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OPINION ARTICLE for *Trends in Biotechnology*
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Engineering of 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 emergent lignocellulose biorefineries in processes that range from the production of bioenergy to that of biomaterials. To ensure that these versatile enzymes meet industrial 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.

KEY WORDS: Lignin, directed evolution, ligninases, white-rot yeast, ancestral resurrection, *Saccharomyces cerevisiae*.

HIGHLIGHTS:

- Ligninases are situated at the front line of green chemistry.
- Directed ligninase evolution is crucial for emergent lignocellulose biorefineries.
- Synthetic secretomes and evolved ancestral ligninases are the next engineering goals.

OPINION ARTICLE

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 of producing 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 Tns/year, it is not surprising that the US Department of Energy has invested funds to sequence over 80 fungal genomes due to their potential application in lignocellulose biorefineries. In fact, ligninolytic enzymes -also known as ligninases- could offer a broad repertory of solutions in different industrial settings, such as in the sustainable production of renewable chemicals, materials and fuels (including 2^{nd} generation biofuels like 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), food industry (beverage and bakery processing), pulp biobleaching and textiles industries, to name just a few [4, 5]. However, to meet market demands, these versatile biocatalysts need to be produced and manipulated so that their properties can be adapted to non-natural industrial environments, or to improve their catalytic capacities [6].

The current article summarizes the most significant advances in engineering the ligninolytic consortium by directed evolution and hybrid approaches (see **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.

Case studies of ligninolytic oxidoreductase engineering

i) Functional expression, activity and stability

Laccases (see the description of the main types of ligninases in **Box 1**): Early pioneering work on this group of enzymes was carried out with the medium-redox potential laccase from the thermophile ascomycete *Myceliophthora thermophila* (MtL), subjecting it to 10 generations of directed evolution to enhance its secretion to 18 mg/L in *Saccharomyces cerevisiae* (see **Box 3**), whilst notably improving its catalytic efficiency along the way [15]. Among the beneficial mutations introduced, a cleavage site was generated in the C-terminal tail of the protein that favored its processing in the Golgi compartment, promoting successful packaging of the mutant into vesicles and

hence, its exocytosis. This evolved variant was later tailored by combinatorial saturation mutagenesis (CSM) that helped reveal the possible role of this C-terminal tail in the catalytic efficiency of ascomycete laccases [19]. Although MtL is not a true high-redox potential ligninase, the results of these efforts inspired important advances in the field. Accordingly, two high-redox potential laccases (HRPL) from white-rot fungi, PM1 (CECT 2971) and *Pycnoporus cinnabarinus* (PcL), were evolved to improve their secretion, activity and thermostability in *S. cerevisiae* [20-22]. In both cases the native signal peptides were replaced by the α-factor prepro-leader from *S. cerevisiae*, and the corresponding fusion genes were subjected to multiple rounds of random mutagenesis and *in vivo* DNA recombination, as well as several semi-rational strategies. Through this approach, the two genetic elements (the α-factor prepro-leader and the mature laccase) were evolved jointly, fostering rapid adaptation to the subtleties of the yeast secretory pathway whilst improving the kinetics on phenolic and non-phenolic compounds (including some redox mediators from natural and synthetic sources [23]).

Although both PM1L and PcL share around 77 % of amino acid sequence identity, their distinct evolutionary trees reflect the broad fitness capacity of these types of ligninases. Reconstructing such synthetic fossil records opened the way to create new improved variants by mutational exchange, introducing beneficial mutations from one laccase scaffold into another, or through laccase chimeragenesis, thereby enhancing sequence diversity. Indeed, up to 6 crossover events were seen to occur in each chimera, generating several hybrid laccases with combined properties in terms of activity, pH profile and thermostability [24]. Laccase chimeragenesis has also been reported for other HRPL, highlighting the potential of homologous recombination within different ligninolytic templates [25]. Interestingly,

directed laccase evolution was coupled to computational molecular dynamics in attempting to improve the activity of an HRPL from *Pleurotus ostreatus* [26-28]. This approach provided useful information and insights into the physical and electrochemical properties following evolution, and it was recently applied to assess the robustness of the evolved PM1L protein *in silico* [29].

