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Biovalorization of Lignin

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Lignocellulosic biomass comprises three components – cellulose, hemicellulose and lignin. Biorefining is defined as the separation, isolation and conversion of cellulose, hemicellulose and lignin from lignocellulosic biomass into fuels, chemicals, materials and energy. While a number of processes have been developed over the years to produce valuable products from cellulose and hemicellulose, processes utilizing lignin have been few and far between, largely owing to the severe recalcitrance of lignin. Our inability to utilize lignin is a lost opportunity for the green economy. Lignin is abundant and can provide a myriad of chemicals that could be used as building blocks for life-saving pharmaceuticals or even as flavours and food ingredients. The development of greener conversion platforms that can efficiently convert lignin into valuable products is highly desired. This paper summarizes recent progress made towards the development of a family of biocatalytic processes that convert lignin to pharmaceutical building blocks, flavouring agents and drug delivery platforms. Biocatalytic processes are greener, emit lesser carbon dioxide and are more energy efficient than other alternatives. The first family of biocatalysts selectively degrades lignin to its monoaromatic constituents and the second family of biocatalysts then modifies the monoaromatic constituents to desired target molecules. In particular, this paper describes the novel use of functional metagenomics and whole-cell biosensors for the discovery of 147 new biocatalysts that convert lignin into vanillin and syringaldehyde, and the identification of a novel transaminase that catalyzes the asymmetric synthesis of chiral amines from vanillin, syringaldehyde and 12 other monoaromatic aldehyde and ketone degradation products of lignin. Recent progress on the assembly of a metabolic pathway for the biosynthesis of the spice molecule capsaicin from vanillylamine, and the integration of the entire pathway that valorizes lignin to capsaicin into the metabolic network of *E. coli* has also been discussed. This work will eventually lead to the development of consolidated bioprocesses that convert lignin into value-added chemicals.

1. Introduction

Biorefining is loosely defined as the separation, isolation and conversion of cellulose, hemicellulose and lignin from lignocellulosic biomass into fuels, chemicals, materials and energy (Luo et al., 2010). A number of biocatalytic (Heux et al., 2015) and thermocatalytic (Wettstein et al., 2012) processes have been developed for biorefining. However, regardless of the mode of conversion employed, biomass comprising a higher content of cellulose and hemicellulose is generally preferred as a feedstock compared to biomass with a higher proportion of lignin (Parajuli et al., 2015). The degree to which a resource can be valorized by a process is defined as the valorization or V-factor (Patankar et al., 2019). Although a variety of operating and market parameters influence the V-factor of a process, it is directly proportional to the ratio of the value of all products that it can manufacture from that resource to the value of the resource itself. This implies that the greater the number of useful products that can be manufactured from a resource, the greater is the V-factor; and lignin's poor valorizability can be attributed to its recalcitrance to most biocatalytic or thermocatalytic transformations (Ragauskas et al., 2014; Yamaguchi et al., 2017). The inability to efficiently valorize lignin represents a lost opportunity for biorefining. Lignin has a higher carbon and lower oxygen content compared to both, cellulose and hemicellulose (Rinaldi et al., 2016), which makes it an attractive feedstock for the development of green chemical processes. It is especially appealing as a feedstock for the manufacture of aromatic specialty and fine chemicals that are, among other applications, widely used in pharmaceutical

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synthesis. Aromatic chemicals are presently derived from petroleum but they could be sourced from lignin at substantially higher atom and energy economies. Moreover, the V-factors for the biovalorization of lignin to monoaromatic compounds, which can be biovalorized subsequently to pharmaceutical intermediates or products are orders of magnitude higher than the V-factors of processes that manufacture lignocellulosic biofuels (Figure 1). The development of conversion platforms that can efficiently valorize lignin is highly desired, particularly biocatalytic processes that are greener, emit lesser CO_2 and are more energy efficient than thermocatalytic alternatives (Clark et al., 2012; Keller and Zengler, 2004; Lorenz and Eck, 2005; Truppo, 2017; Whitman et al., 1998; Yadav and Stephanopoulos, 2014).

