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*Review*

# Perspectives on the use of transcriptomics to advance biofuels

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**Abstract:** As a field within the energy research sector, bioenergy is continuously expanding. Although much has been achieved and the yields of both ethanol and butanol have been improved, many avenues of research to further increase these yields still remain. This review covers current research related with transcriptomics and the application of this high-throughput analytical tool to engineer both microbes and plants with the penultimate goal being better biofuel production and yields. The initial focus is given to the responses of fermentative microbes during the fermentative production of acids, such as butyric acid, and solvents, including ethanol and butanol. As plants offer the greatest natural renewable source of fermentable sugars within the form of lignocellulose, the second focus area is the transcriptional responses of microbes when exposed to plant hydrolysates and lignin-related compounds. This is of particular importance as the acid/base hydrolysis methods commonly employed to make the plant-based cellulose available for enzymatic hydrolysis to sugars also generates significant amounts of lignin-derivatives that are inhibitory to fermentative bacteria and microbes. The article then transitions to transcriptional analyses of lignin-degrading organisms, such as *Phanerochaete chrysosporium*, as an alternative to acid/base hydrolysis. The final portion of this article will discuss recent transcriptome analyses of plants and, in particular, the genes involved in lignin production. The rationale behind these studies is to eventually reduce the lignin content present within these plants and, consequently, the amount of inhibitors generated during the acid/base hydrolysis of the lignocelluloses. All four of these topics represent key areas where transcriptomic research is currently being conducted to identify microbial genes and their responses to products and inhibitors as well as those related with lignin degradation/formation.

**Keywords:** transcriptomics; RNA sequencing; fermentation; lignocellulose; lignin; plant hydrolysates; ethanol; butanol; bioreporters; stress response

## 1. Introduction

Humankind is facing several different crises primarily due to our own devices and activities. One major crisis is global warming and, integrally related with this problem, is the energy crisis. Petroleum has its origins in decaying and decomposing plant and animal remains that have remained dormant and locked within the earth's crust for eons. As such, this resource is limited and human dependence upon it has led to the release of significant amounts of carbon into the environment in the form of carbon dioxide. This byproduct of petroleum combustion, along with other pollutants, contributes to the warming of the environment being observed currently world-wide. To off-set this negative impact, there is a push globally within governments, economies and amongst scientists to evaluate, establish and implement processes, technologies and perspectives that are either carbon neutral or negative [1].

Carbon negative technologies, by their definition, will lead to an overall reduction in the atmospheric carbon load over time. To achieve this, therefore, a process must be in place that can absorb carbon dioxide and assimilate it or store it long-term. However, as noted above, petroleum is a limited resource and by some estimates we have already reached or passed the peak oil stage [1,2]. Consequently, it is projected that we will see a decline in the amount of petroleum produced within the coming years. Thus, a renewable energy source is also required. Perhaps it is fortuitous that nature has provided both a carbon sink and a renewable energy source in the form of plants. Through photosynthesis, plants naturally fix and assimilate atmospheric carbon dioxide, generating their biomass. Although they vary somewhat, the amount of plant biomass generated annually is significant [3–5]. The majority of this is in the form of cellulose, a polymer of glucose formed through β-1,4 linkages. As glucose is the preferred substrate for fermentative organisms, photosynthesis not only addresses carbon dioxide removal but also provides an optimal renewable feedstock sugar for the production of biofuels. However, plant cell walls do not only contain sugars but also include lignin, a polymer formed by the oxidative radicalization of monolignols, such as coniferyl alcohol, and their subsequent uncatalyzed coupling. Lignin contributes to the rigidity of the plant [6] but also acts to prevent rotting due to its recalcitrant nature.

It is this resilient characteristic of lignin that makes the conversion of plant biomass to biofuels so difficult and necessitates pretreatment steps using acid or base hydrolysis. During these processes, the lignin is broken down and exposes the cellulose, making it affable to enzymatic hydrolysis. However, acid or base hydrolysis of the lignin generates a variety of aromatic acids and aldehydes, including 4-hydroxybenzoic acid and ferulic acid [7,8]. When present at sufficient concentrations, these compounds are inhibitory and significantly reduce both the growth of the fermentative organisms as well as their solvent yields [8–11]. In response to this, researchers have sought to remove the inhibitors from the plant hydrolysates through over-liming [12,13], the use of an absorbent such as activated carbon or charcoal [14–17] or with ion exchange resins [15,18].