H_2O_2 enzymes: The directed evolution of heme-peroxidases/peroxygenases is also generating much interest. In a recent study, the evolution of a versatile peroxidase (VP) from *Pleurotus eryngii* was directed towards secretion and activity in *S. cerevisiae*, while further improving its kinetic thermostability [30, 31]. The mutational loads were finely adjusted so that only one mutation was included per generation, avoiding the accumulation of neutral and deleterious mutations. The α factor prepro-leader processing at the Golgi compartment changed due to the overexpression (21 mg/L), retaining an additional N-terminal tail that enhanced secretion (the same effect was detected during PM1L evolution, the secretion of which increased to 8 mg/L [21]). In addition, the enzyme´s stability at alkaline pH was significantly increased while its affinity for H_2O_2 decreased, allowing VP variants to display high specific activities under saturating conditions. Harnessing this system, the VP was recently evolved for oxidative stabilization, a mechanism-based inactivation process caused by H_2O_2 that is common to all heme peroxidases. The ultimate variant of this study accumulated 8 new mutations that increased the enzyme's half-life up to 35 min in the presence of 3,000 equivalents of H_2O_2, whilst revealing key structural determinants implicated in the suicide inactivation process [32]. Rational studies aside [33], other attempts to enhance the stability of ligninolytic peroxidases in the presence of H_2O_2, include the directed evolution of MnP from *Phanerochaete chrysosporium* using an *in vitro* expression system [34], and the evolution of

LiP from *P. chrysosporium* to enhance turnover rates and stability by yeast surface display [35, 36].

Unspecific peroxygenases (UPO) are considered by many as the missing link between heme-peroxidases and P450s, yet unlike the latter they require neither “expensive” co-factors nor auxiliary flavoproteins as they are soluble, extracellular and very stable [37]. In a recent work, the UPO from *Agrocybe aegerita* was evolved to improve its expression and activity in *S. cerevisiae* [38]. The four mutations in the native signal peptide and the five mutations in the mature protein gave rise to a 27-fold improvement in secretion (up to 8 mg/L) and 18-fold better catalytic efficiency, respectively. Taking advantage of these mutations, a tandem yeast-expression system was developed that enabled the UPO variant to enhance production over 200 mg/L in *Pichia pastoris*, a suitable point of departure for future large-scale applications.

Research is currently focusing on the H_2O_2 supplying enzymes, paying special attention to the members of the glucose-methanol-choline (GMC) superfamily. Recently, the aryl alcohol oxidase (AAO) from *P. eryngii* was functional expressed in *S. cerevisiae* by engineering a set of chimeric signal peptides composed of segments of the α-factor prepro-leader and the toxin K_1 Killer [39]. The preαproK-AAO fusion gene (containing a chimeric leader formed by the α-factor pre-leader fused to a truncated version of the γ segment of the Killer) was subjected to focused domain mutagenesis in three different protein segments, notably enhancing the activity of the enzyme. Another member of the GMC superfamily, the cellobiose dehydrogenase (CDH) from *Myriococcum thermophilum*, was expressed and subjected to CSM in *S. cerevisiae* in order to improve H_2O_2 production with a view to its potential applications in cotton bleaching, laundry detergents and antimicrobial functionalization of medical devices [40]. The best CDH variant was produced

in *P. pastoris* showing improved oxygen and substrate turnover, reflecting a modification in the oxidative and reductive FAD half-reaction.

ii) Adaptation to non-natural environments

In nature, the ligninolytic secretome works in a wet and acid environment (pH ~3) because of the release of organic acids by the white-rot fungi. Thus, the conditions found during biological delignification are far from those required in a given biotechnological application. However, the good news is that we can force these enzymes to adapt to non-natural environments through directed evolution. One of the most exciting applications for HRPL is the engineering of 3D-nanobiodevices for biomedical use, given that these ligninases are capable of accepting electrons directly from a cathodic compartment [41]. Indeed, PM1L was recently tailored to be active in human blood [42]. The inherent inhibition of HRPL by the combined action of the high NaCl concentrations and the alkaline pH of blood was surpassed by screening mutant libraries in a buffer that simulated the biochemical composition of blood, albeit in the absence of coagulating agents and red blood cells. The ultimate mutant enzyme was tested on real human blood samples (after over-production in *P. pastoris* [43]), revealing the mechanisms underlying this unprecedented improvement. The re-specialization required to adapt PM1L to such inclement conditions affected only 0.4 % of the amino acid sequence. This was the first successful example of laboratory evolution to optimize an enzyme for enhanced catalysis in blood, a milestone that will be applicable to an array of exciting biomedical applications. In fact, the mutant has just been incorporated into the first wireless and self-powered biodevice for carbohydrate and oxygen monitoring [44]. From a more general point of view, this variant is of broad interest to a wide range of sectors, including new energy sources: This laccase mutant was recently shown to be capable of oxidizing H_2O to O_2

during water splitting under alkaline conditions, an issue that meets the requirements for sustainable large-scale production of H_2 and O_2 [45].

