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Engineering the ligninolytic enzyme consortium

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The ligninolytic enzyme consortium is one of the most-efficient oxidative systems found in nature, playing a pivotal role during wood decay and coal formation. Typically formed by high redox-potential oxidoreductases, this array of enzymes can be used within the emerging lignocellulose biorefineries in processes that range from the production of bioenergy to that of biomaterials. To ensure that these versatile enzymes meet industry standards and needs, they have been subjected to directed evolution and hybrid approaches that surpass the limits imposed by nature. This Opinion article analyzes recent achievements in this field, including the incipient groundbreaking research into the evolution of resurrected enzymes, and the engineering of ligninolytic secretomes to create consolidated bioprocessing microbes with synthetic biology applications.

The ligninolytic armory

The degradation of lignin is fundamental for carbon recycling in the biosphere and, as such, it is being studied intensely to be incorporated into emergent lignocellulose biorefineries (see Glossary) [1,2]. Among the microbes involved in natural ligninolysis, basidiomycete white-rot fungi have a wide array of high redox-potential oxidoreductases with a broad substrate range and the ability to catalyze the complete mineralization of lignin to CO_2 and H_2O (Box 1) [3]. Bearing in mind that lignocellulose biomass is the most abundant feedstock on the earth, with an estimated production of ~200 billion tons/year, it is not surprising that the US Department of Energy has invested funds to sequence over 80 fungal genomes in view of their potential application in lignocellulose biorefineries. In fact, ligninolytic enzymes – also known as ligninases – could offer a broad repertoire of solutions in different industrial settings, such as in the sustainable production of renewable chemicals, materials, and fuels (including second-generation biofuels such as bioethanol and biobutanol), organic synthesis (antibiotics, polymers, building blocks), nanobiotechnology (biofuel cells and biosensors for biomedical applications), bioremediation [removal of polycyclic aromatic hydrocarbons (PAH), dioxins, halogenated compounds and many other xenobiotics], the food industry (beverage and bakery processing), and the pulp biobleaching and textiles industries, to name but a few [4,5]. However, to meet market demands these versatile biocatalysts need to be produced and manipulated such that their properties can be adapted to non-natural industrial environments, or to improve their catalytic capacities [6].

The present article summarizes the most significant advances in engineering the ligninolytic consortium by directed evolution and hybrid approaches (Box 2), as well as the trends in the foreseeable future of this exciting research field. For the sake of clarity, the information is organized around two case studies: (i) engineering to improve functional expression, activity, and stability, and (ii) adaptations to non-natural environments. Finally, the development of future prospects is briefly outlined, including 'time-travel' back and forth along the evolutionary timeline and the incipient engineering of synthetic white-rot yeasts.

Glossary

Directed evolution: a protein-engineering strategy to improve or create catalytic attributes mimicking the algorithm of natural selection. It couples random mutagenesis and DNA recombination with high- and ultrahigh-throughput screening assays. When combined with semi-rational and computational approaches, the exploration of the protein sequence space can be notably reduced.

Enzyme resurrection: heterologous expression of ancestral enzymes from extinct organisms in modern microorganisms. The ancestral genes must first be reconstructed by phylogenetic/inference methods based on bioinformatics computations supported by sequence databases.

Lignin: a highly-recalcitrant biopolymer that strengthens the plant cell wall and forms a complex matrix that protects cellulose and hemicellulose fibers from microbial attack. Lignin is mainly formed of dimethoxylated, monomethoxylated, and non-methoxylated phenylpropanoid moieties derived from *p*-hydroxycinnamyl alcohols (coniferyl, coumaryl, and sinapyl alcohols). Lignin represents one third of the carbon fixed in terrestrial ecosystems as lignocellulose.

The ligninolytic secretome: a set of ligninases and H_2O_2-supplying enzymes secreted by white-rot fungi during natural wood decay. Highly active, extracellular, and soluble, the ligninolytic secretome is fairly heterogeneous and its enzyme composition varies depending on the species, stage of growth, and environmental conditions.

