

Title

Secretion of recombinant lignin peroxidase in the yeast *Kluyveromyces lactis*

Authors

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Extended Abstract

Persistent organic pollutants (POPs) such as PCBs, DDT, TNT, have been extensively used as pesticides and fungicides. In a recent report, more than 75% of POP-related poisonings were caused by either POPs transported beyond where they were applied or by pesticide residues. DDT and PCBs discharged into the ocean near Los Angeles, CA have contaminated numerous fish species. Physical decontamination methods can prove to be too costly, impractical or even generate additional problems. Bioremediation presents a cleaner and more economical alternative. One promising enzyme for bioremediation is the metalloprotein lignin peroxidase (LiP). LiP is able to degrade the biopolymer lignin as well as a large number of recalcitrant environmental pollutants such as TNT, PCBs, azo dyes, etc.

LiP is secreted as a group of isozymes with overlapping substrates of which isomers H2 and H8 are most common. Incorporation of a heme into the catalytic site, glycosylation of side chains and presence of veratryl alcohol and hydrogen peroxide are critical for LiP activity. The native fungus *Phanerochaete chrysosporium* secretes LiP under stringent conditions of nutrient limitation after 4 – 6 days, making large scale production unviable. Homologous overexpression has been achieved with a yield of only 2mg/liter. Expression and secretion in numerous prokaryotic and eukaryotic hosts have been met with numerous problems such as inability to post-translationally modify the protein (*Escherichia coli*), secretion of inactive protein (*Aspergillus niger*) and incorrect glycosylation (insect cells). Recently, a *Saccharomyces cerevisiae* system was developed that expresses LiP only as a surface bound protein with reduced activity.

Our research aim is to engineer yeast for large scale production of this valuable protein. Two successful industrial yeast species, *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, were investigated for the synthesis and secretion of LiP. In initial tests, several strains and plasmid constructs were screened to determine the best candidates for LiP secretion. Only the extracellular fraction of *K. lactis* was bound by the LiP antibody indicating this species' ability to secrete the enzyme. LiP was also produced in active form. To obtain increased levels of the enzymes, several strategies were employed. A gratuitous *K. lactis* strain allowed high levels of induction with minimal quantities of inducer, and a high-copy partial pKD1 plasmid reduced structural instability. Further, culture and medium conditions were optimized for maximum synthesis and secretion of the protein product. Replacement of the initial *S. cerevisiae* *GAL1* promoter with the tightly-regulated native *K. lactis* *LAC4* promoter substantially reduced plasmid instability, increasing secreted LiP levels. LiP is a complex protein to make and induction in early batch culture resulted in very low plasmid stability. To decouple the growth and protein production stages,

cultures were grown to late exponential phase and then induced. This increased protein yield an additional 2-5 fold. Preliminary tests reveal over-glycosylation of the recombinant enzyme; however, the protein is in active form in the supernatant. The combination of genetic and cultivation strategies has resulted in substantial improvements in extracellular LiP production. To the best of our knowledge, this is the first report of secretion of active LiP from a microbial host.

Current efforts are two-pronged – chromosomal integration and directed evolution. Integration of the LiP cassette into the host chromosome will allow for earlier induction without fear of losing the expression cassette. This should further increase secreted LiP levels. Directed evolution will select for variants that are easier to secrete, have increased protein activity towards a particular substrate, or have improved intrinsic properties such as thermal stability. Traditionally, LiP has been used for producing better quality paper by degrading lignin in the wood used. Current applications have expanded its use to biotransformations and biosensors. Given such myriad uses of this efficacious protein a large scale source of LiP would be very welcome.