The use of non-conventional media (organic solvents, ionic liquids) is required for many biotransformations in which the substrates are relatively insoluble in water, such as in the remediation of xenobiotics, and in the organic synthesis of pharmaceuticals, lignin intermediates and biopolymers. The MtL mutant evolved for secretion and activity [15] was adapted to the presence of high concentrations of organic co-solvents [46]. To furnish the final mutant with co-solvent promiscuity, directed evolution was performed in the presence of increasing concentrations of co-solvents of different chemical nature and polarity. The final variant was fairly active and stable at concentrations as high as 50 % (v/v) of the co-solvents. Of the 4 mutations accumulated in this variant after five generations of evolution, 2 were located at the C-terminus of the laccase that is cleavage upon maturation, suggesting a possible role during protein folding. Mutations in the mature protein were mainly situated at the surface of the protein, establishing new interactions with the surrounding residues through H-bonds or salt bridges that eventually lead to structural reinforcement against denaturation in the presence of co-solvents. The adaptation of laccases to ionic liquids was recently demonstrated for the HRPL from *Trametes versicolor* expressed in *S. cerevisiae* [47]. This enzyme was subjected to directed evolution in the presence of 1-ethyl-3-methylimidazolium ethyl sulfate ([EMIM] [$EtSO_4$]). The IC_{50} (the concentration of inhibitor at which the enzyme´s activity is reduced by half) of the mutant was enhanced by ~100 mM after only two rounds of random mutagenesis and the mutations were analyzed by molecular docking to define the change in kinetics in the presence of 15 % (v/v) [EMIM] [$EtSO_4$].

Alkalophilic fungal laccases are important to the textile and paper industries. In the course of the 15 generations when MtL was evolved to enhance secretion and organic co-solvent tolerance, a shift in the pH profile for activity towards more basic pH values was detected that was valuable to specifically tailor an alkalophilic laccase. After 5 additional rounds of evolution, the new MtL variant displayed improved kinetic parameters and significant activity at alkaline pH values, both against phenolic and non-phenolic substrates [48]. Mutations were mapped in the surroundings of the T2/T3 trinuclear Cu cluster, tempering the interaction with OH^- to avoid enzyme inhibition.

Prospects for future research

i) Travelling back and forth along the temporal scale of evolution

There is increasing interest in ancestral protein reconstruction and resurrection, travelling back in time by phylogenetic analysis and ancestral inference to recreate ancient enzymes with greater stability and broader substrate promiscuity than their extant counterparts [49-53]. To date, the only studies that have combined directed evolution and ancestral studies have focused on the reconstruction of evolutionary adaptive pathways for the creation of small but functional libraries [54] and the engineering of ancestral libraries by combinatorial spiking based on ancestor-consensus mutations [55]. Very recently, it has been demonstrated that the introduction of consensus mutations in extant enzymes yields lower stabilities and promiscuous activities than those obtained from laboratory resurrected enzymes, becoming the latter more suitable departure points for further engineering [56]. Through fungal genome reconstruction, recent studies linked the formation of coal deposits during the Permo-Carboniferous period (~260

million years ago) with the birth and evolution of white-rot fungi, and their lignin-degrading enzymes [57]. Indeed, the interface between natural and artificial evolution can be explored by travelling back and forth along the evolutionary timeline with extant and ancestral enzymes. This approach is based on enzyme re-specialization and it promises to provide a deeper understanding of the principles underlying the evolution of new functions for the ligninolytic consortium -and many other enzymatic systems- while rescuing latent promiscuous activities (**Box 4**).

ii) Engineering of a white-rot yeast

Another emergent field of research deals with the development of full autonomously consolidated bioprocessing microbes (CBM, [58]). In fact, obtaining customized microorganisms capable of cost-effectively producing chemicals and biofuels from plant biomass is one of the most important goals for emergent lignocellulose biorefineries [1, 2, 59]. With several efficient minicellulosomes displayed by *S. cerevisiae*, the development of a synthetic ligninolytic secretome is the next milestone. The first successful example of co-expressing two ligninolytic genes in yeast was reported this year: a HRPL and a VP evolved in the laboratory were readily secreted by *S. cerevisiae* at high titers without interfering with the cell's metabolism [60]. However, the engineering of a more complex self-sufficient secretome, including other ligninases and H_2O_2 supplying enzymes, will answer the doubts as to whether or not *S. cerevisiae* can tolerate the secretion of a set of ligninolytic enzymes without suffering excessive metabolic burden and expression constraints.

Concluding Remarks

The courtship between synthetic biology and metabolic engineering has been quite productive in the last decade. By combining these disciplines, we

are ever closer to reproducing in the laboratory one of nature's best hidden secrets: how lignin is degraded. Indeed, the past five years have seen an increasing growth of robust and reliable directed evolution platforms for ligninolytic genes. For the first time, this approach has allowed us to design different features of biotechnological interest, improving activity, thermostability and catalysis in non-natural environments. Current advances in genomics, metagenomics and transcriptomics are supplying us with inexhaustible resources to discover new ligninolytic genes, whilst trying to reduce the production costs of establishing these enzymes as industrial biocatalysts. More ambitious steps will include the directed evolution of CBM with a self-sufficient ligninolytic secretome and a displayed minicellulosome that can act directly on raw materials for the production of biofuels and chemicals. Meanwhile, new ligninases of ancestral and extant origin will be engineered to develop sustainable and competitive biotechnologies in a drive towards green chemistry.