Lignin is not the only inhibitory compound that bacteria and microbes are exposed to during the fermentative process. Both ethanol and butanol are secondary metabolites that are useful to humans and produced by these organisms but their production also leads to reduced activity and culture viability due to their chaotropic nature. Chaotropic solutes have been found to cause water stress in bacteria and fungi, leading to a reduced water activity [19–21]. One way for microorganisms to respond to these conditions is to produce or to use available compatible solutes, such as trehalose,

glycerol and erythritol [22–24]. A nice review covering chaotropicity and its impacts on biofuel-producing microorganisms was recently published by Cray et al. [25]. The stress responses within bacteria, however, have generally been classified as being genotoxic [26], membrane damaging [27,28], heat-shock [29,30] and oxidative damage [26,31], as well as others. In each case, the stress induced by the compound or condition tested was categorized according to the specific macromolecule impacted and the mode of toxic action. However, in some cases, the impact of a compound cannot be defined so easily, such as when it binds to a vital protein and blocks its activity, as is seen with the non-productive and irreversible binding of cellulases with plant lignin [32].

In response to this limitation and to delve deeper into established toxic mechanisms, researchers have applied transcriptomics to understand the sub-cellular responses of microbes, particularly when they were exposed to model compounds. Transcriptomics is a high-throughput analysis of the transcriptional responses, *i.e.*, RNA profiles, of an organism and is often performed in a comparative study between a control group and a test group. This analysis can be performed to determine the genes that are differentially expressed when a specific substrate is provided, helping researchers to identify genes and proteins related with a specific metabolic pathway. When applied within the scope of toxicogenomics, it allows one to identify select biomarkers that can be used to understand the stresses imparted during an exposure to various compounds and potentially how to improve upon the organism through genetic manipulation. The relatively recent application of transcriptomics to biofuels research has expanded the means by which researchers and industry can approach potential problems and pitfalls, including the issues related with lignin. Consequently, this review article will discuss four areas of biofuel research where transcriptomic analyses have been applied with varying degrees of success. It should be noted that throughout this review, the term “toxic” refers specifically to the nature and activity of the compound or solute in question while the term “stress” is used as a reference to the biological responses elicited by the compound or solute.

### *1.1. Applying transcriptomics to understand microorganisms and their responses during fermentations*

With increasing concerns about the use of fossil fuels, bio-based production of fuel and chemicals has been suggested as one of the more promising substitutes. Through research efforts around the world, considerable advancement has been made with regards to the overall processes, including biomass pretreatment, fermentation and product recovery [33–35]. However, one of the highest priorities still lies in finding an optimal microbial host for the efficient and economical production of biofuels. Factors that need to be considered include their abilities to utilize lignocellulose and its resulting sugars, to tolerate cellular stress caused by lignocellulose-related inhibitors and fermentative end-products, and so on. Along these lines, it is also essential to choose properties to be introduced or improved within the strains to overcome limitations that we currently face. To this end, characterization of genes playing key roles in these functions at the transcriptome level would be helpful.

#### 1.1.1. Analysis of the transcriptome related with carbon utilization and solvent formation

Within nature, and plants in particular, glucose is hardly the only sugar present. Plant biomasses can contain several different sugars, including cellobiose, arabinose, galactose and xylose, as well as

others. Therefore, the biofuel yield is initially impacted by how much and which sugar is taken up and metabolized by the microbes employed. In this respect, *Clostridia* are capable of utilizing a broad range of carbon substrates, including mono- and disaccharides [36,37]. Related with this, a transcriptional analysis of *Clostridium acetobutylicum* was used to identify the mechanisms responsible for differential carbohydrate utilization [38]. When *C. acetobutylicum* was cultured using the media containing 11 different substrates, including pentoses (xylose and arabinose), hexoses (glucose, mannose, galactose and fructose), disaccharides (sucrose, lactose, maltose and cellobiose) and a polysaccharide (starch), the genes involved in sugar specific transport and metabolism were identified. As a result, they showed that pentose sugars are transported by symporters and ATP-binding cassette (ABC) transporters, whereas uptake of hexoses and disaccharides is through the phosphotransferase system (PTS) and gluconate: $H^+$ (GntP) transporter. Also, the gene transcripts were expressed in response to both specific sugars and several sugars simultaneously. However, despite *Clostridia*'s flexibility in sugar utilization, they do have a carbon catabolite repression (CCR) system, as has been reported in several previous studies [39,40]. Accordingly, DNA microarray analysis was used to elucidate CCR brought on by glucose preferences in *C. acetobutylicum* [41]. The culture grown on a mixture of glucose and xylose was monitored and two putative operons related to xylose metabolism were identified. The first operon, which includes genes encoding for a transporter, a xylulose-kinase and a putative xylose isomerase, was induced by xylose, but was shown to be repressed in presence of glucose. Another operon expressed by xylose, but not affected by glucose, was identified and included a xylulose-kinase, a hypothetical protein and another putative xylose isomerase. Another strain that is attractive for biofuel production due to the range sugars it can utilize is *C. thermocellum*, a thermophile that produces a protein complex called a cellulosome that allows this bacterium to degrade and utilize cellulose directly [42,43]. With its genome sequence recently identified [44], researchers have used transcriptomic analyses to elucidate several functions important to this process. For instance, two recent papers reported on the global gene expression patterns in *C. thermocellum* cultures grown on cellulose and cellobiose [45,46], helping them to identify the gene cluster related with the cellulosome, including several hydrolytic enzymes.