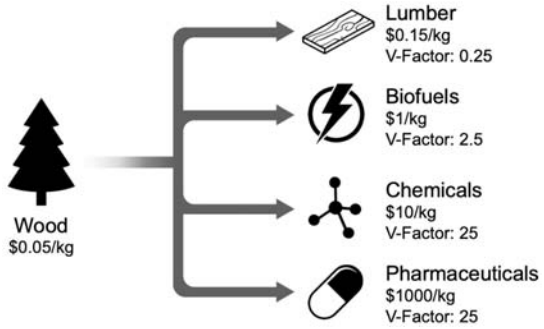

Figure 1: The V-factors of wood products

2. Recent progress

Functional metagenomics is poised to be a major driver of the discovery of superior biocatalysts for the valorization of lignin. Functional metagenomics provides reliable and easy access to the entire catalogue of microbial biocatalytic transformations (Armstrong et al., 2015; Taupp et al., 2011). Briefly, the typical workflow for functional metagenomics presently proceeds by extracting, purifying, fragmenting and ligating the DNA into specialized vectors such as fosmids (Taupp et al., 2009). The DNA fragments typically range between 30- to 50-kilo base pairs in length. The fosmids are linearized, packaged into phage particles, and subsequently transduced into a suitable host such as phage-resistant *E. coli* to produce a library of fosmid-bearing clones. The clones are later recovered and cultured on selected substrates to evaluate their catalytic potential using a variety of detection schemes (Armstrong et al., 2015; Mewis et al., 2013; Taupp et al., 2011). Clones exhibiting the desired phenotype are investigated further to unambiguously determine the identity of the biocatalyst or biocatalytic cluster. Among the numerous detection schemes that are currently employed, of the use of whole-cell biosensors comprising regulatory elements of one-component transcriptional regulators has emerged as a favoured tool, especially for high-throughput metabolite detection (Armstrong et al., 2015). Whole-cell biosensors can detect lignin transformation at microliter volumes, which facilitates high-throughput screening of the fosmid clones at detection sensitivities that are otherwise unattainable by macro-scale analytical methodologies such as gel permeation chromatography. The biosensor is typically expressed on a plasmid and comprises a fluorescent or luminescent reporter. The gene encoding the reporter is transcribed under the control of the promoter of a transcriptional regulator that is either endogenous to the biosensor host or is heterologously transferred to the host from a different bacterium. In the absence of the product in the intracellular milieu of the screening host, the regulator binds to its cognate promoter and represses transcription of the reporter gene. Binding of the product to the regulator induces a conformational change in the protein that de-represses transcription of the reporter gene. As a consequence, the biosensor functions by transducing intracellular product concentrations to a fluorescent or luminescent signal (Fernandez-López et al., 2015; Marijuán et al., 2010; Ulrich et al., 2005).

We had previously employed functional metagenomics to identify 147 novel, whole-cell biocatalysts that selectively degrade lignin into vanillin and syringaldehyde when they are expressed in *E. coli* (Ho et al., 2018) (Figures 2A & 2B). We employed a whole-cell biosensor comprising the transcriptional fusion of the green fluorescent protein (GFP) with the promoter of the *emrRAB* operon to screen 42,520 fosmid clones comprising the environmental DNA of archaea and bacteria from coal beds. The *emrRAB* operon encodes a multi-drug efflux pump EmrAB under the transcriptional control of the regulator EmrR. We also tuned the configuration of the biosensor with the aid of mathematical model to improve its sensitivity, selectivity and detection range. We then further improved the V-factor of lignin biorefining by re-employing functional metagenomics to discover a novel transaminase (TA) that catalyzes the asymmetric synthesis of chiral amines from vanillin, syringaldehyde and 12 other monoaromatic aldehyde and ketone degradation products of lignin (Pawar et al., 2018) (Figures 2C & 2D). It is estimated that roughly 40% of all new chemical entities (NCEs) approved by the