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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 makes the plant cell wall more consistent, while forming a complex matrix to protect 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). One third of the carbon fixed in terrestrial ecosystems as lignocellulose comes from lignin.

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 quite 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: It 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 laccases (up to 0.8 V); the latter compensate 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: Basidiomycetes and ascomycetes fungi 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 remove of lignin in advance of cellulose degradation.

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 combustion, 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 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)), **Figure I**. The general characteristics of main ligninases are described below.

Laccases (EC 1.10.3.2): Enzymes belonging 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 by-product. 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 (below 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, with catalytic traits similar to LiP, MnP and generic low-redox potential peroxidases (GP).

Unspecific peroxygenases (UPO, EC 1.11.2.1 -also referred as to aromatic peroxygenases, APO): A new type of heme-thiolate enzyme with self-sufficient mono(per)oxygenase activity and a high redox potential. With over

300 positive substrates tested, UPO 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 (AOO, EC 1.1.3.7): Monomeric flavoproteins that supply H_2O_2 to peroxidases and peroxygenases during lignin degradation, whilst displaying a high enantioselectivity through a hydride abstraction process. AAO have recently been classified as auxiliary activity (AA) families, along with glyoxal oxidases, pyranose oxidases and methanol oxidases.

BOX 2: Directed evolution and hybrid approaches: Tools and strategies that contribute to the biotechnological rainbow

Over recent years, directed evolution has been comprehensively applied to improve the characteristics of ligninases and many other enzymes, and to create new functions, while employing rational approaches to understand the mechanisms underlying each newly evolved property. Typically, although not exclusively, ligninolytic mutational landscapes are examined by an adaptive/standard evolution strategy (*i.e.* searching for beneficial mutations that accumulate and recombine with each other in the course of in vitro evolution [7]). In many cases, a gradual increase in selective pressure is employed to tailor mutant offspring to the desired attributes. However, an emergent strategy based on neutral genetic drift has also been applied to enhance a protein's capacity to evolve: the gradual accumulation of neutral mutations opens unexplored adaptive avenues by increasing enzyme stability and substrate promiscuity [8, 9]. Among the semi-rational approaches, the use of focused directed evolution methods (*e.g.* combinatorial saturation mutagenesis -CSM- and domain mutagenesis) helps to direct the exploration

of the vast protein space, which only becomes applicable when structural information is available. Moreover, computational tools for random or focused mutagenesis and recombination are fundamental to create smart mutant libraries, streamlining resources and research time [10, 11]. Along these lines, computational simulations (Quantum mechanics/molecular mechanics QM/MM, molecular dynamics and Monte-Carlo algorithms) may be useful to study the interactions between different ligands and the enzyme so that specific residues and regions can be targeted for mutagenesis and recombination, whilst discerning the principles behind each improved trait [12]. Finally, the directed evolution of genes isolated from extremophile environments or generated by ancestral resurrection may facilitate the use of higher mutational loads whose tolerance is directly related to protein robustness [13, 14]. Taken together, directed evolution harnesses other well-established areas of biotechnology (including genomics, metagenomics, rational and computational approaches, and paleoenzymology), at the same time as contributing to the development of tools for the generation of DNA diversity (including a variety of methods for DNA recombination, random and/or focused mutagenesis), fostering progress in growing disciplines from synthetic biology and metabolic engineering to systems biology; all of which fall within the biotechnological rainbow formed by the white (industrial) red (biopharmaceuticals) green (agricultural) and blue (marine) biotechnologies, **Figure II**.

BOX 3: Why perform directed evolution of ligninases in *S. cerevisiae*?

Ligninolytic oxidoreductases are hardly functionally expressed in heterologous hosts suitable for directed evolution experiments (typically bacteria or yeast). During maturation, ligninolytic polypeptides undergo several post-translational modifications (mostly glycosylation, N- and C-