To improve biofuel yields, it is also important to know the metabolic flux of the sugars utilized during fermentation. In case of *Clostridium* strains, the metabolic pathway is complicated and includes a phase switch from an acidogenic lifestyle to one that is solventogenic. Wang et al. analyzed the transcriptome of *C. beijerinckii* NCIMB 8052 during growth using RNA-sequencing [47] to identify the transcriptional activity of important genes, including those involved in acid and solvent formation as well as chemotaxis, motility, transcriptional regulation and fatty acid biosynthesis. As such, their study provided key information about the culture during fermentation, such as gene functions and regulatory mechanisms involved in glycolysis and primary alcohol production. Similarly, global transcriptional changes occurring in *C. acetobutylicum* cultures have also been studied, especially during the shift from acidogenesis to solventogenesis [48]. One key finding was confirming the importance of the megaplasmid pSOL within *C. acetobutylicum* as a majority of the genes on the plasmid showed an increased expression during the solventogenic phase. Analysis of the genes involved in sporulation, such as *spo0A* and *sigF* operon, was also performed in comparison with *Bacillus subtilis*, with both showing conserved expression patterns. A similar analysis during the growth switch was reported using continuous cultures of *C. acetobutylicum* [49]. The authors chose to use a continuous culture since it provided a more detailed analysis focusing on

phase switch without concern for sporulation and other growth-phase dependent parameters. The result showed that expressions of acetone decarboxylase and pyruvate decarboxylase were increased before and after the switch, respectively, and that two alcohol dehydrogenase genes (*adhE1* and *adhE2*) and two paralogs of the thiolase genes (*thlA* and *thlB*) antagonistically regulated each other.

In addition to single strain analyses, comparative analyses have also been performed using hyper-butanol producing mutants and their parental strains during fermentation to help elucidate the factors responsible for the higher yields seen with the mutants [50,51]. Transcripts of *C. beijerinckii* BA101, a butanol hyper-producing mutant, and its parent strain *C. beijerinckii* NCIMB 8052 were examined during the shift from the acidogenic phase to solventogenic phase [50]. The comparison identified several differences in genes related with sporulation, PTS-mediated sugar transport and also in primary metabolic genes and chemotaxis/motility genes, which showed elevated expression levels within the mutant. Another transcriptomic comparison was performed using the hyper-butanol producing *C. acetobutylicum* EA 2018 and its parent strain *C. acetobutylicum* ATCC 824 [51]. In this study, the two strains were compared both in terms of their butanol production as well as their xylose utilization. Consequently, in *C. acetobutylicum* EA 2018 a putative transcriptional regulator involved in xylose utilization was found, helping to explain the accelerated xylose consumption seen with this strain.

### 1.1.2. Characterization of Clostridia and their responses to end-products

One of the major constraints on microbe-driven production systems is the inhibition of the microorganisms by their own products. For example, *Clostridium* strains, regardless of their catabolic versatility, are only able to tolerate only up to 2% (w/v) butanol [52,53]. *E. coli* has been shown to be completely inhibited by butanol at concentrations below 1% (w/v) [54]. Although there have been studies on chaotropic stresses induced by ethanol or butanol [25,55], it is still important to understand how microbes respond to their own metabolic by-products both when added extracellularly and during their production by the microbe, especially in sub-cellular responses.

DNA microarray-based investigation of *C. acetobutylicum* to elucidate the chaotropic activity of butanol began in the early 2000s. First, Tomas et al. reported on their transcriptional analysis of *C. acetobutylicum* ATCC 824 and a recombinant strain harboring a *groESL*-overexpressing plasmid [56]. A comparative analysis of these two strains when exposed to butanol revealed differences in the expression of genes involved in motility, chemotaxis and other stress responses. A later study from the same group helped to identify genes related with butanol stress and tolerance, including major stress proteins and the genes involved in butyrate/solvent formation and those related with fatty acid biosynthesis and sporulation [57]. One key finding, though, was the higher expression of genes encoding amino acid binding proteins and ABC-type transporters in only the control strain. Through their analyses, the authors finally suggested that the stress caused by butanol induces expression of the genes related with solvent formation, and so butanol induces its own production.

A similar comparative transcriptional study was performed to identify a role for *spo0A* in the cell's response to butanol [58]. In this study, DNA microarray data from 824(pMSPOA), a *spo0A*-overexpressing strain of *C. acetobutylicum* ATCC 824, was compared with data from a control strain and a *spo0A*-deleted mutant. Within the two strains bearing an intact *spo0A* gene, the genes involved in fatty acid metabolism, chemotaxis, heat shock protein, and cell division proteins were differentially expressed, but not in the latter, implying that these genes play some important

role in the process of sporulation and butanol stress. Moreover, several genes that were highly induced by over-expression of *spo0A* were also up-regulated in *groESL* over-expressing strain [57], further affirming their role in enhanced butanol stress.

