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Bacterial Carbon Storage to Value Added Products

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Suggested Reviewers:	
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Response to Reviewers:	Reviewer #1: The manuscript by Brigham et al presents a development and description of carbon storage molecules production for biofuels using bacterial cultures. I find this to be a very important study that advances the field. This field is complicated, and this review contributes to clarify it. The review has a very clear general introduction on the theme but also there are

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	discussions on practical aspects all over the text. The authors performed well supported analyses and experiments and point out the major problems dealing with the whole idea of the topic and also giving interesting aims. This is a carefully organized, analyzed and discussed work, from which good methods for byopolymers production are proposed. >The authors would like to thank the reviewer for the kind remarks. - Line 20: Delete the second "grown on" >We have removed the words, as suggested by the reviewer - I would add some more explanations about the potential improvement from '90 to nowadays on this field, giving practical hopes for a future use of these techniques. >We have added some explanations regarding the logical advancement of biopolymers and biofuels production from these organisms, and implications of scaling up processes. In particular, the authors feel that the future lies in utilization of low cost organic wastes as feedstocks for producing these materials.

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Bacterial Carbon Storage to Value Added Products

REVIEW

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Keywords: carbon storage, polyhydroxyalkanoate, triacylglycerol, carbon/nitrogen ratio

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Abstract

Microorganisms have evolved different systems for storing carbon during times of stress. In the cell's natural environment, the stored carbon can then be utilized for growth when other nutrients are in better supply. Storage of carbon and other nutrients is ubiquitous throughout the prokaryotic and eukaryotic domains of life. These carbon storage molecules have great industrial importance. They can be useful as value-added products, as either biopolymers or biofuels, and cells are grown in large quantities and these compounds are harvested, usually as a replacement for a petroleum-based product. Nowadays, entire industries have been generated based on the production and utilization of these compounds. We focus on two bacteria that could be considered paradigms of their particular carbon storage strategy: *Ralstonia eutropha* and *Rhodococcus opacus*. *R. eutropha* has been well-studied as a polyhydroxyalkanoate (bioplastic) producer and *R. opacus* is a model bacterium for high yield triacylglycerol (TAG) production for biofuels. Both species produce carbon storage molecules that can potentially diminish our reliance on fossil-based petroleum. However, in both cases, there are challenges that must be overcome before profitable production schemes are established using these organisms. We explore the previous and current works to address these challenges in this review.

Introduction

Concerns about dwindling petroleum reserves have sparked worldwide concern, especially considering that the largest oil reserves tend to be located in unstable regions of the globe. As consumers of petroleum products for fuel and chemical needs, it is in our best interest to develop an inexpensive, renewable process for synthesis of bio-based fuels and other chemicals (*e.g.* plastics). As fuels and polymers are usually carbon-based, we can turn to organisms that are masters of carbon storage for this endeavor. Bacteria are capable of storing carbon in various forms during stress conditions. A well-studied family of carbon storage molecules is the polyhydroxyalkonoates (PHAs), which are known to exhibit properties of petroleum-based plastics [1,2]. Bacteria are also capable of storing carbon in the form of triacylglycerols (TAGs) [3,4], which is a less well-known process, but is rapidly gaining recognition as a biofuel production scheme. To compete with petroleum products, biofuel and bioplastics production must be efficient and cost-effective. Many researchers in both academia and industry have produced pilot plant or industrial scale PHA production processes, but the cost of the polymer product is still high when compared to petroleum-based plastics. In many of these cases, *Ralstonia eutropha*, the model organism for PHA biosynthesis, or a recombinant *Escherichia coli* strain are used. Less is known about industrial TAG productions using bacteria, although one organism *Rhodococcus opacus* strain PD630 stands out as being an efficient TAG accumulating organism. In this paper, we discuss the state of PHA and TAG production processes, namely how we turn bacterial carbon storage molecules into value-added products. Since the study of bacterial TAG production is still in its early stages, we offer some recent data in support of the quest to find an inexpensive process to produce the target molecule, with the goal of competing with petroleum products.

1. Polyhydroxyalkanoates

Ecology of valuable storage polyesters

Nutrient storage systems have evolved throughout nature as a stress survival mechanism. Prokaryotes can store carbon for later use in different forms, as glycogen [5], as polyhydroxyalkanoates (PHAs) [6], and as triacylgycerols (TAGs) [7]. In nature, organisms store carbon when other nutrients are in short supply (*i.e.* unbalanced growth) and utilize these stores in carbon-sparse conditions. Since early in history, humans have made use of other organisms' carbon stores as food (plant oils and starches), fuel (whale oil), cosmetics (coconut oil, palm kernel oil), and other applications. In recent times, we are turning to microorganisms to rapidly produce carbon storage products for our own use. One well-studied example of carbon storage molecules becoming value added products is the aforementioned PHA. Several species of microorganisms have been characterized to express PHA production enzymes [2,8,9]. Intracellular PHA stores assist in survival of the organism when nutrients are sparse. In some cases, free-living, PHA-producing bacteria can outcompete non-PHA producers for the same niche [10]. PHA is polymerized by microbial cells and stored in dense, protein-covered inclusion bodies termed granules. In general, there are two types of PHAs, based on the monomer content: short chain-length, or scl-PHA, containing the 4 carbon 3-hydroxybutyrate (3HB) and/or the 5 carbon 3-hydroxyvalerate (3HV) monomers; and medium chain-length, or mcl-PHA, containing monomers of chain lengths greater than 6 carbons, including 3-hydroxyhexanoate (3HHx, 6 carbons), 3-hydroxyoctanoate (3HO, 8 carbons), and other monomers of longer chain length than 3HHx. There are at least two distinct metabolic pathways for microbial PHA biosynthesis. For scl-PHA like polyhydroxybutyrate (PHB), two acetyl-CoA molecules are ligated to form acetoacetyl-CoA, and the acetoacetyl-CoA is reduced to form β-hydroxybutyryl-CoA. The β-hydroxybutyryl-CoA molecule then acts as a monomer substrate for the PHA synthase, and is incorporated into the nascent polymer chain by a thioesterase reaction taking place at the active site of the enzyme, and the Coenzyme-A is released [11]. Figure 1 shows a schematic of PHA production and intermediates starting with acetyl-CoA. The polymerization pathway shown here is typical for PHB production in the bacterium *Ralstonia eutropha* strain H16 (wild type), using a variety of carbon substrates. *R. eutropha* is a soil and fresh water dwelling bacterium that has been considered the model organism for PHA biosynthesis, as it can produce up to 80% of its cell dry weight as PHA during nitrogen limitation [6,12]. Monomers for PHA production can also be produced from intermediates of fatty acid β-oxidation. Fatty acid breakdown via β-oxidation produces 3-hydroxyacyl-CoA which can be used as a monomer for mcl-PHA production. However, since 3-hydroxyacyl-CoA, the β-oxidation intermediate, is the *(S)* form, it is unusable by the PHA synthase. The polymerizable *(R)*-3-hydroxyacyl-CoA is produced by conversion of enoyl-CoA using an *(R)*-specific enoyl-CoA hydratase, often termed PhaJ [13,14]. For both scl- and mcl-PHA, cells synthesize and store polymer in intracellular inclusion bodies, called granule, surrounded by proteins (Figure 2). These proteins facilitate PHA metabolism, protect the granule from coalescence, and separate the hydrophobic polymer from the aqueous cytoplasm. By far the most abundant protein present on the PHA granule is the phasin (PhaP1), so named because of its analogous function to olesins that surround TAG inclusion bodies in plants [15]. The PhaP1 phasin has been shown to cover anywhere from 27-54% of the PHA granule surface in *R. eutropha* [16]. Other granule associated proteins include: the PHA synthase, PhaC [17]; the regulatory protein, PhaR [12,18,19]; and depolymerase enzymes, PhaZs [20]. Figure 2 illustrates PHA granule formation in *R. eutropha.* Recently, additional granule associated proteins have been discovered in *R. eutropha* [21], including a dual-function granule associated protein that also binds to the nucleoid region of the cell [22]. This newly discovered protein, PhaM, appears to have functional homology to the lipid body associated protein TadA from *Rhodococcus opacus* [23]. PhaM was independently discovered by our laboratory (Cho, *et al*. manuscript in preparation) attached to residual PHB in a highly purified sample of epitope tagged PhaC protein, isolated from recombinant *R. eutropha.* The association of many proteins, each having different functions in the PHA production cycle, suggests that the PHA granule is a complex organelle that allows for optimal carbon sequestration and mobilization, depending on nutrient availability in the extracellular milieu [8].