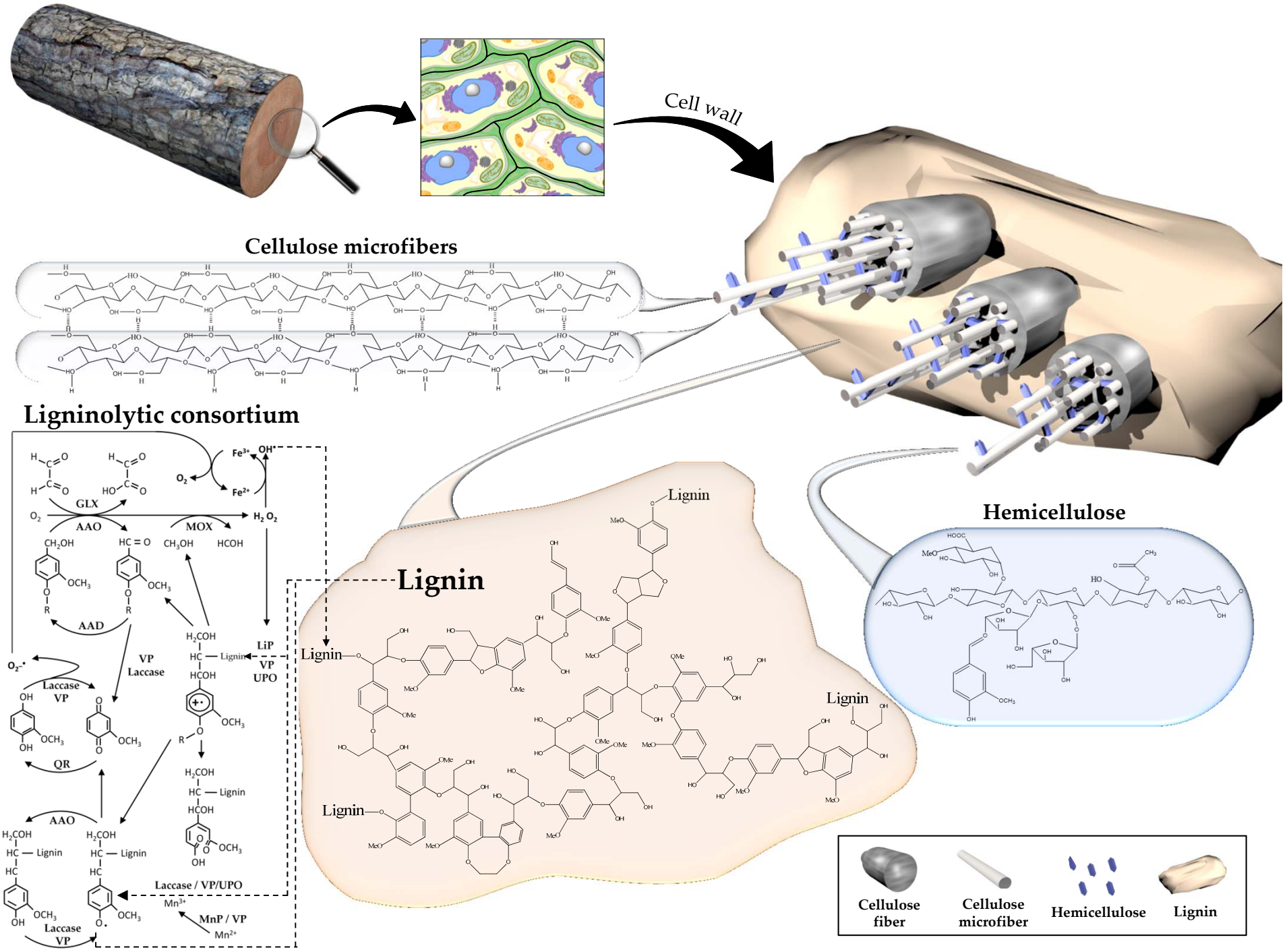
terminal processing), which are readily performed by the native wood-rooting fungi but that are quite difficult to accomplish in a foreign microorganism. Indeed, attempts to express these genes in the bacteria *Escherichia coli* led to misfolding and the formation of inclusion bodies. It is highly likely that differences in codon usage and missing chaperones, along with the absence of a more sophisticated machinery to perform complex post-translational processing, hampers the proper folding of these enzymes. By contrast, the physiology of *S. cerevisiae* is much closer to that of the native fungi, although the functional expression of ligninolytic genes in this yeast is still not an easy task. In most cases, inherent hurdles during trafficking, processing and exportation through the endoplasmic reticulum, Golgi and cell membrane mean that additional protein engineering work is necessary to fine-tune secretion and enhance or maintain activity. Other yeasts like *Pichia pastoris* or *Yarrowia lipolytica* have also been considered for the directed evolution of ligninases, although the absence of robust episomal vectors and low transformation efficiencies hinder their use. Thus, *S. cerevisiae* has been consolidated as the best organism to evolve ligninolytic genes, as well as to develop a full set of protocols to enhance genetic diversity due to its high frequency of homologous *in vivo* DNA recombination, a useful characteristic to construct complex metabolic pathways using *in vivo* splicing expression cassettes [16-18]. Indeed, the wealth of possibilities offered by *S. cerevisiae* to create DNA diversity, and from a more general point of view, the outstanding role played by this yeast in synthetic biology studies cannot be underestimated. Finally, the increasing use of tandem-expression systems (*e.g. S. cerevisiae* for directed evolution and *P. pastoris* or *A. oryzae* for overproduction) ensures large scale production and adaptation to industrial applications. **Figure III** depicts a typical directed evolution round using *S.*

