Journal of Biotechnology & Biomaterials
Mahan et al., J Biotechnol Biomater 2017, 7:2
DOI: 10.4172/2155-952X.1000262
Review Article
OMICS International

A Review on The Bioconversion of Lignin to Microbial Lipid with Oleaginous *Rhodococcus opacus*

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Abstract

Rhodococcus opacus produces intracellular lipids from the biodegradation of lignocellulosic biomass. These lipids can be used to produce biofuels that could potentially replace petroleum-derived chemicals. Current studies are focusing on deconstructing lignin through efficient and cost-effective pretreatment methods and improving microbial lipid titers. *R. opacus* can reach high levels of oleaginicity (>80%) when grown on glucose and other aromatic model compounds but intracellular lipid production is much lower on complex recalcitrant lignin substrates. This review will discuss recent advances in studying *R. opacus* lignin degradation by exploring different pretreatment methods, increasing lignin solubility, enriching for low molecular weight lignin compounds and laccase supplementation.

Keywords: *Rhodococcus opacus*; Oleaginous organisms; Microbial lipids; Lignin; Biofuels

Introduction

Transportation consumes a vast amount of energy in the United States [1-3]. In 2016, the total U.S. primary energy consumption was about 97.4 quadrillion Btu, of which, 86% was consumed from energy produced domestically (83.9 quadrillion Btu). Petroleum, natural gas and coal, accounted for the majority of the nation's energy production, with the following breakdown: 28% petroleum, 33% natural gas, 17% coal, 12% renewables (including wind and solar) and 10% nuclear electric power [4]. With the world population growing, identifying sustainable fuel alternatives is imperative due to the finite amount of global petroleum, rural development challenges and environmental concerns [5]. Lignocellulosic biomass is a renewable carbon source for the production of biofuels and other products. Interest in producing renewable biofuels has been increasing over the last few decades [5-13]. Lignocellulosic biomass is an ideal resource for biofuel production, because it can reduce fossil fuel dependence, greenhouse gas emissions, and is an abundant resource. Lignin sources can be carefully selected as to not compete with food sources [14-16]. Identifying biodegradable, renewable, substitute fuels with properties similar to petroleum diesel will allow for compatibility within the existing transportation infrastructure. Prices fluctuate based on many factors but currently biodiesel production costs currently range from $105-$115 per barrel while crude oil is currently selling at $45 per barrel [17-19]. The cost associated with the development of biofuels remains challenging; therefore the development of novel lignocellulosic biomass deconstruction strategies and fermentation platforms to reduce the cost of biorefining biomass to biofuels will be vital to establishing sustainable biofuels [2].

Using lignocellulosic biomass to produce biofuels

Lignocellulosic biomass (i.e., wood, energy crops and agriculture residues) is an ideal renewable feedstock for the production of biofuels [20]. However, bioconversion of these substrates to sugars and subsequently cellulosic ethanol and other fungible fuels is hindered by lignin which often contributes to the recalcitrance of biomass and does not contribute to fuels production with today's biological conversion platform [21]. Lignocellulosic biomass consists of cellulose, hemicellulose, and lignin, which form a complex, rigid, and recalcitrant structure that is resistant to biological and chemical degradation [22,23]. The organic compound compositions vary depending on the particular lignin feedstock (i.e., switch grass (% dry basis): cellulose (42%), hemicellulose (25%), and lignin (18%) [24]. Compared with plant polysaccharides, lignin is generally regarded as a more complex polymer and has received much less attention as a resource for biofuel production.

Lignocellulosic biomass pretreatment strategies

There are a variety of pretreatment methods to reduce lignin recalcitrance, however not all the products or residues of these pretreatments have been used in lipid production fermentations, and therefore have not been studied in-depth to know the effect on oleaginicity/lipid yield. To be effective, pretreatment methods must disrupt the plant cell wall to enhance access of hydrolytic enzymes to plant polysaccharides to yield the desired microbial metabolism products. Current methods include alkali treatment, acid treatment, steam explosion, oxygen delineation, organosolv pretreatment among others [8,25-30]. Some of these pretreatment strategies enrich lignin oligomers and have resulted in sugar yields of 90% [31]. Dilute acid pretreatment followed by simultaneous saccharification and fermentation is a commonly employed pretreatment strategy to produce sugars for bioethanol production [32,33]. Dilute acid is sufficient to hydrolyze hemicelluloses; however, hydrolysis of cellulose requires more extreme conditions. In ethanol production, dilute-acid pretreatment is commonly coupled with simultaneous saccharification and fermentation to convert sugars to ethanol [21,34-36]. The released sugars can then be metabolized by yeast and the resulting lignin-rich residue can be utilized by aromatic-metabolizing oleaginous organisms. The thermal and chemical (alkaline and/or oxidative) pretreatments result in the degradation of β-O-4 moieties and aromatic ring openings.