Polyhydroxyalkanoates as a value added product: biodegradable plastics

Since before the first patents for commercial PHA production were issued to W.R. Grace and Company in the 1960's, many individuals have recognized the commercial potential of these biopolymers. Several types of PHA biopolymers have thermal and mechanical properties that rival those of petroleum-based thermoplastics. A summary of thermal and mechanical properties of various PHAs is found in Table 1. These PHAs can substitute for petrochemical plastics in many different applications. The company Metabolix, based in Cambridge, MA, USA, is currently the world's largest industrial producer of PHA (www.metabolix.com). Telles, a joint venture between Metabolix and Archer Daniels Midland, will produce large quantities of the copolymer poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate), or P(3HB-*co*-4HB), from corn sugar using engineered bacterial strains. Other PHA producing companies are in operation all over the world, such as Tianan Biologic Material in China and Biocycle in Brazil. Recently, a pilot scale bioplastic production plant was opened in Malaysia to produce PHA from palm oil products using engineered *R. eutropha* [24].

What makes a biopolymer suitable for industrial production? The PHA polymer must have favorable thermal and physical properties, so it can replace petroleum-based plastics like polypropylene. As seen in Table 1, the polyhydroxybutyrate (PHB) homopolymer is very stiff and brittle, suggesting a limited range of applications. PHA copolymers exhibit preferable properties (Table 1), likely due to decreased crystallinity as a result of interactions of two different chain-length monomers in the polymer [25]. Copolymers P(3HB-*co*-4HB), P(3HB-*co*-3HHx) (3HHx = 3-hydroxyhexanoate), and P(3HB-*co*-3HV) (3HV = 3-hydroxyvalerate) have been well-studies for their potential to replace petrochemical polymers in many different applications. These polymers are bio-based, biodegradable, and biocompatible [26,27,28,29,30]. Each type of copolymer is attractive as petroleum-based polymer substitutions, and large quantities are being produced commercially today.

Fermentative production of PHA copolymers: progress

Production of PHAs on an industrial scale requires that many challenges be overcome. Importantly, high cell density cultivation is a prerequisite to maximizing volumetric productivity of microbial fermentation. In many cases, an *Escherichia coli* strain is employed for polymer biosynthesis [31,32,33]. As wild-type *E. coli* is not capable of producing 3-hydroxyacyl-CoAs (3HA-CoA) de novo for polymer synthesis, the PHA production pathway must be supplied heterologously. Some advantages of using a recombinant *E. coli* strain are the rapid growth rate and the fact that PHA biosynthesis is not controlled by nutrient limitation in a recombinant strain, and thus cells will produce PHA concomitant with growth. An *E. coli* strain expressing PHA biosynthesis genes and overexpressing the cell division protein FtsZ, accumulated PHB to a concentration of 104 g/L in fed batch conditions [33]. A PHB concentration of >140 g/L was obtained using *E. coli* expressing PHA production genes from *Alcaligenes latus*, in fed batch culture [32]. The same strain was used in fed batch culture with propionic acid feeding and P(HB-*co*-HV) was produced with a productivity of >2.8 g/L/h [31]. Recombinant *E. coli* has also been utilized for P(HB-*co*-HHx) production, with final productivities of ~0.5 g PHA/L/h [34]. *R. eutropha* is also an attractive species for industrial PHA production. Since the bacterium is a native PHA producer, the cellular machinery and regulatory systems are already in place to produce large quantities of PHA. Also, *R. eutropha* is capable of utilizing a wide array of carbon sources for growth and polymer biosynthesis, including sugars [16,35,36], organic acids [37,38], plant oils and fatty acids [39,40,41,42,43], and CO_2 [44,45]. Thus, inexpensive feedstocks, such as agricultural and food processing wastes, unrefined natural products (*e.g.* plant oils), or concentrated CO_2, can potentially be used to produce large amounts of polymer at competitive prices. High productivity (*i.e.* space time yield) of PHA is critical in industrial polymer production. Over the past decade, researchers have made many attempts to increase yields of biomass and with it, PHA. While for TAG production (see below), culture carbon/nitrogen (C/N) ratios are paramount for maximizing productivities, high PHA productivity cultures can result from nitrogen or phosphate (or other nutrient) limitation in cultures. PHB productivities of over 1.0 g/L/h were observed when *R. eutropha* was grown in fed batch culture using corn steep liquor [46,47]. Two-stage culture systems have also been examined for maximization of PHB production by *R. eutropha*, where the initial stage served as cell growth, producing maximum biomass, and the second stage constituted PHB accumulation. These two stage cultures exhibited a maximum productivity of 1.2 g/L/h with >70% PHB per cell dry weight [48]. Typically with PHA production in *R. eutropha* strains, nitrogen plays the role of limiting nutrient to trigger polymer biosynthesis. High productivity has been seen using phosphate limitation, also, with >1.5 g PHB/L/h [49].