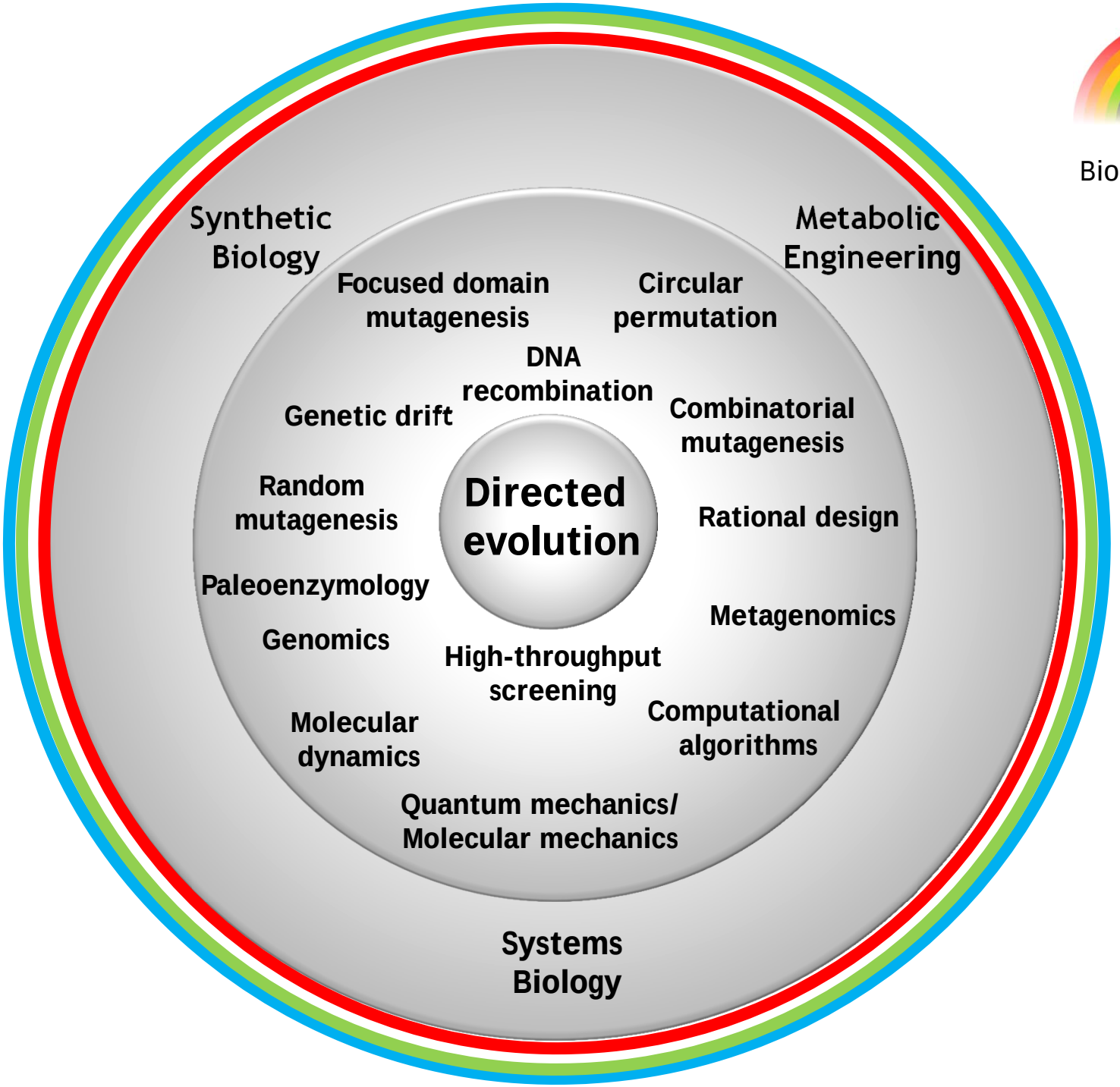
cerevisiae as DNA-tool box for in vivo recombination, cloning and expression. The engineering of overlapping overhangs to favor in vivo splicing and recombination between product fragments is a rule of thumb to create DNA diversity protocols [16].