Since previous studies reporting on butanol stress could not obviate the impact of the cell's growth from their results, one group's solution was to study the impacts of butanol stress using steady state acidogenic chemostat cultures of *C. acetobutylicum* [59,60]. When solvent stress was applied using a transient butanol pulse, a total of 358 genes were differentially expressed, including general stress response genes, as well as those involved in amino acid and nucleotide synthesis, and specific transport systems [59]. One noteworthy finding was an increase in the glycolipid biosynthetic pathway when under butanol stress, suggesting that the cells modified their cytoplasmic membrane composition in response to this stress. In a parallel study, *C. acetobutylicum* was serially adapted to n-butanol up to 1% (v/v) [60]. When they performed a transcriptomic analysis with this strain, no changes in the solvent formation gene expression levels were obtained, but genes encoding heat shock proteins, proteins related to membrane composition and ABC transporters encoded for within the chromosome were up-regulated while a few genes on the megaplasmid pSOL had altered expression levels, including rare lipoprotein and membrane protein genes.

#### 1.1.3. Characterization of other microbes and their responses to end-products

Although *Lactobacillus brevis* is not a natural biofuel-producing strain, its potential use in industry and as an alternative host for biofuel production has increased due to its higher butanol tolerance than common butanol fermentative strains [53]. To understand its resistance mechanism, consequently, researchers assessed butanol stress in *L. brevis* using transcriptomics [61]. Addition of n-butanol to growing cultures of *L. brevis* at a concentration of 2% (w/v) resulted in general and oxidative stress adaptations, as shown by induced expression of the genes related with these stresses. The main finding of this transcriptomic study, though, was that the entire fatty acid biosynthetic pathway was induced, and that the proportion of 19:1 cyclopropane fatty acids within the membrane decreased. To test if this can account for the higher resistance to butanol, the authors tried to improve butanol tolerance within *E. coli* by over-expressing acetyl-coA carboxylase and deleting the gene encoding for cyclopropane fatty acid synthase. However, no enhancement of butanol tolerance was achieved, implying that other stress responses are responsible for the n-butanol resistance seen in *L. brevis*.

Many species of yeast are also attractive for use within biotechnological processes due to their intrinsic alcohol tolerance, particularly to ethanol [62,63]. One report by Alexandre et al. (2010) assessed the global gene expression patterns of *Saccharomyces cerevisiae* during a short term 7% (v/v) ethanol stress [64]. DNA microarray analyses of *S. cerevisiae* exposed for 30 minutes revealed that 3.1% and 3.2% of the yeast genome was up-regulated and down-regulated, respectively. Of the genes altered by the ethanol stress, a large number are involved in ionic homeostasis, heat protection, trehalose synthesis and antioxidant defenses. However, as numerous genes related to energy metabolism were highly induced, it was suggested that *S. cerevisiae* was attempting to manage its energy pool under the stressful conditions.

Another promising host for biofuel is cyanobacteria [65,66], but one severe defect is it's weakness with product inhibition. To improve the butanol tolerance of a *Synechocystis* sp. strain, its transcriptome was investigated using RNA-sequencing when exposed to butanol at two different

concentrations, 0.4 and 1 g/L [67]. Under these conditions, 80 and 280 genes were differentially expressed, respectively, and they were primarily involved in cell membrane function, photosynthetic electron transport and biosynthesis genes. Another group that was impacted was the heat shock response, and consequently the authors chose to over-express a small heat shock protein gene, *hspA*, which successfully improved *Synechocystis*' tolerance to butanol.

Several additional microbes are also known to be more tolerant to useful solvents showing chaotropic activities, such as ethanol and butanol. For example, *Pseudomonas putida* has been tested as a host strain for butanol production despite the absence of an intrinsic metabolic pathway to generate butanol [68]. The rationale was *P. putida* is resistant to chaotropic solvents [19,20] and still grows considerably in the presence of 3% (v/v) of butanol [68]. This resistance increased up to 6% (v/v) butanol after adaptation. Similarly, *Bacillus subtilis* showed the highest tolerance to butanol out of several host microbes tested, including *S. cerevisiae* and *Z. mobilis* [69]. Another example is the isolation of an *E. coli* that is capable of producing more than 60 g/L ethanol and is six times more tolerant to butanol than wild type *E. coli* strains [70]. To date, however, transcriptomic studies of these strains have not been reported. Once performed, the results may provide information about key genes conferring high stress tolerance and allow researchers to apply these findings to other bacterial strains as well.