US Food & Drug Administration (FDA) contain chiral amines building blocks(Aleku et al., 2017) such as vanillylamine that are produced by Tas (Frodsham et al., 2013; Green et al., 2014; Guo and Berglund, 2017; Lichman et al., 2015; Richter et al., 2015, 2014; Saville et al., 2010; Sehl et al., 2013) and related enzymes (Abrahamson et al., 2012; Ghislieri et al., 2013; Heberling et al., 2013; Leipold et al., 2013). As before, we screened an in-house library of *E. coli* fosmid clones that exhibit activity towards monoaromatic degradation products of lignin, and each fosmid once again ranged between 30- to 50-kilo base pairs in length. In this deployment of functional metagenomics, however, we utilized a colorimetric assay utilizing *o*-xylylenediamine as the amino donor, and the TA that was discovered utilizes the canonical cofactor, pyridoxal 5'-phosphate (PLP). Saliently, we achieved a discovery rate of 0.03% (on a clonal basis) or 1 per 122.9-mega base pairs. This rate is significantly higher than the median rate that is observed for functional metagenomic screening. We also identified the optimal conditions for enzyme activity, including temperature, pH and choice of co-solvent. We also evaluated the specificity of the enzyme towards a variety of amino donors, as well as optimal concentration of the most favoured amino donor. Significantly, the novel enzyme is markedly smaller than typical transaminases, and it is stably expressed in *E. coli* without any modifications to its amino acid sequence.

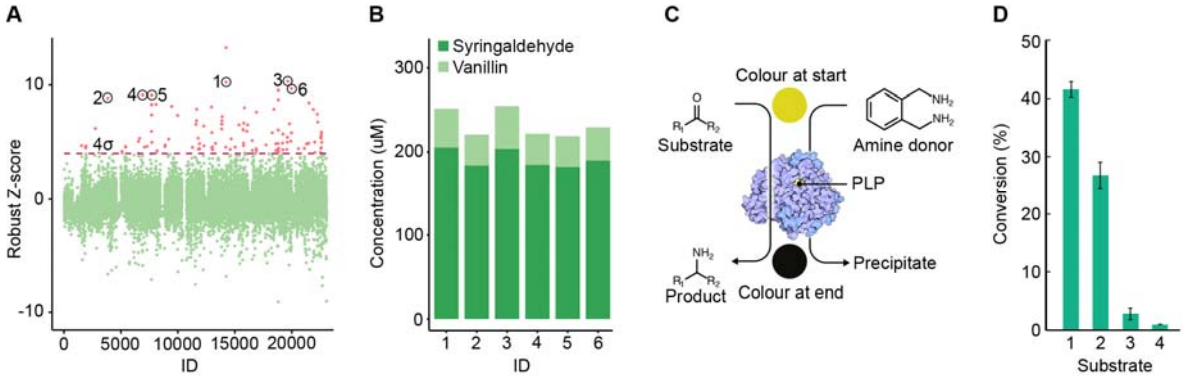

Figure 2: Asymmetric amination of vanillin and syringaldehyde derived from lignin. ***A:*** *Metagenomic screening of a coal-bed fosmid library using a whole-cell biosensor comprising GFP and the transcriptional promoter of the emrRAB operon yielded numerous clones (coloured in red) that can selectively degrade lignin to vanillin and syringaldehyde. Incidentally, clones having a robust Z-score in excess of 4σ were catalogued as positive hits.* ***B:*** *GC-MS confirmation of vanillin and syringaldehyde production by 6 clones identified through metagenomic screening. The clone IDs in the chart correspond with those highlighted in panel A.* ***C:*** *Scheme for the colorimetric screen for transaminase discovery. The reaction between the amine donor, o-xylylenediamine, and an intermediate of the reaction between PLP and the enzyme produces pyridoxamine phosphate (PMP) and 1H-isoindole. PMP then reacts with the monoaromatic compound to produce the desired product. H-isoindole is released into solution, where it tautomerizes to 2H-isoindole. The latter then rapidly polymerizes to yield a black precipitate.* ***D:*** *The transaminase derivatizes 14 monoaromatic compounds, including benzaldehyde (**1**), acetophenone (**2**), vanillin (**3**) and syringaldehyde (**4**). Reaction conditions: 5 mM of the individual substrates, 25 mM of o-xylylenediamine and 2 mM PLP in 100 mM potassium phosphate solution buffered at pH 7.5 and maintained at 30°C for 48 hours.*