While high productivity PHB production is a good method by which to demonstrate that efficient fermentations can be performed using *R. eutropha*, PHA copolymers are the more ideal fermentation product, given their more favorable properties (Table 1). Using an alternating feeding of sugar and propionic acid in *R. eutropha* fed batch culture, Madden and Anderson [50] show that a P(HB-*co*-HV) productivity of >1.5 g/L/h can be obtained. The final 3HV content was ~7.5 mol% in this study [50]. Using a strain of *R. eutropha* expressing a heterologous PHA synthase (from *Aeromonas caviae*), P(HB-*co*-HHx) was produced using soybean oil as the sole carbon source, with a productivity of just over 1.0 g/L/h. In this case, the 3HHx fraction of the polymer was ~5 mol% [41]. Recently, a novel strain of *R. eutropha* was designed that expressed a heterologous PHA synthase (from *Rhodococcus aetherivorans*), as well as a *phaJ* gene (from *Pseudomonas aeruginosa*) for conversion of fatty acid β-oxidation intermediates into *(R)*-3HA-CoA substrates for PHA biosynthesis [24]. Growth of this strain in fed batch fermentations using palm oil as the sole carbon source and urea as the nitrogen source resulted in P(HB-*co*-HHx) biosynthesis with a productivity of > 1.0 g/L/h, and a 3HHx content of ~ 17mol%. In this work, PHA productivities of different feeding strategies were compared, with fed batch fermentation being the ideal fermentation procedure [43]. Other high density fermentations resulting in efficient production of P(HB-*co*-HHx) have been performed. Cultures of *Aeromonas hydrophila* produced P(HB-co-HHx) with a productivity of ~1.0 g/L/h, using fatty acids as the sole carbon source [51]. Large scale (20,000 L) fermentations were performed using *A. hydrophila* grown on glucose, with a P(HB-*co*-HHx) productivity of slightly greater than 0.5 [52]. In production of mcl-PHA, a productivity of 0.8 g PHA/L/h was observed in fed batch culture of *Pseudomonas putida* grown on mixed sugars [53]. A productivity of 2.0 was achieved with high density *P.*

putida cultures using phosphate limitation [54]. A summary of high yield PHA production studies from the current literature is shown in Table 2.

PHA production challenges

While researchers have shown robust, scalable PHA production in several systems, there are still challenges that must be overcome. The challenges largely relate to producing PHA in an inexpensive manner so the price of the final product competes with petrochemical plastics. First, readily available and inexpensive feedstocks must be used for carbon substrates in growth and PHA production. CO_2 is readily available and has been used as the sole carbon source for producing PHA [44,45,55,56], but concentrating CO_2 for use as carbon feedstock in an autotrophic fermentation, as well as the fermentation parameters themselves, present major challenges [55]. Recently, waste streams have been sought for use as nutrient sources in production of value added products, such as PHA [57,58,59,60,61] and TAGs [62]. Hassan and coworkers have constructed a method for producing PHA from organic acids resulting from digestion of processing sludge from palm oil mill effluent [60]. Polymer has also been produced from pure cultures using whey [61,63], beet molasses [64], inedible jatropha oil [42], and waste glycerol [58]. Many studies have been performed on PHA production using mixed cultures with bacterial species and strains often obtained from the same waste streams [65,66]. This production process typically involves enriching for PHA-producing cultures of microorganisms and propagating stable cultures before harvesting polymer [65]. Enriching a mixed culture for PHA producers can be performed by a microaerophilic-aerobic system, controlling oxygen content of the culture to select for PHA accumulating bacteria over those that accumulate glycogen [66,67,68,69] or "feast/famine" cycling (a.k.a. aerobic dynamic feeding), where PHA accumulating organisms are selected on the basis of their ability to utilize polymer as a nutrient

[66,70]. The advantage to mixed cultures is that cultivation conditions do not necessarily have to be sterile, which will save on energy costs. Feedstocks also do not need to be pure, although acetate is often used as a carbon source to enrich for PHA producing bacteria [65,66]. However, there are challenges associated with mixed culture PHA production, including development of culture selection strategies towards higher PHA yields and productivities [65,66,71]. Since mixed cultures involve wild-type and some unknown or uncharacterized organisms, the type of PHA produced (*e.g.* PHB) may not be ideal for most applications, and the outcome of the process is at the mercy of the microbial input.

There are other downstream challenges for industrial PHA production. The harvesting of biopolymer from cells presents problems in the formulation of a cost-effective production process. Many different chemicals have been tested for polymer recovery, including NaOH [72], sodium hypochlorite [73], chloroform [74], methyl ethyl ketone (MEK), methyl isobutyl ketone (MIBK), ethyl aetate [25,75], and aqueous detergent solutions [76,77]. The most ideal compounds for PHA recovery are those that can be recycled, reused and easily separated from aqueous solutions. Of the chemicals listed above, MEK and MIBK show the most promise in recovering highly pure PHA from biomass (Riedel, *et al*., manuscript in preparation). Not all solvents will successfully recover all types of PHA. For example, MIBK is better suited for PHA containing longer chain-length monomers, and less effective with PHB (data not shown). Other recovery methods, such as: enzymatic digestion [78], controlled autolysis [79], and dissolved-air flotation [80], have been performed to extract PHA from biomass. It is doubtful that any of these alternative recovery methods would be preferable in an industrial setting.

Polyhydroxyalkanoates – outlook

Currently, PHAs are on the market as renewable, biodegradable alternatives to conventional plastic. PHA is being used in many household, industrial, and medical applications. Although PHA produced in large quantities is relatively inexpensive, it is still costlier than petroleum-based plastic. Production of PHA using waste streams, such as agricultural waste, milling waste, food processing waste or even concentrated CO_2 emissions, will potentially help drive down costs and make PHA a more economically competitive polymer, compared to the traditional plastics. Furthermore, environmentally conscious methods of polymer recovery from cells, *i.e.* use of non-halogenated and recyclable solvents, are required to make bioplastic production a greener process. Since polyhydroxyalkanoates can be tailor-made to exhibit similar properties to petroleum-based plastic, a robust and cost-effective production process is needed to compete in the current plastics market. In some cases, this type of process is already in practice, advancing the biplastics industry on a global scale.

2. Triacylglycerols

Triacylglycerols (TAGs) are storage lipids with a neutral and nonpolar nature that allows them to be stored in anhydrous environments, and the major storage molecules of fatty acids for energy utilization and the synthesis of membrane lipids in living organisms [81]. TAGs are esters in which three molecules of fatty acids are linked to glycerol, and these fatty acids may be all the same kind, all different kinds, or only two the same, and may include saturated or unsaturated fatty acids. The chain lengths of the fatty acids in naturally-occurring TAGs vary, but most contain 16 or 18 carbon atoms. Natural fatty acids found in animals and plants are typically composed of only even numbers of carbon atoms, reflecting the pathway for their biosynthesis from the two-carbon building-block acetyl CoA [82,83]. Bacteria, however, possess the ability to synthesize odd- and branched-chain fatty acids [4]. The physicochemical properties of TAGs depend on the nature of the fatty acids present, chain length and the degree to which their fatty acids are desaturated. TAGs have been exploited in versatile materials, such as oleochemicals, cosmetics and food applications, and have furthermore recently garnered attention due to an increasing interest in alternative fuels [84].