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Received May 10, 2017; **Accepted** June 22, 2017; **Published** June 29, 2017

Citation: Mahan KM, Le RK, Yuan J, Ragauskas AJ (2017) A Review on The Bioconversion of Lignin to Microbial Lipid with Oleaginous *Rhodococcus opacus*. J Biotechnol Biomater 7: 262. doi: 10.4172/2155-952X.1000262

Specifically, for alkaline pretreatments, the lignin degradation is achieved by cleavage of aryl ether bonds, Caliphatic-O-Caromatic or Caromatic-O-Caromatic resulting in ring opening, degradation, and solubilization [37]. Pretreatment effluents and lignin that are highly soluble in solution or result in low molecular weight fractions of lignin are more easily utilized by the microorganisms.

Oleaginous lipid accumulation in *Rhodococcus opacus*

Oleaginicity refers to the ability of a microorganism to accumulate oil contents in excess of 20% of its cell dry weight [38]. Examples of oleaginous microorganisms are yeast, fungi, bacteria, and microalgae, which can produce microbial oils, often referred to as single cell oils [39]. Yeast and microalgae synthesize triacylglycerides (TAG), while prokaryotic bacteria generate specific lipids. Most oleaginous organisms produce TAG, which are the main component of biodiesel production, achieved by reacting the TAG with an alcohol in the presence of an acid or alkaline catalyst. This process is called transesterification, which generates fatty acid methyl esters (FAME, biodiesel) and glycerol as a by-product [40]. To determine oleaginicity, the total FAME detected by GC-MS is related back to the original mass of cells (dry cell weight) used for the transesterification process.

Oleaginous bacterial accumulation of lipids for biofuel production from industrial waste is being studied extensively [41-47]. Transcriptomic and proteomic studies have also identified TAG biosynthetic genes and proteins [48-53]. In previous years, the Gram-positive, oleaginous, soil bacterium *Rhodococcus opacus* has been studied for its ability to accumulate intracellular lipids >20%, based on cell dry weight (CDW) [54-60]. Some Rhodococcus strains exhibit oleaginicity above 80% CDW, when glucose is utilized as a carbon source under nitrogen-limited conditions [61,62]. When glucose is not used, Rhodococcus strains can degrade aromatic compounds commonly found in lignocellulosic biomass [54].

Fermentations using different lignin substrates or pretreatments

The oleaginous soil bacterium of the family Rhodococcus is an emerging ideal candidate due to minimal cultivation conditions and broad substrate specificity [47,62,63]. *R. opacus* can metabolize and degrade aromatic compounds found in lignocellulosic biomass [64] and accumulate intracellular single cell oils [54-59]. Rhodococcus strains have been engineered to express enzymes to degrade cellulose [65] and xylose [66]. Co-fermentations using wild-type and engineered strains have also been successfully employed [67]. Rhodococcus is a model organism for lignin degradation because it can tolerate, grow on, and adapt to inhibitory compounds (furan and phenol derivatives) produced from thermal and chemical pretreatments of lignin [68,69].