### *1.2. Transcriptomic analyses of bacteria exposed to plant hydrolysates*

Lignocellulosic biomass definitely offers several benefits as a possible carbon substrate for biofuel production in that it is the most abundant material available on the earth and is not in competition with food supplies. Due to these advantages, many researchers have sought to develop methods for the fermentation of various types of plant biomass, such as grass, agricultural residues and wood wastes [71–73]. However, the sugars present within the biomass are mostly tied up as cellulose and hemicellulose and can only be converted to useful chemicals by fermentative bacteria after being liberated from their complicated association with lignin. During this process, several by-products can be generated, such as phenolic compounds and furan derivatives, which inhibit metabolic activity of the fermentative bacteria and, thus, the production of biofuels [7,8,74]. Several hypotheses have been raised to explain the activity of inhibitory compounds on bacteria [75,76] and the toxic activity of vanillin, a lignocellulose-derived compound, has been recently reported [77], but details on how bacteria counteract these stresses are only now being characterized.

#### 1.2.1. Transcriptional investigation of stress derived from lignocellulosic hydrolysates

Ferulic acid, a hydroxycinnamic acid derived from lignin, has some pharmaceutical benefits for humans [78] but is one of the most toxic compounds when it comes to fermentative bacteria, showing inhibitory effect at a concentration as low as 0.3 g/L [7,8]. To understand its mode of toxicity, the impact of ferulic acid on *E. coli* was recently investigated [79]. From DNA microarray analysis with *E. coli* str. BL21(DE3) exposed to 0.25 and 0.5 g/L of ferulic acid, several genes were highly up-regulated (more than 4-fold) including the *mar* regulon, which encodes for efflux system-related genes. Based on the microarray and real-time quantitative PCR results, five genes were selected as biomarkers that are responsive to lignin hydrolysate-related compounds and then validated using five additional compounds, such as ferulaldehyde, coumaric acid and furfural.

Another transcriptional response to ferulic acid was performed with *Lactobacillus brevis*, which was mentioned above as a good candidate for n-butanol production [61]. *L. brevis* challenged with ferulic acid was analyzed both in terms of the time-dependent responses and according to the cluster of orthologous genes (COGs). As with *E. coli*, various transporter-related genes were induced. Potentially more important, however, several genes encoding for uncharacterized hypothetical proteins showed higher expression levels. Moreover, owing to its superior tolerance to phenolic compounds when compared with other lactic acid bacteria [80,81], understanding the resistance mechanisms of *L. brevis* may help researchers engineer other bacteria.

Like *E. coli* and *L. brevis*, the responses from *Clostridium spp.*, have been also studied when exposed to several lignin-derived inhibitory compounds. Most recently, the impacts of ferulic acid on *C. beijerinckii* NCIMB 8052 was determined [82]. In that study, not only was the growth and solvent production monitored, but also the transcriptional level changes. For this, the authors performed DNA microarray analyses according to the *C. beijerinckii* NCIMB 8052 metabolic states when cultured in presence of 0.5 g/L of ferulic acid. They found several gene ontology (GO) terms and Kyoto Encyclopedia Genes and Genomes (KEGG) pathways were prominent throughout, with down-regulation of two-component systems and strong up-regulation of efflux systems, heat shock proteins and proteins related with redox reactions. Based upon their data, they selected a heat-shock protein for over-expression and demonstrated improved growth and solvent production by the recombinant strain when grown in the presence of ferulic acid.

Zhang et al. (2013) performed a similar transcriptional analysis to elucidate *C. beijerinckii*'s response to furfural [83]. Furan-derivatives, such as furfural and hydroxymethyl furfural (HMF), were shown to affect *C. beijerinckii* favorably at concentrations lower than 3 g/L [7,8]. However, it is essential to understand the effect of furfural on the physiology of this strain since it is still an inhibitor commonly generated during the pretreatment and hydrolysis of lignocellulosic biomass [8]. When furfural was added during the acidogenic and solventogenic phases respectively, about 111 and 721 genes within *C. beijerinckii* NCIMB 8052 were found to be differentially expressed [83]. Notably, furfural was shown to be more potent during the solventogenic phase than acidogenic phase, as evidenced by highly induced membrane transporter- and chemotaxis-related genes and a greater ABE production. When the effects of furfural were analyzed using *C. thermocellum*, the results were completely different than *C. beijerinckii*, with a strongly decreased cellobiose consumption and ethanol production in the presence of furfural [84]. The lower production of ethanol by this strain was attributed to the down-regulation of the alcohol dehydrogenase and adjacent genes.

Although the studies performed above with one compound contribute to the understanding about how the organisms counteract stresses, they are limited in their applicability as plant hydrolysates are complex chemical mixtures. Consequently, actual hydrolysate samples have been also tested to determine their impacts on bacterial transcription. For instance, the five biomarker genes identified in *E. coli* BL21(DE3) using individual phenolic compounds were also characterized when *E. coli* was exposed to a spruce hydrolysate sample [79]. Of the five biomarkers, *inaA*, *htpG* and *marA* were all highly up-regulated, showing a maximum induction of 12.9-fold despite the low concentrations of the phenolic compounds (*i.e.*, 0.12 g/L) within the hydrolysate samples, suggesting that analogous stress responses might also be present. Moreover, Jin et al. (2015) have recently reported the transcriptional analysis of *C. acetobutylicum* by RNA sequencing during a fermentation of wheat straw hydrolysates [85]. The biomass used in their study was prepared by milling and enzymatic hydrolysis, generating considerable amount of destructive compounds including 4.48 g/L