Triacylglycerols for biofuel production

It is known that the fatty acyl chains of TAGs are chemically similar to the aliphatic hydrocarbons that make up the bulk of the molecules found in gasoline and diesel [85,86]. Vegetable oil, composed primarily of triacylglycerols, was used to run the early diesel engines when it was invented over 120 years ago. With the advent of inexpensive and abundant petroleum, the development of the diesel engine has been based on the efficacy of petroleum-derived diesel fuel, and vegetable oil as a fuel source was sidelined for decades. In 1973, the Arab oil embargo signaled the start of a new era of petroleum shortages. Suddenly, with a four-fold increase in petroleum prices, the international interest in biofuels has since been rejuvenated. Since 1973, much of the development of alternative bio-based fuels was being enhanced in countries that have little to no internal petroleum resources [87]. Then, between 2003 and 2008, the price of oil steadily rose. The price of a barrel of crude oil on the New York Mercantile Exchange was $30 in 2003, reached $60 by 2005, and peaked at $147 in 2008 (http://tfc-charts.com/chart/QM/W). Thus, the instability of petroleum fuel costs, depleting petroleum reserve and heightened concern about the effects of increasing atmospheric CO_2 levels are intensifying the research for renewable biofuels that could reduce our current consumption of fossil fuels [88]. In the last few decades, efforts in the development of bio-ethanol as an alternative fuel have resulted in significant success [89]. However, bio-ethanol has some limitations, such as low energy density, corrosiveness and high vapor pressure, which prevent its widespread utilization given the existing infrastructure [90]. One possible solution to the issue can be the exploitation of TAGs for the production of lipid-based biofuels. Different TAG-based bioprocesses can generate biofuels with different compositions and properties [84]. A representative of those is biodiesel, which is typically manufactured by transesterification of TAGs with an alcohol, usually methanol, in the presence of an alkaline catalyst and therefore constitutes monoalkyl esters of long-chain fatty acids such as fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs). From the mid-1980s to early 2000s, most of the research on biodiesel production has focused on vegetable oils from oleaginous plants [91]. It has been reported that using raw vegetable oils in diesel engines leads to the progression of many engine-related problems such as deposits, injector coking and piston ring sticking, and these effects can be reduced or eliminated through transesterification of vegetable oil to form methyl or ethyl esters [92]. Most of the biodiesel that is currently produced uses a varied range of vegetable oils (edible and non-edible), animal fats, used frying oil and waste cooking oil. Detailed reviews about biodiesel production are available in the literature [87,91,93,94,95]. The biodiesel has environmental advantages, such as low amounts of suspended particulate matter and low levels of sulfur dioxide in emissions when burned, and can be used in most diesel engines with little or no modification. However, some physical limitations have been pointed out when these molecules are used as the sole fuel and not as a blendstock due to the cold-flow properties [94,95,96], and reliable implementation standards from public and government agencies are still lacking. Another kind of biofuel, probably best termed “renewable diesel”, which is produced from TAGs by a hydrodeoxygenation reaction in the presence of a catalyst, has been garnering much attention since the early 2000’s [95]. The notable advantage of the process is its feedstock flexibility, showing that renewable diesel can be processed from a great variety of TAG-containing feedstocks — weedy plants, animal fats, waste oils, algae and oleaginous microorganisms [97]. TAGs are converted to products such as kerosene, gasoline, jet and diesel fuels all comprising paraffinic hydrocarbons whereby the hydrocarbon chain length is controlled to provide a distribution that is identical in virtually all respects to commercially available petroleum-derived fuels [98]. Recently, an F-22 Raptor successfully flew at supercruise (*i.e.* supersonic speed without using afterburners) on a 50/50 blend of hydrotreated renewable jet fuel and conventional petroleum-based JP-8 [99]. From these points of view, presently, energy-rich TAG molecules have attracted great attention for developing environmental-friendly and high-quality lipid-based biofuels.

Triacylglycerol production in bacteria

TAG biosynthesis is widely distributed in nature and the occurrence of TAG as reserve compounds is widespread among plants, animals, yeast and fungi. In contrast, however, TAGs have not been regarded as common storage compounds in bacteria. Biosynthesis and accumulation of TAGs have been described only for a few bacteria belonging to the actinomycetes group, such as genera of *Streptomyces*, *Nocardia*, *Rhodococcus, Mycobacterium*, *Dietzia* and *Gordonia*, and, to a minor extent, also in a few other bacteria, such as *Acinetobacter baylyi* and *Alcanivorax borkumensis* [3,100]. The presence of TAGs as vacuoles in bacteria has already been reported in *Mycobacterium* and *Streptomyces* in the 1940s to 1960s [4]. The systematic study on the formation of TAGs during growth has been reported in the 1990s with *Streptomyces* sp [101]. Since the mid-1990's, TAG production in hydrocarbon-degrading strains of those genera has been frequently reported [102]. TAGs are stored in spherical lipid bodies as intracellular inclusions, with the amounts depending on the respective species, cultural conditions and growth phase. Commonly the important factor for the production of TAGs is the amount of nitrogen that is supplied to the culture medium. The excess carbon, which is available to the culture after nitrogen exhaustion, continues to be assimilated by the cells and, by virtue of oleaginous bacteria possessing the requisite enzymes, is converted directly into lipid. The compositions and structures of bacterial TAG molecules vary considerably depending on the bacterium and on the cultural conditions (especially carbon sources). The pioneering work of TAG production in bacteria has been published by Steinbüchel *et al.* [3,4]. In recent years, aspects of the physiology and biochemistry of bacterial TAG accumulation, and the molecular biology of the lipid inclusion bodies are being investigated by many researchers [62,103,104,105].

Triacylglycerol production of *Rhodococcus opacus* PD630

Many bacterial species do not usually accumulate significant amounts of TAGs, and the content is generally about 20-40% of dry mass. Among the oleaginous bacteria, Alvarez and coworkers have demonstrated that *R. opacus* PD630 (DSMZ 44193) grown on defined medium containing olive oil is capable of accumulating TAGs accounting for up to 87% of the cell dry weight (CDW) [7]. The strain was isolated from a soil sample collected at a gas-works plant in Germany, as an oleaginous hydrocarbon-degrading bacterium, and was also able to grow on long-chain-length alkanes, gluconate, acetate, fructose, propionate, phenyldecane and phenylacetic acid (among others) and could produce remarkably high amounts of TAGs intracellularly; more than the other bacteria when the cells were cultured on similar substrates under nitrogen-limiting conditions. It has been reported that *R. opacus* PD630 cultivated in fed-batch conditions on media containing sucrose and sugar beet molasses reached a cell density of 37.4 g^{-1} CDW with fatty acid content of 51.9% of the CDW, suggesting that the fermentation on carbon sources from agricultural products can be applied for the biotechnological production of

TAGs [106]. In order for "Second generation biofuel technologies" to be produced in a sustainable manner and to avoid the food-fuel conflict, lignocellulosic biomass must be developed as feedstocks for TAG production [107,108]. Lignocellulosic biomass embraces cellulose, a glucose polymer. However, TAG production of *R. opacus* PD630 on glucose as a carbon source had not been shown until recently, when we discovered that *R. opacus* PD630 has the rare capability of accumulating large amounts of TAGs in batch-cultivation containing high concentrations of glucose under defined conditions [109]. Hereinafter, we briefly describe the notable capability of this strain of establishing a cost-effective "consolidated bioprocess" for TAG production from lignocellulosic biomass.