3. Ongoing work

We are presently developing additional biocatalytic platforms for the synthesis of active pharmaceutical intermediates and flavouring agents. Specifically, we are working on three primary objectives. The first focuses on identifying new biocatalysts or biocatalytic clusters that can selectively degrade lignin to cinnamyl alcohol, cinnamaldehyde, coniferyl alcohol and coniferyl aldehyde. This work is similar in scope to our previous work on identification of biocatalysts that degrade lignin to vanillin and syringaldehyde. We are also enhancing the activity of the previously discovered transaminase towards vanillin and syringaldehyde, as well as tailoring cytochrome $P450_{BM3}$ to enantioselectively cyclopropanate cinnamyl alcohol, cinnamaldehyde, coniferyl alcohol and coniferyl aldehyde through enzyme engineering. We have previously developed and implemented a computational methodology to assess the activity of transaminases (Figure 3). Cytochrome $P450_{BM3}$ has been previously engineered to diastereo- and enantioselectively cyclopropanate styrenes using diazoester reagents (Coelho et al., 2013; Gober et al., 2015). Chiral cyclopropane derivatives of cinnamyl alcohol, cinnamaldehyde, coniferyl alcohol and coniferyl aldehyde can be employed as building blocks for the synthesis of histamine receptor ligands (HRLs) (Kobayashi et al., 2010). These ligands bind to and modulate

the activity of the histamine H_3 and H_4 receptors that, in turn, mediate homeostatic processes in neurons and neuronal tissue (Esbenshade et al., 2008). For example, antagonists of the histamine H_3 receptor have been shown to be promising candidates for treating Alzheimer's disease, schizophrenia, depression, dementia, epilepsy and attention-deficit/hyperactivity disorder (ADHD) (Leurs et al., 2005). Similarly, antagonists of the histamine H_4 receptor have been used to effectively treat allergies (Jablonowski et al., 2003). Presently, chiral cyclopropanated olefins are synthesized in the pharmaceutical industry via the Simmons-Smith reaction (Simmons and Smith, 1959). The reaction typically utilizes a zinc-copper couple (or diethylzinc) and diiodomethane. These reagents react to form iodomethylzinc iodide, an organozinc carbenoid, which then reacts stereoselectively with the alkene to produce cyclopropane. Not only are these reagents expensive and hazardous to work with, but the reaction also requires prolonged reflux and downstream separation in order to remove the metals. In contrast, cytochrome $P450_{BM3}$ and other enzymatic alternatives such as myoglobin utilize safer and cheaper reagents such as α-diazoacetate, function at milder temperatures and ambient pressures, and do not require downstream processes such as metal removal, which greatly simplifies the manufacturing process (Bajaj et al., 2016; Bordeaux et al., 2015; Coelho et al., 2013; Gober et al., 2015; Hernandez et al., 2016).

