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Feasibility of triacylglycerol production for biodiesel, utilizing *Rhodococcus opacus* as a biocatalyst and fishery waste as feedstock

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ABSTRACT

Triacylglycerols (TAGs) can be produced via bacterial fermentation by the oleaginous Gram-positive microorganism *Rhodococcus opacus* strain PD630 in regulated, nutrient-deprived conditions with sufficient carbon supply. Microbially produced TAGs may be further refined via transesterification into biodiesel and glycerol, with 3 mole of biodiesel and 1 mole of glycerol produced from every 1 mole of TAG by chemical conversion. Large-scale industrial production of biodiesel has been conducted for over a decade, yet microbially derived biodiesel has been, up to this point, absent from the biodiesel market. The use of a novel feedstock, chitin, from New England fishery waste may present a viable, cost-effective, unexplored carbon feedstock source for local biodiesel development. Availability and implementation of chitin as a feedstock, along with analysis of potential fuel characteristics, yield promising results for future industrial development of biodiesel production from *R. opacus* PD630 TAGs in regional locations with large lobster, shrimp, and crab harvesting operations around the world. With declining resources of fossil fuels and increased societal awareness of carbon emissions and climate change, an analytical review of this nature is critically relevant.

Contents

1. Introduction

With increased social awareness regarding climate issues and carbon emissions, the volatility in price of fossil-derived fuels, and large percentages of fossil fuel-derived energy imported from international sources, it is imperative for the United States to develop alternative sources of domestically-derived fuels with

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lowered greenhouse gas emissions [1]. Microbial production of liquid fuel, namely diesel and gasoline, has been demonstrated by various organisms from refined carbon feedstocks [2,3], while photosynthetic cyanobacteria have been utilized to produce diesel by harnessing photon energy as a saccharide-free energy feedstock in controlled photobioreactors or shallow, open-air ponds [4]. In order to develop the most controversy-free and economically feasible domestic fuel source, it is necessary to ensure a life-cycle greenhouse gas emission reduction in comparison with fossil fuels and to have little or no competition with food production, thus avoiding the "food vs. fuel" debate [5]. Chitin provides an industrial waste stream carbon source available in large quantities from the exoskeletons of the most economically relevant crustaceans (i.e. lobster, crab, shrimp), as 40–50% of these crustaceans, by weight, go to waste streams [6,7]. While chitin and its monomeric components, 2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine) and 2-amino-2-deoxy-D-glucose (D-glucosamine), have been explored as beneficial food additives and within the field of medicine [8], chitin remains largely an untapped waste-stream carbon resource.

Triacylglycerols (TAGs) are common lipid storage molecules in many multicellular and unicellular eukaryotes, while many prokaryotes tend to store carbon in intracellular bodies known as polyhydroxyalkanoate granules [9,10]. However, specific strains belonging to the order Actinomycetales have demonstrated significant accumulation of intracellular TAGs [9], with *Rhodococcus opacus* strain PD630 commonly considered as the model oleaginous prokaryotic organism for TAG production [3]. *R. opacus* PD630 has many desirable industrial traits, including rapid, high density culturing, high substrate tolerance, and the ability to accumulate up to 80% of its cell dry weight (CDW) as storage lipids [3,11,12]. Additionally, *R. opacus* PD630 has the natural ability to utilize both *N*-acetylglucosamine and D-glucosamine as sole carbon sources [3], yet appears to lack the natural ability to depolymerize chitin into these monomeric subunits based on preliminary BLAST searches for chitinase and chitobiase homologs.

TAGs isolated from a variety of organisms may be transesterified via chemical methods in an alkali environment for the development of biodiesel, a process that has been utilized on an industrial scale for decades, yet lacks the economic feasibility for widespread commercialization and implementation [13]. Other methods of biodiesel development from TAGs have received significant attention in recent years, specifically, enzymatic conversion of TAG to biodiesel, yet industrial implementation of this strategy will likely require significant advances in solvent engineering, lipase immobilization, and proper acyl acceptor and reactor system selections [13]. Utilization of inexpensive feedstock will likely benefit both strategies of biodiesel production from microbially synthesized TAGs, yet the more traditional chemical process will be the sole option considered in this analysis.