BOX 4: Directed evolution of extant and ancestral enzymes

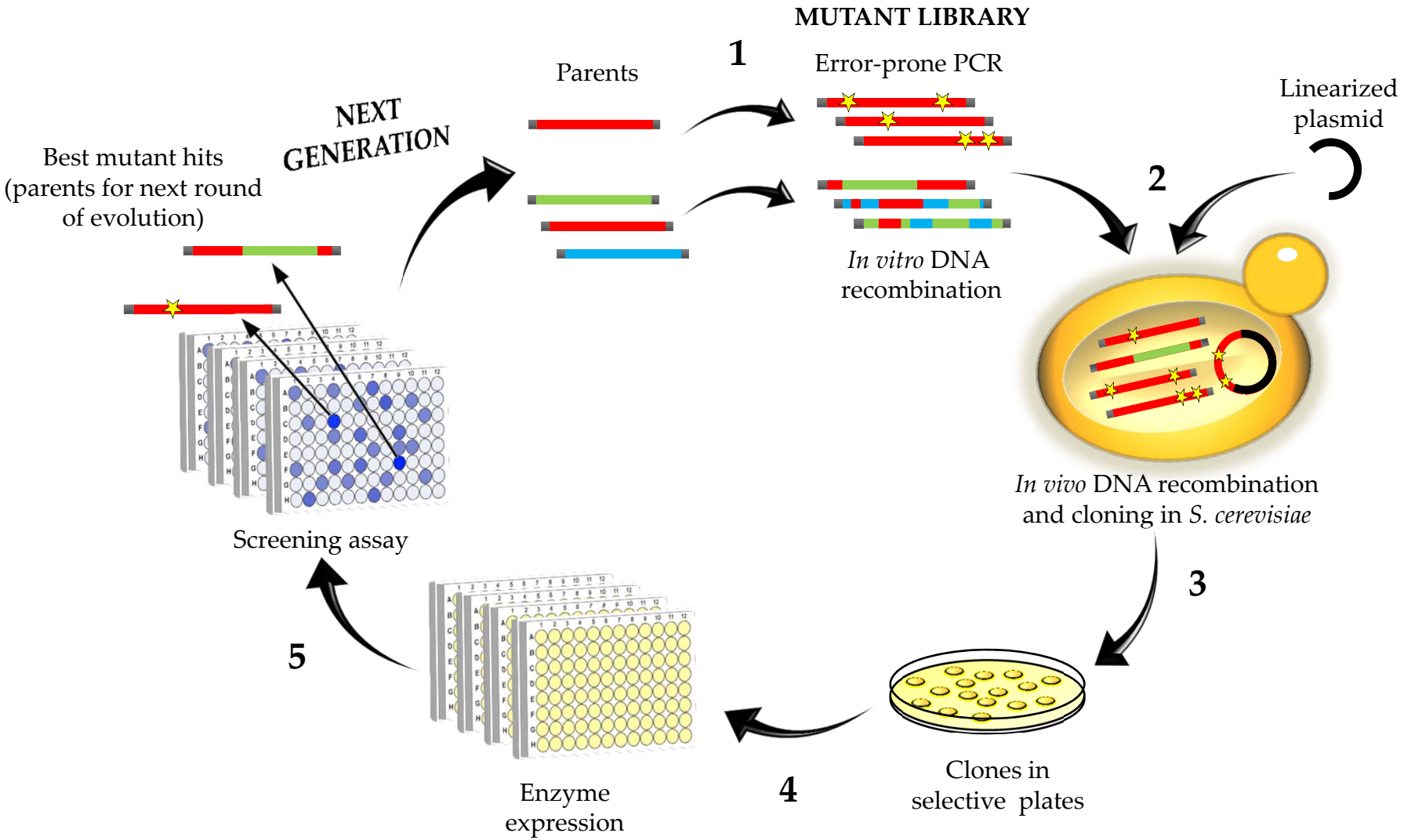
In general terms, it is believed that primitive cells relied on only a small set of enzymes, such that ancient enzymes displayed wide substrate specificities and high stability. Ancestral reconstruction and resurrection allows enzyme evolution to be recreated in the laboratory, providing information that is not easy to obtain by studying extant enzymes. Accordingly, enzymes resurrected usually show higher catalytic promiscuity and stronger stability, making them a suitable and more evolvable departure point for the engineering of new traits not preserved in extant enzymes. **Figure IV** represents a future trend in enzyme engineering driven by directed evolution and ancestral resurrection. Latent "promiscuous" activities (represented as a panel of colors) appeared in ancestral "generalist" enzymes (**1**), which over the course of natural evolution (solid black arrow) became more specialized and adopted specific catalytic functions (*e.g.* from yellow in (**1**) to dark red, in (**2**)). An approach to rescue some promiscuous activities that are no longer present in extant enzymes is shown in the diagram. Based on the in vitro evolution of ancestral and modern enzymes, two different evolutionary pathways could be followed: Strategy (**A**) (red dotted arrow) represents the directed evolution of an ancestral enzyme. In this case, the enzyme is first reconstructed and resurrected (*i.e.* functionally expressed) giving rise to a protein similar to (**1**). Afterwards, the enzyme can be subjected to adaptive evolution (*i.e.* the selection of the fittest) travelling forwards in time to improve the desired promiscuous activity (*e.g.* from light green in (**1**) to dark