Fermentation of *R. opacus* PD630 with high glucose concentrations

High-cell-density cultivation is a prerequisite to maximizing volumetric productivity of microbial fermentation [110]. In this case, the successful execution of the fermentation depends on the ability of the bacterial strain used to deal with stress imposed by high sugar concentrations [111]. The growth kinetics of *R. opacus* PD630 in flask cultures on a defined medium with initial glucose concentrations of 200, 250, 300 and 350 g l^{-1} were examined at an initial inoculum of 1.0 OD_{660}. The strain grew well on media containing up to 300 g l^{-1}, reaching stationary phase after 48 h on 200 g l^{-1}, 72 h on 250 g l^{-1} and 96 h on 300 g l^{-1}, although growth was inhibited at the highest glucose concentration of 350 g l^{-1}, as shown in Figure 3. Thus, *R. opacus* PD630 can be an idealized candidate for industrial fermentations in which high concentrations of glucose are utilized, whereas our preliminary studies have shown the presence of high concentrations of $(NH_4)_2SO_4$ in the media results in a concomitant decrease of the storage of TAG in the cells. Previous reports have demonstrated that carbon storage in various bacteria is heavily influenced by the ratio of carbon to nitrogen (C/N). The effects of altering the C/N ratio on TAG production of *R. opacus* PD630 were tested at the combination of various concentrations of glucose and $(NH_4)_2SO_4$, varying the amounts of glucose and $(NH_4)_2SO_4$ in the medium from 5 to 60 g l^{-1}, 0.3 to 2.8 g l^{-1} respectively. As shown in Figure 4, when the $(NH_4)_2SO_4$ concentration was increased from 0.3 to 1.4 g l^{-1} at concentrations of glucose from 20 to 40 g l^{-1}, the CDW and fatty acid production proportionally increased from 1.9-2.2 to 7.8-9.2 g l^{-1} and 1.0-1.1 to 4.0-4.9 g l^{-1}, respectively, corresponding to a cellular fatty acid content of 50-55% CDW. Meanwhile, when the $(NH_4)_2SO_4$ concentration of the medium was increased further, to 1.7 g l^{-1}, the CDW and the fatty acid content decreased to 58-73% and 52-59%, respectively, of those of the 1.4 g l^{-1} $(NH_4)_2SO_4$ cultures, resulting in a 57-67% drop in fatty acid production. When the final pH of the culture was measured, increasing $(NH_4)_2SO_4$ concentrations corresponded to lower final pH. The broth supernatants of *R. opacus* PD630 grown in a defined medium containing more than 1.7 g l^{-1} $(NH_4)_2SO_4$ had a final pH value of 4.2-4.8. The results suggested that a decrease in pH inhibited growth and consequently decreased lipid production when *R. opacus* PD630 was grown under uncontrolled pH cultivations. Batch-fermentations under controlled pH conditions allowed for an increase of the concentrations of glucose and $(NH_4)_2SO_4$ in the medium, resulting in a dramatic increase in TAG production [109]. When the critical operational C/N ratio for maximum production of fatty acids was optimized using a response surface methodology based on the Box-Wilson Central Composition Design, the design predicted that growing *R. opacus* PD630 in a defined medium with a C/N of 17.8 containing 240 g l^{-1} glucose and 13.4 g l^{-1} $(NH_4)_2SO_4$ would result in the maximum production of 25.1 g l^{-1} of fatty acids (Figure 5). *R. opacus* PD630 grown in batch-fermentations with the predicted optimal conditions yielded 25.2 g l^{-1} of fatty acids corresponding to 38% of the cell dry weight after 147 h of cultivation. The accumulated fatty acids in the cells consisted primarily of palmitic acid (28%), oleic acid (25%)

and *cis*-10-heptadecenoic acid (16%). These results suggest that *R. opacus* PD630 has great potential as a TAG producer for developing industrial lipid-based biofuels on starchy lignocellulosic biomass that consist primarily of glucose polymers.

Fermentation of *R. opacus* PD630 on starchy lignocellulosic biomass-derived sugars

The growth and lipid accumulation properties of *R. opacus* PD630 on saccharified solutions derived from corn silage were investigated. The corn silage homogenized by acid treatment was provided by Sweetwater Energy Inc. (Rochester, NY, USA). The undigested feedstock was adjusted to a pH of 5.0 and commercial enzymes (Novozymes, Bagsvaed, Denmark) consisting of 2 ml Viscozyme L and 0.5 ml Celluclast were added into 100 ml of the suspension containing the silage of 67 g l^{-1} as the dried mass. The saccharification was performed at 45^{o}C with a rotational speed of 200 rpm. After 72 h of incubation, as shown in Figure 6, the sugar of the hydrolysate was composed of 32.2 g l^{-1} glucose, 3.1 g l^{-1} xylose, 0.7 g l^{-1} arabinose and 3.6 g l^{-1} other unidentified sugars, indicating that we are able to convert approximately 50% of the feedstock to monosaccharides. As it is known that the feedstock contains large quantities of starch, 0.5 ml of glucoamylase (AMG 300L, Novozymes) was added into 100 ml of the feedstock in a separate treatment, and the suspension was incubated at 45^{o}C for 72 h at 200 rpm (data not shown). The HPLC data of the hydrolysate showed the presence of glucose as a principal sugar with more than 96% selectivity. Considering that 28 g l^{-1} of glucose was detected in the supernatant when glucoamylase alone was added into the feedstock suspension (67 g l^{-1}) and saccharified, it appeared that the feedstock contained approximately 35% starch.

The effects of concentrations of the saccharified corn silage solution on growth of *R. opacus* PD630 in flasks were tested. Depending on the desired conditions, the saccharified solution after

72 h of incubation shown in Figure 6 was adjusted to pH 7.2 with 1 M NaOH and either diluted with deionized water or concentrated by freeze-drying. In examination of cell growth on the saccharified solution, use of a 100% stock for feeding resulted in growth inhibition, thus we used diluted stocks of 1:1 saccharified solution/water (termed “50%”) or 3:1 saccharified solution/water (termed “75%”). As shown in Figure 7, PD630 started growing in a defined medium containing 18 g l^{-1} glucose and 1 g l^{-1} $(NH_4)_2SO_4$ and in media containing 50% (20 g l^{-1} of fermentable sugars) and 75% (30 g l^{-1} of fermentable sugars) levels of the saccharified solution after 24 h cultivation, and in undiluted solution (40 g l^{-1} of fermentable sugars) after 72 h cultivation. Use of a concentrated solution (50 g l^{-1} of fermentable sugars) resulted in a growth inhibitory effect on *R. opacus* PD630 (data not shown). The results suggested that the hydrolysate of corn silage apparently contains certain growth inhibitory compounds, as *R. opacus* cells are typically capable of growth using sugars as carbon sources. In addition, a clear difference in the percentage of fatty acids per CDW was observed between the defined medium and the saccharified solution fermentation. In the fermentation of defined medium containing 18 g l^{-1} glucose and 1 g l^{-1} $(NH_4)_2SO_4$, the fatty acid content was more than 50% of the CDW at the stationary phase of growth after 96 h of cultivation; whereas in fermentation of the 50% saccharified solution composed of 16.1 g l^{-1} glucose and 3.7 g l^{-1} other sugars, the fatty acid content represented 34.3 (±4.0) % of CDW at 96 h of cultivation and gradually decreased after reaching the maximum fatty acid accumulation. This result using the saccharified solution suggests that the C/N ratio of the solution was unbalanced, with an excess of nitrogen source over carbon source.