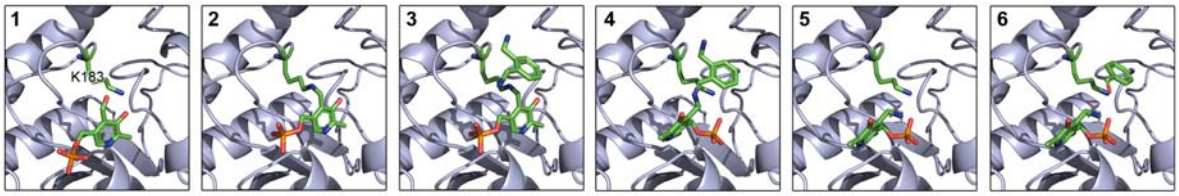

Figure 3: Computational pipeline for model-guided enzyme engineering of transaminases. We applied a variety of computational tools to simulate the progression of the reaction within the active site of the enzyme. ***Panel 1:*** *Binding of PLP within the active site and the close proximity of the K183 residue;* ***2:*** *reaction between the PLP and the K183 residue to form the internal aldimine;* ***3:*** *docking of o-xylylenediamine within the active site of the aldimine-functionalized TA;* ***4:*** *formation of the ketamine intermediate on account of the reaction between o-xylylenediamine and PLP;* ***5:*** *formation of PMP in the active site;* ***6:*** *docking of substrate (benzaldehyde, in this instance) within the active site of the TA alongside PMP.*

Previous work has shown that a variant of cytochrome $P450_{BM3}$ that bears the T268A mutation almost exclusively produces both diastereomers of the *trans* cyclopropanated derivative of styrene using ethyl diazoacetate (EDA) as the source of the carbene and sodium dithionite ($Na_2S_2O_4$) or nicotinamide adenine dinucleotide phosphate (NADPH) as the reducing agents, and alteration of the selectivity towards the two *cis* diastereomeric products necessitates as many as 12 additional mutations to this enzyme, namely V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, A290V, L353V, I366V and E442K (Coelho et al., 2013). Moreover, air was observed to inhibit cyclopropanation of styrene by as much as 50%. We are using these results as starting points for engineering $P450_{BM3}$ to derivatize cis-trans isomers of cinnamyl alcohol, cinnamaldehyde, coniferyl alcohol and coniferyl aldehyde.

We are also establishing a *E. coli*-based production platform for the synthesis of capsaicin from lignin-derived vanillin through pathway and metabolic engineering. Capsaicin is a secondary metabolite of pepper plants that is widely used as a spice and flavouring agent (Ochoa-Alejo, 2006). It has also been employed as an active ingredient in cosmetics, as an antimicrobial agent, as a therapeutic against chronic pain, overactive bladder and diabetes, and in weight loss pills (Fattori et al., 2016). Capsaicin is currently manufactured through direct extraction from chili peppers. However, yields are quite low since the natural abundance of the compound in the fruit is typically lower than 0.1 wt.% on a dry mass basis (Huang et al., 2000). Moreover, capsaicin is one of several capsaicinoids produced by pepper plant. Examples of other capsaicinoids include dihydrocapsaicin, nordihydrocapsaicin and homocapsaicin. The structural similarity between the capsaicinoids greatly complicates separation and purification, and the use of simulated moving bed chromatography is necessitated to purify these compounds. The low abundance and challenging purification of capsaicin combine to push the price of the molecule upwards of $3000 per kg. The development of a bacterial capsaicin biosynthetic platform could deliver significantly higher titres of a single product that can be easily purified using solvent extraction. Our previous discovery of a transaminase that asymmetrically aminates vanillin to vanillylamine is important to this objective (Figure 4).

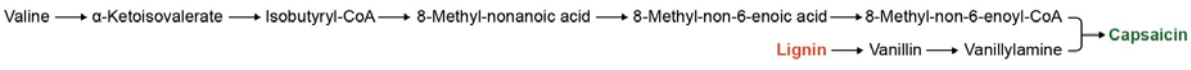

Figure 4: Biosynthetic pathway for production of capsaicin from lignin

4. Conclusions

The pulp and paper industry in Canada is presently in economic strife owing to its inability to effectively and economically deal with their lignin-rich waste streams. The inability to efficiently valorize lignin represents a lost opportunity. The development of green and economical biovalorization platforms such as the ones described herein that can convert lignin into value-added chemicals will not only facilitate monetization of a recalcitrant and hitherto worthless waste stream, but will also reduce the environmental footprint of pulp and paper manufacturing and revitalize that sector.

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