Kosa and Ragauskas demonstrated that *R. opacus* could bioconvert native Loblolly pine ethanol organosolv lignin (EOL) to lipids with limited oleaginicity (~4%) and low lipid titers (0.02 mg/ml) [56] (Table 1, #1). While these numbers are low compared to yields on lignin model compounds [55], EOL did support growth and lipid production. Ultrasonicating the EOL substrate did increase solubility but did not increase lipid production. These results suggest that lignin must be first converted into aromatic compounds via a pretreatment to increase oleaginicity and lipid titers in *R. opacus*. Wells et al. used the effluent from EOL as the carbon source in *R. opacus* fermentations to produce organism with increased oleaginicity (27%) [3] (Table 1, #2). Typically, effluent fractions are discarded as industrial wastewater but here it was shown to be a viable feedstock for the production of microbial lipids possibly due to the increased solubility of lignin compounds. Wei showed that oleaginicity can be improved (28%) when substrates are detoxified by the removal of inhibitors such as hydroxymethyl-furfural [70] (Table 1, #3). Fermentations of Kraft lignin from black liquor by *R. opacus* also resulted in poor lipid titers (<0.01 mg/ml) and strain oleaginicity [71] suggesting that pretreatments were necessary to improve the properties of Kraft lignin. It was hypothesized that lowering molecular weight via various pretreatments would result in higher lipid titers. Lignin depolymerization generates diverse low-molecular weight compounds which can be more readily processed [72]. Oxygen-delignification (O_2-delignification) is a pretreatment strategy that uses oxygen and alkali to remove the residual lignin from cellulose fibers at increased temperatures [73]. Significant structural changes appear in the lignin after O_2-delignification [74-77] resulting in a decrease in molecular weight. To obtain smaller molecular weight Kraft lignin, oxygen-pretreatment (O_2-pretreatment) was carried out on Kraft lignin under alkaline conditions which lowered the molecular weight of the lignin and when treated with *R. opacus* resulted in increased oleaginicity (14%) and an increase in lipid titers (0.07 mg/ml) [57] (Table 1, #4). Alternatively, Zhao supplemented *R. opacus* fermentation with Kraft lignin with laccases which resulted in an increase in lipid titers (0.15 mg/ml) [78] (Table 1, #5). Laccases aid in lignin depolymerization allowing for selective degradation of different lignin functional groups probably improving the usage of the lower molecular weight lignin molecules [78].

Pyrolysis which uses heat to decompose wood and grass biomass is a promising pretreatment for production of biofuels from lignocellulosic resources. The liquid products from pyrolysis are known as pyrolysis oils which separate into two immiscible phases: heavy oil and light oil [79]. A pyrolysis light oil fraction of switch grass used as the sole carbon source in a fermentation with *R. opacus* resulted in increased oleaginicity (22-26%) and improved lipid titers (0.06-0.12 mg/ml) (Table 1, #6). The pyrolysis resulted in low molecular weight water-soluble substances in light oil fraction which seems to play a role in oleaginicity and improved lipid titers [79].

To date, the pretreatment strategy and lignin substrate that has resulted in one of the highest oleaginicity and production of lipid titers in *R. opacus* is a two-stage alkali/alkali-peroxide pretreatment of corn stover [80]. The rigorous and chemically-efficient two-stage chemical

#	Lignin substrate	Oleaginicity (%)	Lipid Yield (mg/ml)	Ref.
1	Pine ethanol organosolv lignin (EOL) or ultrasonicated EOL	4	0.02	[56]
2	Effluent from Pine EOL	27	NR	[3]
3	Detoxified pine autohydrolysate	28-29	0.25-0.31	[70]
4	Kraft lignin (O_2-delignification)	14	0.07	[57]
5	Kraft lignin supplemented with laccases	NR	0.15	[78]
6	Pyrolysis light oil fraction from switch grass	22-26	0.06-0.12	[59]
7	Corn stover (alkali/alkali-peroxide pretreatment)	42	1.3	[80]

Table 1: Summary of studies involving *R. opacus* fermentations using different lignin substrates or pre-treatments.

pretreatment provided higher concentrations of solubilized glucose and lower molecular weight lignin degradation products thereby promoting improved oleaginicity (42%) and lipid titers (1.3 mg/ml) [80] (Table 1, #7).

Conclusion

R. opacus is a model oleaginous organism capable of producing single cell oils from numerous aromatic compounds and lignocellulosic biomass. Research is ongoing in efforts to efficiently deconstruct complex lignin molecules in order to allow maximum lipid production and lignin degradation. Current studies have shown that *R. opacus* can grow and produce intracellular lipids from unmodified lignin substrates (i.e., pine EOL: 4% oleaginicity) but those lipids can be greatly increased (effluent from pine EOL: 27% oleaginicity) using effluents which are composed of soluble lignin compounds. Currently, *R. opacus* fermentations with a two-stage alkali/alkali-peroxide pretreatment of corn stover results in the largest improvement of oleaginicity (42%) to date. Engineered strains are currently being evaluated for increased oleaginicity under these improved fermentation conditions and novel pretreatment strategies are currently underway to determine the best conditions for maximum production of single cell oils.

Acknowledgement

The research team acknowledges the Department of Energy (DE-EE0006112) for funding the microbial conversion results reported in this manuscript.

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