The effect of the addition of glucose on lipid production by *R. opacus* PD630 grown on the saccharified solution in flasks was also examined. PD630 was grown on the 75% saccharified solution supplemented with 10, 20 or 30 g l^{-1} of glucose, or without, and a time course of fatty acid production in flask cultures was investigated. As shown in Figure 8, fermentations following amendment with additional glucose into the 75% solution containing 30 g l^{-1} of sugars resulted in a significant increase in the fatty acid production and the fatty acid content of the CDW as compared with that on the solution without additional glucose. After 120 h of cultivation, the CDW and the fatty acid production of PD630 grown in the solution without additional glucose were 14.2 (±1.3) g l^{-1} and 4.4 (±0.7) g l^{-1} representing a fatty acid content of 31.0 (±2.0) % CDW, respectively, whereas the CDW of 16.9 (±1.1) g l^{-1} and the fatty acid production of 8.9 (±0.3) g l^{-1} corresponding to a fatty acid content of 52.7 (±2.1) % CDW were maximum when 20 g l^{-1} of glucose was added into the solution. The fatty acid content in the solution supplemented with 20 g l^{-1} of glucose were equal to that [55.0 (±3.0) % CDW] in a defined medium containing 18 g l^{-1} glucose and 1 g l^{-1} $(NH_4)_2SO_4$ (Figure 5). The identity of the lipids and the fatty acid composition profile of *R. opacus* PD630 grown in the saccharified solutions were very similar to those of the strain grown in the defined medium (data not shown). Figure 9 shows lipid body morphology of *R. opacus* PD630 grown in a 75% saccharified silage solution supplemented with 20 g l^{-1} of glucose for 120 h in flask cultures. *R. opacus* PD630 grown in the saccharified solution has a multitude of small lipid bodies which almost completely fill the cytoplasm of the cells, which is similar to what is seen in cells grown in defined medium.

These results indicated that saccharified solution from corn silage contains sufficient nutrients for TAGs production by *R. opacus* PD630, although the carbon to nitrogen ratio (C/N) in the solution composition is optimized for high lipid production.

Perspectives of bacterial TAG for lipid-based biofuels

Many countries are navigating their attention to the development of clean and sustainable energy sources [88,112]. Among the various feasible sources of renewable energy, advanced liquid (lipid-based) fuels such as biodiesel and bio-jet fuel are of greatest interest and are expected to play a crucial role in the global energy infrastructure in the future [94,95,96]. TAGs are utilized as precursors for the production of lipid-based biofuels, and currently the main sources for TAG are vegetable oils, animal fats or waste cooking oils. The biofuels produced from crop seeds have come under major scrutiny due to the food vs. fuel competition problem [107]. Microalgae are currently viewed as an attractive feedstock for lipid-based fuels due to their ability to produce substantial amounts of TAG. However, current studies with TAG production by microalgae, using the best available strains and cultivation methods, have resulted in considerably lower yields than the theoretical maximum [113,114,115]. Presently, the limited supply of bioresources for obtaining TAGs is a major bottleneck for production of lipid-based biofuels, in spite of the favorable impacts that commercialization of TAG-based biofuels could provide. One alternative method to produce TAGs is to utilize heterotrophic organisms which produce TAGs from lignocellulose-derived sugars. Consequently, bacteria are now being considered as one of the more promising TAG sources for producing lipid-based biofuels since they have the following favorable qualities: a fast growth rate, ease of culturability, and the property of being a renewable source of biomass [100,104,116,117]. Whereas the accumulation of TAGs is a characteristic of few bacteria, one of particular interest would be *R. opacus* PD630, where intracellular lipid contents can reach more than 70% of the total cellular dry weight in cells grown on gluconate or olive-oil as the sole carbon source under growth-restricted conditions. Research on this particular strain has been ongoing since the mid-1990’s [7]. Recently, it has been demonstrated that *R. opacus* PD630 has an acceptable feasibility for industrial fermentations in which high concentrations of glucose are used, as described above. There are global research efforts concerning the accumulation of TAGs in bacteria, and the expression and activities of many genes related to fatty acid synthesis are newly understood as of today [118,119,120,121,122]. It would seem easy to modify a bacterium's performance to improve its TAG accumulation on lignocellulosic biomass and establish a cost-effective consolidated bioprocess. However, there are some difficulties that have hindered the achievement of lower production costs on the large scale. Lignocellulose is an abundant and underutilized renewable feedstock, but it is a complex of rigid cellulose fibers embedded in a cross-linked matrix of lignin and hemicellulose that bind the fibers. For the conversion of lignocellulosic biomass to TAGs, the cellulose and hemicelluloses must be broken down into their corresponding monosaccharides so that bacteria can utilize them for growth and lipid production [123,124,125]. One of the major challenges to overcome is the presence of cell growth inhibitors generated during the treatment step of lignocellulosic biomass [126]. The presence of lignin in lignocellulosic hydrolysates leads to growth inhibition of *R. opacus*. It has been reported that *R. opacus* PD630 is able to break down some lignin-derived compounds, whereas Figure 7 shows that certain components of a relatively high concentration, probably lignin, present in the lignocellulosic hydrolysate inhibits cell growth [127]. Higher starting sugar concentrations in the medium result in maximizing the volumetric productivity and the efficiency of the fermentation, resulting in a lower cost process. At present, it might be difficult to prepare a lignocellulose-derived sugar solution greater than 200 g/L without the overabundance of growth inhibitors in a cost-effective manner [128]. To alleviate this inhibition problem, genetic engineering could be employed to provide increased lignin tolerance to *R. opacus* PD630. A combination of inhibitor-tolerant strains along with the desired properties for detoxification of lignocellulose hydrolysates will likely improve of lignocellulose-to-TAG production process. Furthermore, genetic modifications should be investigated to extend the substrate utilization range of the strain, which could permit the utilization of cheaper, renewable substrates for biosynthesis of TAGs.

Bacterial TAG production at industrial scale is not economically feasible as of now due to its high processing costs and low productivity of the bioprocesses. To overcome these obstacles, highly productive strains need to be engineered and optimized for high oil productivity on lignocellulose-derived sugars, and development of effective and economical cultivation systems as well as separation and harvesting of biomass and oil will have to be investigated intensively. We believe that an engineered *R. opacus* strain can contribute to the ultimate goal of producing scalable and cost-effective advanced liquid biofuels.

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Tables

Table 1: Thermal and mechanical properties of PHA polymers and petrochemical polymers.