of furans and 0.81 g/L of phenolic compounds, and followed by supplying sodium sulfide. Therefore, they performed the comparative analysis at the mRNA level to elucidate the effects of sodium sulfide on enhancing tolerance of this strain to chaotropicity induced by compounds. Supplementation of sodium sulfide led to a higher expression of genes involved in the membrane transport system, such as phosphotransferase system (PTS) and ATP-binding cassette transporters (ABC-transporters) as well as those related with glycolysis and the solvent formation pathways, which finally resulted in a higher sugar consumption and butanol production.

### 1.2.2. Downstream applications of genes characterized by transcriptomic studies

The studies discussed above aimed to provide better insights into microbial physiology and gene regulation when exposed to stresses induced by lignin-related compounds. Using the data, therefore, robust and tolerant microbes can be developed so that more cost-effective biofuel production can be achieved with a minimum loss of sugars. Several mutant strains capable of withstanding inhibitors have been reported, but these were generated via random mutagenesis approaches [86,87]. Although these strains generated higher ethanol or butanol yields in the presence of toxic compounds, implying they can be effective candidates for lignocellulosic materials, the random mutagenesis process is time- and labor-consuming work requiring various mutagenic chemicals, long adaption courses and screening of the variants. On the other hand, genetic manipulation based on data obtained from transcriptomic studies offers a more rational and focused solution. For example, *C. beijerinckii* NCIMB engineered to over-produce GroES and GroEL, two heat shock proteins induced by ferulic acid, has been shown to be more tolerant to this chemical [82]. Also, *Saccharomyces* strains possessing improved ethanol productivities in the presence of ferulic and cinnamic acids were obtained by over-expressing the gene encoding phenylacrylic acid decarboxylase [88].

An additional way of exploiting the data from transcriptomic studies with either individual chemicals or plant hydrolysates has also been explored. Bacterial bioreporter strains have been developed that detect specific toxicities through their stress responses [26,31] or compounds [89,90] using the genes responsive to them. In the same manner, biosensor strains can be constructed to detect hydrolysate-related compounds with the genes showing higher expression by those compounds. Previously, a *nagR-nagAa: lux* fusion strain was constructed to detect salicylic acid and other benzoic acid derivatives [91], including known lignin-derived phenolic compounds. Using the same principle, three bioreporter strains were developed and characterized that detect lignin-derived phenolic compounds [92,93] and furfural [94]. Each gene used in the reporter fusion was originally identified based on DNA microarray analyses of *E. coli* exposed to ferulic acid [79]. Although it may appear simple, biosensing with these bioreporter strains may have great significance for biofuel production in both quantitative and qualitative aspects. The effective concentrations of hydrolysate byproducts are very low (<1 g/L) but the sensitivity of these strains was as low as 5 mg/L, far lower than the inhibitory concentration needed. Their broad specificity is also a positive as hydrolysates contain many different aromatic acids and aldehydes, many of which were detectable alone or in mixtures. Also, the bioreporter strains can provide the extent of toxicity of the compounds and permit the researcher to evaluate whether hydrolysate is ready for fermentation. Taking all these things into account, more bioreporter strains should probably be developed to further improve biofuel production systems.

### *1.3. Transcriptomic analyses of lignin-degrading microorganisms*

One novel way that researchers have sought to deal with the lignin issue is to utilize microbes that are capable of degrading this complex plant polymer. Degradation of lignin is an oxidative process employing hydrogen peroxide and either ligninolytic peroxidases or laccases. Within nature, a plethora of microorganisms exist that harbor these enzymes and metabolic activities necessary to hydrolyze the lignin component within plant biomasses.

#### 1.3.1. Ligninolytic bacteria: a potential waiting to be tapped

The idea of using bacteria to degrade lignin is not new [95–97], but novel strains are still being isolated today [98–101]. These strains are often evaluated for their ability to decolorize dyes, a characteristic that is associated with the production of laccases, lignin peroxidases (LiPs) and/or manganese peroxidases (MnPs). Some of the dyes that are used for these tests are Azure B, Phenol Red and Methylene Blue [100]. The bacterial strains that are capable of degrading lignin and its derivatives are diverse, and include *Bacillus* sp. [98,102], *Brevibacillus agri* [100], *Citrobacter* sp. [103] *Pandoraea norimbergensis* [98], *Paenibacillus* sp. [102], *Pseudomonas* sp. [98], *Sphingobium chlorophenolicum* [104] and *Thermobifida fusca* [105,106], as well as many others [107]. However, to date, none of the transcriptomes of these strains appear to have been studied in the presence of lignin to determine how these strains adapt to growth on this substrate.