2. Feedstock and fermentation

2.1. Global and regional availability

Numerous refined and non-refined feedstocks have been explored for TAG synthesis via natural [3] and engineered [14–16] metabolic pathways of *R. opacus* PD630. However, utilization of chitin from New England fishery waste streams as a potential feedstock has not been explored on an availability or feasibility basis for liquid fuel production, to our knowledge. Chitin is the second-most abundant natural biopolymer on earth, second only to cellulose [17]. Estimated natural annual turnover of chitin in the environment by insects, fungi, and marine organisms is estimated between 10^{10}–10^{11} metric tons [18]. Crustacean processing facilities generate waste on a global scale estimated at around 60,000 metric tons annually [19], yet percentage of waste by weight as chitin varies depending on geographic region, season, and organism [18]. For example, waste from crabs of the Genus *Cancer* can be comprised of up to 72.1% chitin, while crabs of the Genus *Callinectes*, only 14% [18]. Globally, chitin is refined and repurposed on a scale of roughly 1600 metric tons/yr [19], meaning that roughly 93% (22,400 metric tons) of chitin introduced to waste streams goes unutilized, based on an estimate of 40% of total waste as chitin, by weight. Global crustacean industries are localized to specific regions, based largely on Exclusive Economic Zone (EEZ), the range extending from a country's coastline, outwards to two hundred miles, for that country's exclusive use in exploration and exploitation of marine resources (http://www.oecd.org). The United States has the greatest amount of EEZ globally, and is a major processor of chitinous crustaceans, with fishery ports concentrated on the East and West mainland coasts, Alaska, and the Mississippi River delta [20]. Internationally, chitin availability is widespread, from locations in the North Atlantic to the South Pacific, where countries like Australia pay up to $150 USD/ton of shell waste for disposal, while developing nations often landfill shell waste or dump it back into the sea [21].

2.2. Demineralization, deproteination, and isolation

As chitin is very closely associated with natural pigments, proteins, minerals and lipids while in its biological state [22], it is necessary to remove these compounds in order to maximize chitin bioavailability for microbial utilization. This process is generally conducted in a two-step method known as demineralization and deproteination. The specifics of this process can vary for optimum chitin recovery depending on organism, but follow the general scheme as displayed in Fig. 1.

In an industrial purification process developed for strict microbial feedstock use, the bleaching step could likely be circumvented, as this step is simply for obtaining a white final product for future incorporation into foods or medical supplements, in order to satisfy consumer demands. Yet, this still leaves treatment with two harsh chemicals, sodium hydroxide for deproteination, and hydrochloric acid for demineralization. The hydrochloric acid treatment has proven to be a preferred reagent, though it does cause mild deacetylation of *N*-acetylglucosamine monomeric units, thus increasing the percentage of D-glucosamine within the polymer, along with acid hydrolysis of alpha-1–4 linkages [18,22]. Interestingly, as most uses of purified chitin have been as additives to other products, high-degree of polymerization and the resulting properties (e.g. viscosity, solubility) have been a sought after characteristic by most researchers [18,22,23]. Yet, in this context, monomers of both *N*-acetylglucosamine and D-glucosamine are favorable to chitin or chitosan polymers for microbial utilization, thus providing a slight benefit for strong chemical-based demineralization and deproteination processes, over potential biological alternatives.

2.3. Microbial fermentation of N-acetylglucosamine and D-glucosamine

Qualitative data demonstrates a notably greater ability for *R. opacus* PD630 to utilize *N*-acetylglucosamine, in comparison to D-glucosamine, as the sole carbon source [3], however, while both monomers are abundantly present in natural chitin and chitosan, *N*-acetylglucosamine is much more difficult to isolate via the chemical methods described above, as the degree of acetylation is reduced significantly during the chemical deproteination and demineralization processes [8,18]. This raises significant issues in regards to obtaining maximum TAG yield based on feedstock, as current technologies and processes are able to only yield D-glucosamine in high volumes from chemical methods.