Polymer	T_m (°C)	T_g (°C)	Young's Modulus (GPa)	Tensile Strength (MPa)	Elongation to break (%)	Reference
PHB	177	4	3.5	43	5	[129,130]
P(HB-*co*- 10mol% HHx)	127	-1	nd[a]	21	400	[129]
P(HB-*co*- 12mol% HHx)	103	-2	0.5	10	130	[131]
P(HB-*co*- 15mol% HHx)	115	0	nda	23	760	[129]
P(4HB)	53	-48	0.15	104	1000	[132]
P(3HB-*co*- 12 mol% 4HB)	124	-4	0.54	25	630	[131]
LDPE[b]	130	-30	0.2	10	620	[130]
Polypropylene	176	-10	1.7	38	400	[130]
Polystyrene	240	100	nd[a]	nd[a]	nd[a]	[130]

[a]nd = not determined in the indicated study
[b]Low density polyethylene

Table 2: Summary of high PHA yield fed batch fermentation studies

Production organism	Polymer produced	Carbon source	Biomass yield (g/L)	PHA yield (g/L)	Productivity (g/L/h)	Reference
E. coli	PHB	glucose	194.1	141.6	4.6	[32]
R. eutropha	PHB	glucose	164.0	121.0	2.4	[46]
R. eutropha	PHB	corn steep liquor	281.0	232.0	3.1	[47]
R. eutropha	PHB	glucose	208.0	139.0	3.1	[49]
E. coli	PHB	glucose	149.0	104.0	2.1	[33]
R. eutropha	P(HB-*co*-HV)	glucose and propionic acid	84.0	65.5	1.6	[50]
Aeromonas hydrophila	P(HB-*co*-HHx)	oleic acid	95.7	43.2	1.0	[51]
Aeromonas hydrophila	P(HB-*co*-HHx)	glucose and lauric acid	50	25	0.5	[52]
E. coli	P(HB-*co*-HHx)	dodecanoic acid	79.0	21.5	0.5	[34]
R. eutropha	P(HB-*co*-HHx)	palm oil	140.0	104.0	1.1	[43]
Pseudomonas putida	mcl-PHA	sugar cane carbohydrates	50.0	31.5	0.8	[53]
Pseudomonas putida	mcl-PHA	oleic acid	141.0	72.6	1.9	[54]

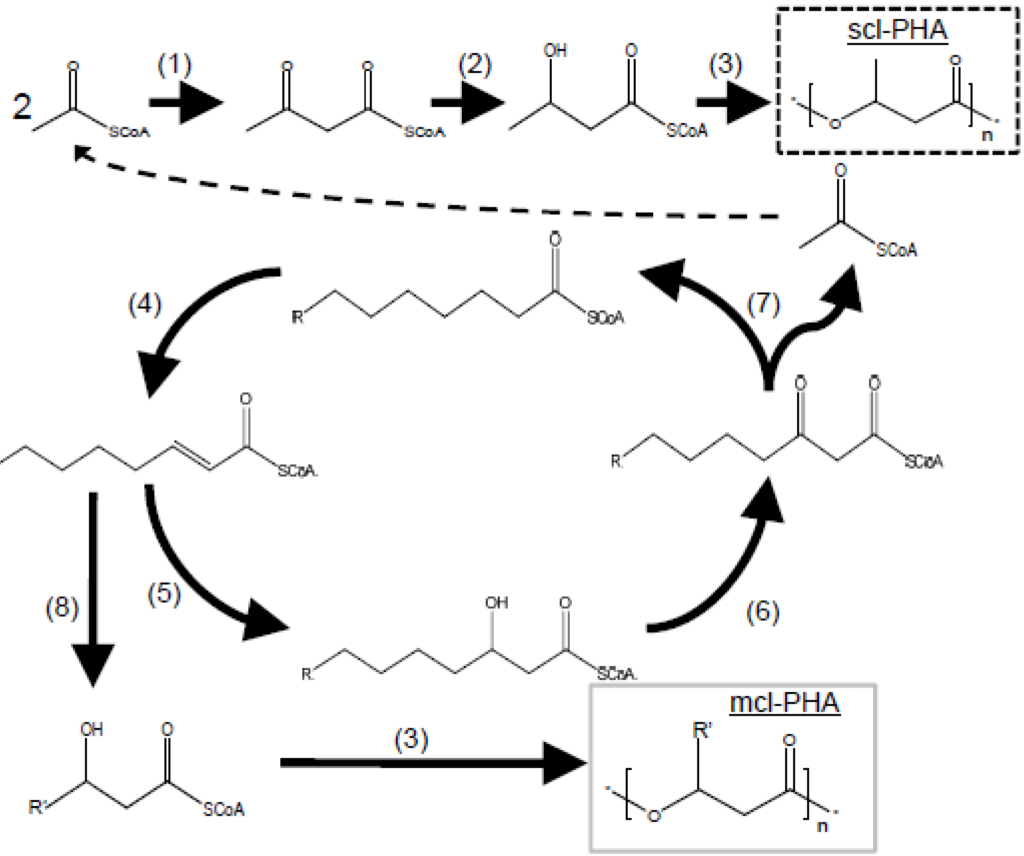

Figure 1: A schematic of microbial PHA production pathways. For scl-PHA, 2 molecules of acetyl-CoA are ligated by a β-ketothiolase (1) to form acetoacetyl-CoA, which is reduced by acetoacetyl-CoA reductase (2) to form 3-hydroxybutyryl-CoA (3HB-CoA). The 3HB-CoA is polymerized by a PHA synthase enzyme (3) to produce PHB (black dashed box). Acetyl-CoA for PHB biosynthesis can be produced by a turn of the fatty acid β-oxidation cycle (enzymes 4-7). Substrates for mcl-PHA can also come from β-oxidation, via an *(R)*-specific enoyl-CoA hydratase (8). These medium chain-length hydroxyacyl-CoA molecules are polymerized by a PHA synthase (3) to produce mcl-PHA (grey box). Medium chain-length monomers can also be produced through fatty acid biosynthesis (not shown). Enzyme designations: fatty acyl-CoA dehydrogenase (4), 2-enoyl-CoA hydratase (5), 3-hydroxyacyl-CoA dehydrogenase (6), β-ketothiolase (7).