Lignocellulose biorefinery: a fully integrated and highly sustainable pipeline that uses lignocellulosic components from plant biomass to synthesize biomaterials (including bio-derived plastics, high-value chemicals, and building blocks), transportation fuels (bioethanol and biobutanol), and direct energy.

Redox potential of ligninases: can be defined as the energy required to capture one electron from the reducing substrate. The higher the redox potential the broader the oxidative capability of the ligninase. Ligninolytic peroxidases show higher redox potentials (up to 1.4 V) than do laccases (up to 0.8 V); the latter compensate for this deficit by using redox mediators (diffusible electron carriers that upon oxidation by the laccase are capable to oxidize higher redox potential and bulky compounds).

Wood-rotting fungi: many basidiomycetes and ascomycetes are capable of degrading wood. They are sorted into white-rot, brown-rot, soft-rot, and stain fungi according to the extent of lignocellulose modification. White-rot fungi mineralize the main components of wood according to two distinguishable patterns of decay: simultaneous rot (the simultaneous degradation of lignin, cellulose, and hemicellulose) and selective rot (the selective removal of lignin in advance of cellulose degradation).

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Box 1. The ligninolytic enzyme consortium

The fungus secretome profile and its sequential production are fundamental to study natural lignin degradation. Strongly connected to the pattern of lignin degradation, the function of this multi-enzymatic cascade is complemented by radicals of aromatic compounds and oxidized metal ions that can act as diffusible electron carriers through a mechanism of action that is not fully understood. Sorted in terms of their oxidative performance, the main ligninases are high redox-potential peroxidases [lignin peroxidases (LiP), manganese peroxidases (MnP), and versatile peroxidases (VP)] and laccases. Other enzymes involved in the modification of lignin at different stages (Figure I) are: unspecific peroxygenases (UPO); hydrogen peroxide-supplying oxidases [aryl alcohol oxidases, AAO), glyoxal oxidases (GLX), methanol oxidases (MOX), glucose oxidases (GO)], and ferric-ion reducing enzymes [cellobiose dehydrogenases (CDH) and quinone reductase (QR)]. The general characteristics of main ligninases are described below.

Laccases (EC 1.10.3.2). These enzymes belong to the group of blue multi-copper containing oxidases capable of oxidizing phenols, polyphenols, aromatic amines, and many other compounds, using oxygen from air and releasing water as the only byproduct. Laccases harbor 4 Cu atoms, one blue Cu at the T1 site where the oxidation of the reducing substrate takes place,and three additional Cu ions clustered in a T2–T3 trinuclear site for the reduction of O_2 to H_2O. Laccases are classified according to the redox potential exhibited at the T1 site as low (<460 mV), medium (≤700 mV), and high (800 mV) redox-potential enzymes.

Ligninolytic peroxidases. Heme-containing peroxidases with high redox potential represented by lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13), and versatile peroxidase (VP, EC 1.11.1.16). The latter contains three catalytic sites for the oxidation of low, medium, and high redox-potential compounds, combining the catalytic traits of LiP, MnP, and generic low redox-potential peroxidases (GP).

Unspecific peroxygenases (UPO, EC 1.11.2.1). Also referred as to aromatic peroxygenases, APO), these are new types of heme–thiolate enzymes with self-sufficient mono(per)oxygenase activity and high redox potential. With over 300 positive substrates tested, UPOs are capable of performing several highly selective oxyfunctionalizations (including hydroxylation of aromatic and aliphatic alkenes, and epoxidation of olefins), which are of great interest in organic synthesis.

Aryl-alcohol oxidases (AAO, EC 1.1.3.7). Monomeric flavoproteins that supply H_2O_2 to peroxidases and peroxygenases during lignin degradation, while displaying a high enantioselectivity through a hydride abstraction process. AAOs have recently been classified as auxiliary activity (AA) families, together with glyoxal oxidases, pyranose oxidases, and methanol oxidases.