#### 1.3.2. Transcriptomic and secretomic analyses of ligninolytic fungi

Probably the best known and studied ligninolytic organism, however, is *Phanerochaete chrysosporium*, one of the fungi commonly referred to as white-rot fungus. This organism utilizes the sugars present to generate hydrogen peroxide, which is then used by its peroxidase enzymes to catalytically cleave the bonds present in the lignin polymer. A study by Wymelenberg et al. (2009) analyzed the transcriptome and secretome of this basidomycete when grown under ligninolytic or cellulolytic conditions [108]. They found numerous genes that were up-regulated under carbon-limited conditions when compared to rich media, including those encoding for oxidoreductases pyranose-2 oxidase (*pox*) and the aryl alcohol dehydrogenase (*aad*). Both of these proteins may be involved in generation of peroxide. They also saw the genes expressing *O*-methyltransferase and phenylalanine ammonia lyase up-regulated and proposed that these genes may play a role in veratryl alcohol generation. This compound is important as the lignin peroxidase protein is too bulky to directly interact with the lignin polymer and so uses mediators, such as veratryl alcohol [109]. A closer look at genes thought to be involved in lignin and aromatic degradation showed many peroxidases were up-regulated, including genes encoding for the lignin peroxidases *lipB*, *lipD* and *lipE* in the carbon-limited media while the nitrogen-limited media induced *lipC* and *lipJ* as well as the manganese peroxidases *mnp1* and *mnp2*. Potentially more important, though, was the identification of nearly 200 genes and proteins with unknown functions that were up-regulated under the conditions tested, many of which may be involved in lignin or cellulose degradation [108].

A subsequent study from the same group looked at the expression patterns and secretome from the same organism when cultivated on various poplar woods, including syringyl-rich transgenic

variants [110]. They found that the lignin composition present within the plant biomass influences the gene expression patterns within *P. chrysosporium*, suggesting that lignin degradation is not simply a single pot reaction. For instance, growth on the syringyl-rich variant line 64 in their study led to significantly increased expression of eleven genes, including two glycoside hydrolases (GH15 and GH55), two flavin-dependent oxidoreductases, a formaldehyde dehydrogenase and phenylalanine ammonia lyase, which was also seen in their previous study [108]. Once again, however, several genes of unknown function (four of eleven) were identified in the microarray analyses, paving the way for further analysis of their function and importance in lignin degradation and possibly the conversion of high lignin biomass.

Several groups have also looked into the transcriptomes of ligninolytic organisms other than *P. chrysosporium*, including *Phlebiopsis gigantea* [111] and *Phanerochaete carnosa* [112,113]. *P. gigantean* is probably best known as a biocontrol agent against *Heterobasidium* [114]. However, many of the enzymes commonly utilized by ligninolytic organisms for the production of hydrogen peroxide, such as aryl-alcohol oxidase, glyoxyl oxidase and methanol oxidase, have not been found in *P. gigantean* cultures. Consequently, Hori et al. (2014) sequenced and annotated the *P. gigantean* chromosome and then analyzed its transcriptome when grown on ground loblolly pine [111]. In their study, they used two wood substrates, one that was extracted with acetone (ELP) and one not extracted (NELP). In comparison with glucose media, numerous genes were significantly expressed, particularly within the NELP media. Once more, the two glycoside hydrolases (GH15 and GH55) were present, as was an *aad*-like oxidoreductase and P450. Of the 54 genes showing stronger expression in ELP or NELP media relative to the glucose media, a full twenty were glycoside hydrolases, implying the need of these enzymes to access the cellulose and hemicellulose within the plant biomass. Interestingly, of the nine genes listed as being specifically up-regulated within the ELP media relative to the glucose media, only three had putatively known functions and the remaining six were all hypothetical with unknown functions. One of the three identified genes encodes for a heroxidase DyP protein, a haem peroxidase that differs structurally from lignin peroxidases (LiPs) and manganese peroxidases (MnPs) [115].

Transcriptomic studies with *P. carnosa* by MacDonald et al. (2011) were performed using a variety of wood substrates, including pine, fir, spruce and maple [112]. Once the mycelial mat reached a diameter of 4 cm, they sampled the central 2.8 cm region for RNA purification and analyses. When grown on ground fir, the *P. carnosa* gene transcript encoding for LiP was 200-fold more abundant than in the control cultures. This strong induction of the peroxidases was also observed with the other woody biomasses tested as MnPs showed the strongest responses, with several having relative abundances that exceeded an average of 700-fold for all four of the woods tested. This strong induction also extended to the glycosyl hydrolases, monooxygenases and oxidoreductases, albeit to a lower degree. A later study by the same group looked into the time-dependence on the expression levels of select genes [113]. They found that as the mycelial mat grew to 4 cm in diameter, the gene expression levels within the 2.8 cm central region increased initially but then tended to drop when the mat grew further and the diameter reached 6 cm. A closer look at specific genes found that expression of the MnP and LiP genes were strongest during the early stages of growth while enzymes related with carbohydrate utilization were stronger towards the later stages. The strong production of the peroxidases in the early cultures illustrates the role of the lignin in protecting the underlying cellulose and hemicellulose and the need to remove it before the fungus could access the sugars.