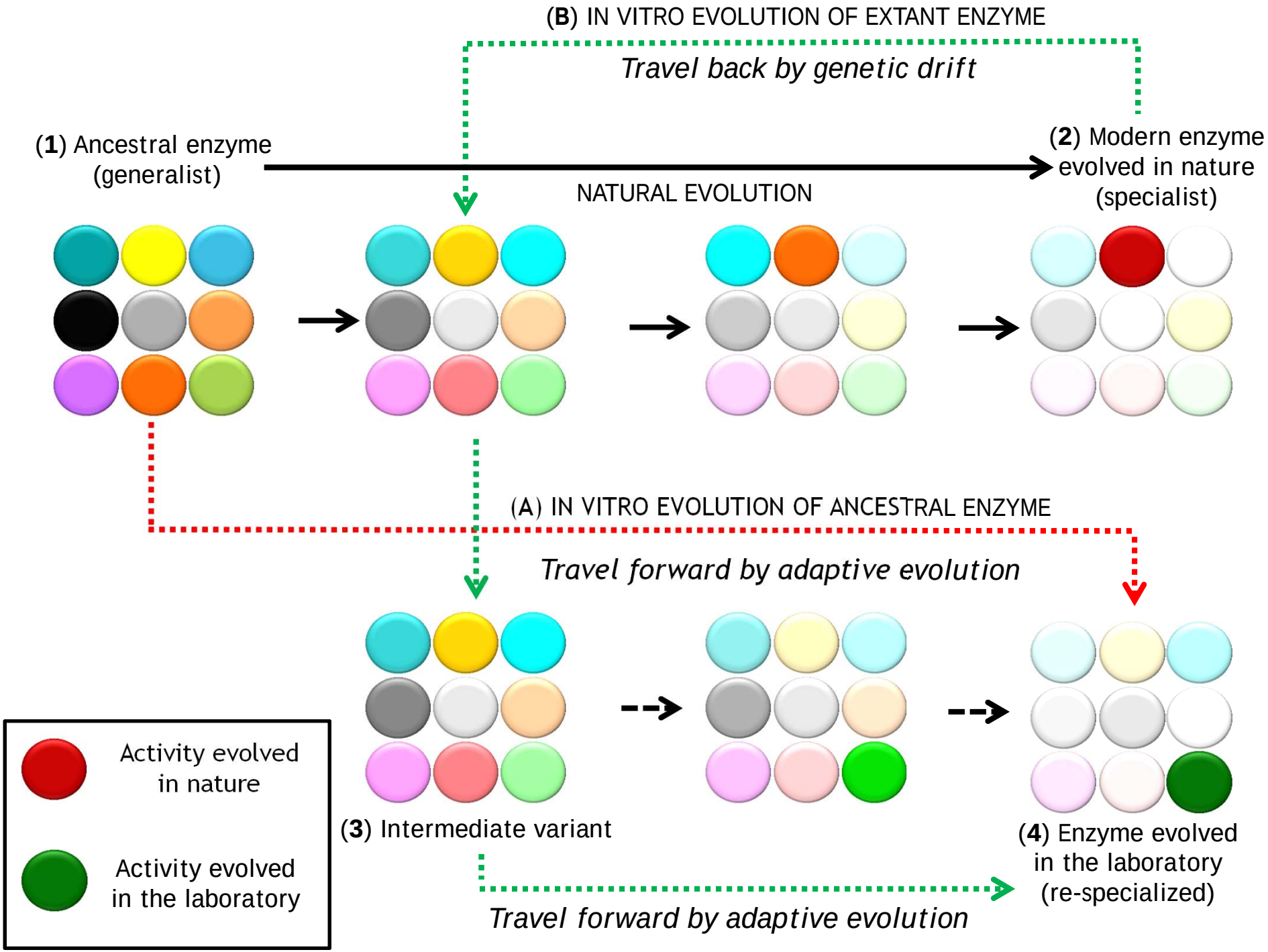
green in (**4**)). Strategy (**B**) (green dotted arrow) shows the directed evolution of an extant enzyme to create novel functions. In this route, an extant "specialized" enzyme (**2**) is subjected to neutral genetic drift in order to reach an intermediate variant (**3**) whose latent activities are to be partially recovered (by creating a polymorphic population whereby intersection regions in the protein sequence space are unmasked). Thereafter the emerged latent activity/ies can be improved by adaptive evolution, *e.g.* from light green in (**3**) to dark green in (**4**).


Cell wall
Cellulose microfibers
Ligninolytic consortium
Lignin
Hemicellulose
Cellulose fiber
Cellulose microfiber
Hemicellulose
Lignin



Biotechnological Rainbow


MUTANT LIBRARY
1
Parents
Error-prone PCR
Linearized plasmid
2
NEXT GENERATION
Best mutant hits (parents for next round of evolution)
In vitro DNA recombination
In vivo DNA recombination and cloning in S. cerevisiae
3
Screening assay
5
Enzyme expression
4
Clones in selective plates


(B) IN VITRO EVOLUTION OF EXTANT ENZYME
Travel back by genetic drift
(1) Ancestral enzyme (generalist)
NATURAL EVOLUTION
(2) Modern enzyme evolved in nature (specialist)
(A) IN VITRO EVOLUTION OF ANCESTRAL ENZYME
Travel forward by adaptive evolution
(3) Intermediate variant
(4) Enzyme evolved in the laboratory (re-specialized)
Travel forward by adaptive evolution
Activity evolved in nature
Activity evolved in the laboratory