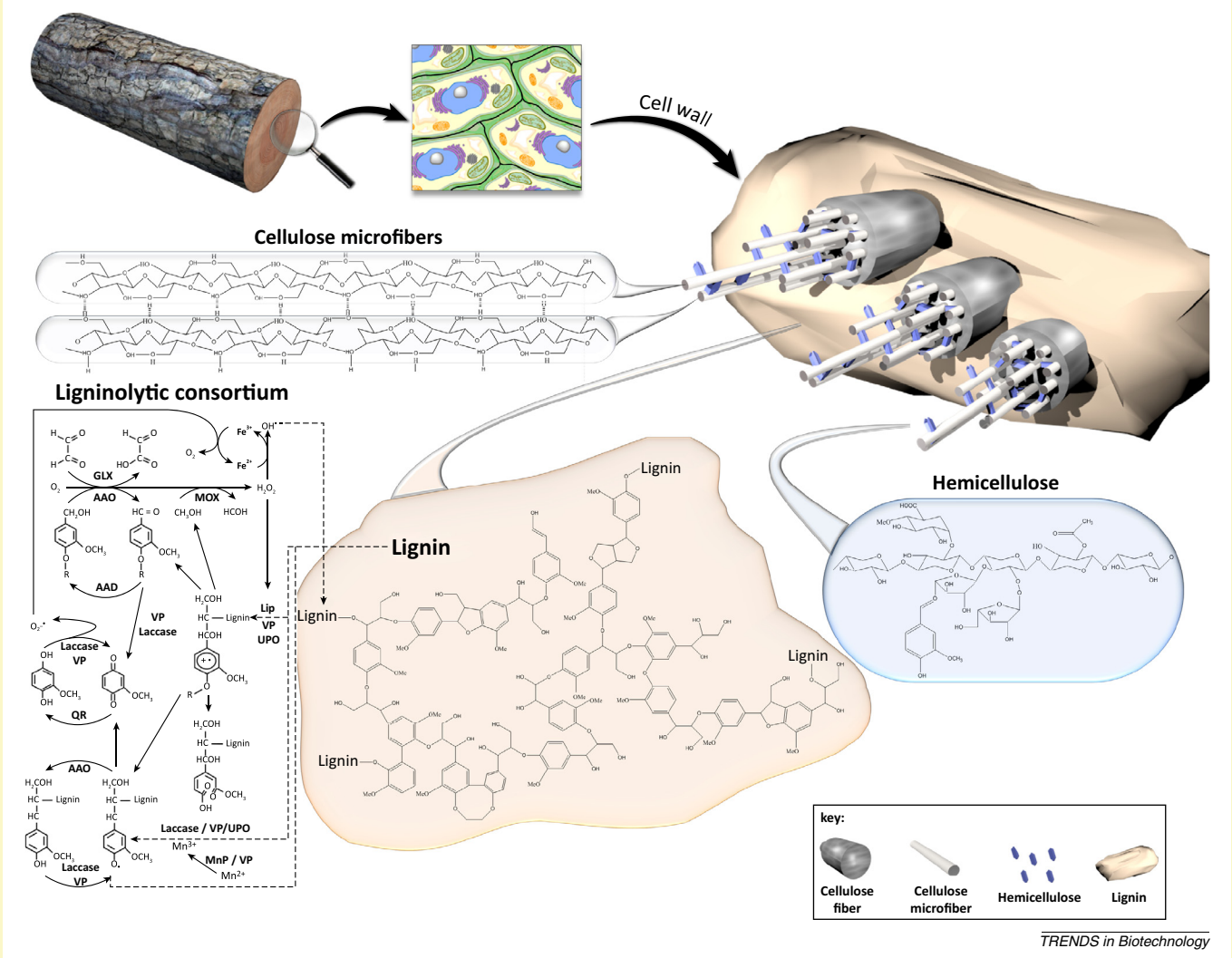

Figure I. Overview of the place of lignin in cellulosic biomass and the reactions catalyzed by ligno enzymes.