### *1.4. Transcriptomics of lignin biosynthesis within plants*

As lignin is the source of many problems and costs associated with the production of biofuels, researchers have also sought to remove it from the equation, almost literally. Probably the first instance of this was published in 2007 by Chen and Dixon [116]. Their study clearly showed an inverse relationship between the lignin content of the plant and the sugar yields obtained after pretreatment. Using antisense RNA within alfalfa, they were able to down-regulate the production of six genes related with lignin production. A pathway analysis found that targeting genes encoding for proteins earlier in the lignin biosynthetic pathway was a more effective strategy and that the sugar yields were nearly doubled when compared with the controls. Moreover, the sugar yields from the untreated transgenic plants were higher than those obtained from the acid-treated controls, implying that lignin reduction may obviate the need for such pretreatment steps. Not only would this reduce potential problems related with the production of inhibitors, but it would also reduce the costs associated with plant biomass conversion.

In the wake of this seminal study, a variety of groups have sought to use transcriptomics to study lignin-production within other plant species and how it potentially relates with bioenergy yields [117–120]. One recent study by Van Acker et al. (2013) [117] studied twenty mutations within *Arabidopsis thaliana* and found similar results as Chen and Dixon in that most of the transgenic plants showed significantly higher saccharification yields both with and without the acid pretreatment steps. Plants bearing mutations in three genes (*c4h-2*, *ccr1-3* or *ccr1-6*) all had cellulose conversion yields that exceeded 50% without the pretreatment, and approximately 80% when the pretreatment was included. This was in stark contrast with the wild-type control plants, where the conversion yields were 16.3% and 18.1% without and with acid pretreatment, respectively. In a subsequent study from the same group, they demonstrated that using antisense RNA against the cinnamoyl-CoA reductase gene (*ccr*) within poplar, resulted in ethanol yields increasing by as much as 161%, from about 3.1 g/l to 8.1 g/l [120]. This is a key example where the selection of a single gene for expression modification leads to significantly improved yields.

The use of RNA sequencing to study gene expression patterns in plants began around the same time as the study from Chen and Dixon (2007) [116] mentioned above, with several preliminary studies published between 2006 and 2007 [121–123]. A few years later, Wong et al. (2011) used RNA sequencing techniques to identify genes related with lignin biosynthesis within *Acacia auriculiformis* and *Acacia mangium* [124]. As the genome sequence for *Acacia* species still remains unknown, the results from their study help to highlight the use of RNA sequencing procedures over microarrays, the latter of which requires the genome to be sequenced and annotated prior to their use. Within the RNA transcripts sequenced, the authors found all ten genes involved in the monolignol biosynthesis pathway, including phenylalanine ammonia lyase, cinnamate 4-hydroxylase and cinnamoyl-CoA reductase, the same gene down-regulated through antisense RNA by Van Acken et al. [120]. Of these ten genes, more than one isoform was discovered for half, while 16 out of 18 isoforms were homologous between *A. auriculiformis* and *A. mangium*.

The above two studies are critical to understanding the power of transcriptomics as together they demonstrate the potential applications of this tool. Wong et al. (2011) [124] demonstrated that transcriptome studies based upon RNA sequencing can be performed on basically any organism, as long as the RNA can be extracted efficiently. Within their analysis of the RNA from both *A. auriculiformis* and *A. mangium*, they identified not only the *ccr* gene but also its sequence.

Consequently, using the same antisense protocol as presented in Van Acker et al. (2014) [120], it is now possible for them to down-regulate the translation of *ccr* within *A. auriculiformis* and *A. mangium* and potentially improve the saccharification of these plants as well. Similar applications with other plant species are likewise feasible.

## 2. Conclusions

As the field of bioenergy continues to grow, the use of transcriptomics, in the form of either microarray analyses or RNA sequencing, presents itself as a key platform for identifying candidate genes for further study and evaluation. As highlighted within this review, researchers in diverse labs are using transcriptomics to not only identify genes that will improve the resilience of the fermentative microorganisms, but also useful genes that are present within ligninolytic organisms and the plants themselves. As such, one strength of transcriptomics is that it can clearly be applied within the various strata involved in the fermentative process, *i.e.*, from the fermentative bacteria employed to the ligninolytic organisms used as well as the plants selected for use as the renewable feedstock. Moreover, with RNA sequencing, researchers are now capable of studying the transcriptome from genetically variant and newly isolated organisms without prior genome sequencing. This alone promises to expand biofuel research tremendously as many ligninolytic bacteria have yet to be characterized fully. In conclusion, bioenergy research has clearly benefited from the use of transcriptomics, but stands to see even more benefits in the near future as the use of transcriptomics expands further into other labs and research areas.

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## Conflict of Interest

The authors declare no conflict of interest.

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