomyces*, only converted 70% of the available PCS cellulose compared to 80% for *Tr*CBHI purified from its native host while peak 1 from *S. cerevisiae* only converted 60% (Figure 3). Despite taking the best fraction for *Saccharomyces* expressed *TeTr*CBHI it still clearly underperforms compared to *L. starkeyi* and *Y. lipomyces* expressed chimeric *TeTr*CBHI. *Tr*CBHI is still more active by reaching a conversion extend of over 80% in 100 hours comparted to around 70% for the other two.

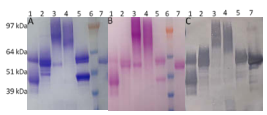


Figure 1. Conversion of PCS cellulose by enzymes purified from S. cerevisiae, L. starkeyi, Y. lipolytica, and T. reesei.

Table I. Yield of purified TeTrCBHI and HPLC data. Higher numbers for the RI/UV area ratio indicate higher glucan content

Protein	Protein yield (mg/L)	HPLC RI peaks mobility (minutes)	HPLC RI/UV area ratio
*TeTr*CBHI in *Y. lipolytica*	1.09	15.07, 19.97	0.43
*TeTr*CBHI in *L. starkeyi*	0.08	15.18, 19.20	0.61
*TeTr*CBHI in *S. cerevisiae* peak 1	0.82	13.30, 20.42	0.67
*TeTr*CBHI in *S. cerevisiae* peak 2A	1.09	13.10, 19.42	0.66
*TeTr*CBHI in *S. cerevisiae* peak 2B	1.08	18.23, 21.20	0.40
*TeTr*CBHI in *S. cerevisiae* combined	2.99	-	-
*Tr*CBHI	-	15.12, 18.68	0.46

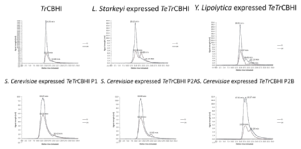


Figure 2. HPLC chromatograms.

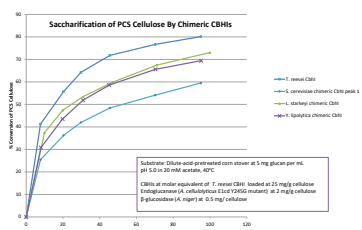


Figure 3. Conversion of PCS cellulose by enzymes purified from S. cerevisiae, L. starkeyi, Y. lipolytica, and T. reesei.

Conclusions

- *Saccharomyces* hyper glycosylates *TeTr*CBHI
  - ➢ Some activity loss but the best yield of active enzyme
- *S. cerevisiae* has the best yield but also the lowest extent of conversion with purified protein
- *L. starkeyi* and *Y. lipomyces* expressed chimeric *TeTr*CBHI have similar extents of conversion but Yarrowia produces over ten times more purified and active chimeric *TeTr*CBHI
  - ➢ *L. starkeyi* is an inferior CBP candidate compared to *Yarrowia* and *Saccharomyces*
  - ➢ Both Yarrowia and Saccharomyces would likely perform well as CBP microorganisms

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