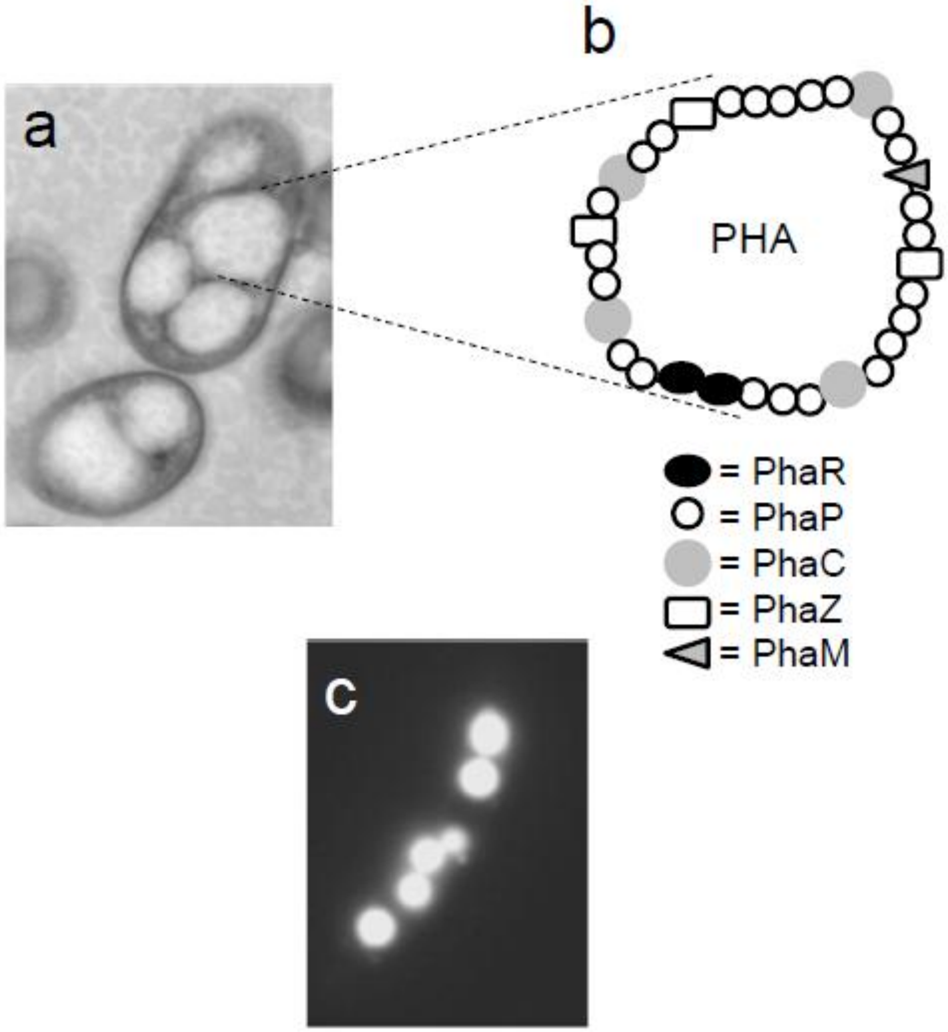

Figure 2: (A) Transmission electron micrograph of *R. eutropha* H16 cells containing PHA granules. (B) Schematic representation of a PHA granule in *R. eutropha*. Enzyme designations: PhaR = Regulator of PhaP expression; PhaP = Phasin protein; PhaC = PHA synthase; PhaZ = PHA depolymerase; PhaM = New DNA-binding, granule associated protein, as described in [22]. (C) Fluorescent micrograph of *R. eutropha* Re2058/pCB113 [24] grown for 24 h in minimal (PHA production) medium with palm oil as the sole carbon source [43]. P(HB-*co*-HHx) granules are stained with Nile red.

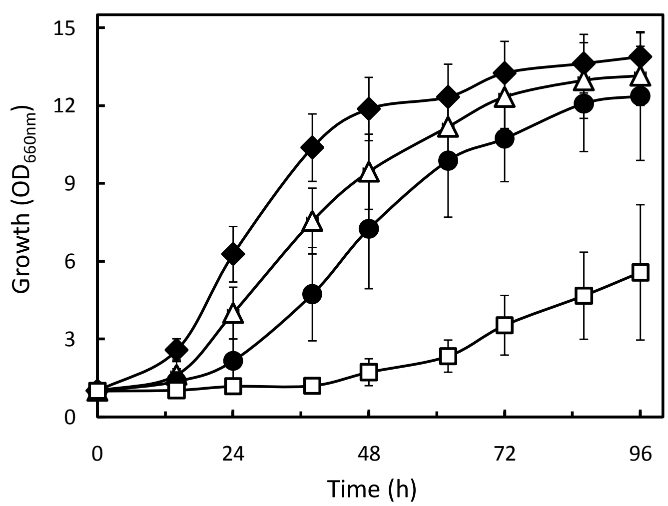

Figure 3: Growth of *R. opacus* PD630 on high glucose concentrations in flask cultures. Glucose concentrations tested in defined medium containing 1.4 g l^{-1} $(NH_4)_2SO_4$ were 200 g l^{-1} (◆), 250 g l^{-1} (△), 300 g l^{-1} (●) and 350 g l^{-1} (□). Initial inoculum density was adjusted to obtain an OD_{660} of 1.0. The error bars represent the standard deviation of three separate replicates of each experiment.

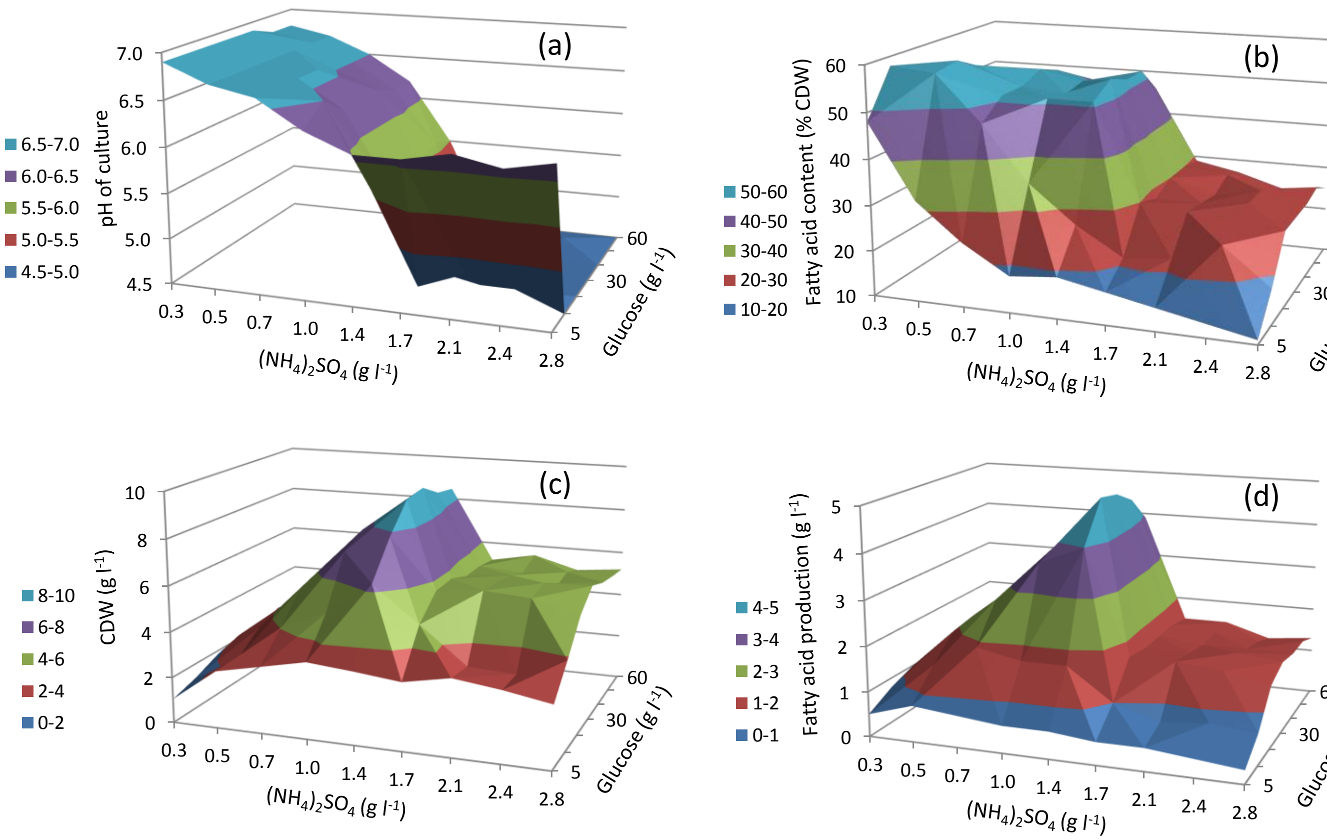

Figure 4: The effects of glucose and $(NH_4)_2SO_4$ concentrations on pH of culture (a), fatty acid content (b), CDW (c), and fatty acid production (d) by *R. opacus* PD630 in flask cultures.

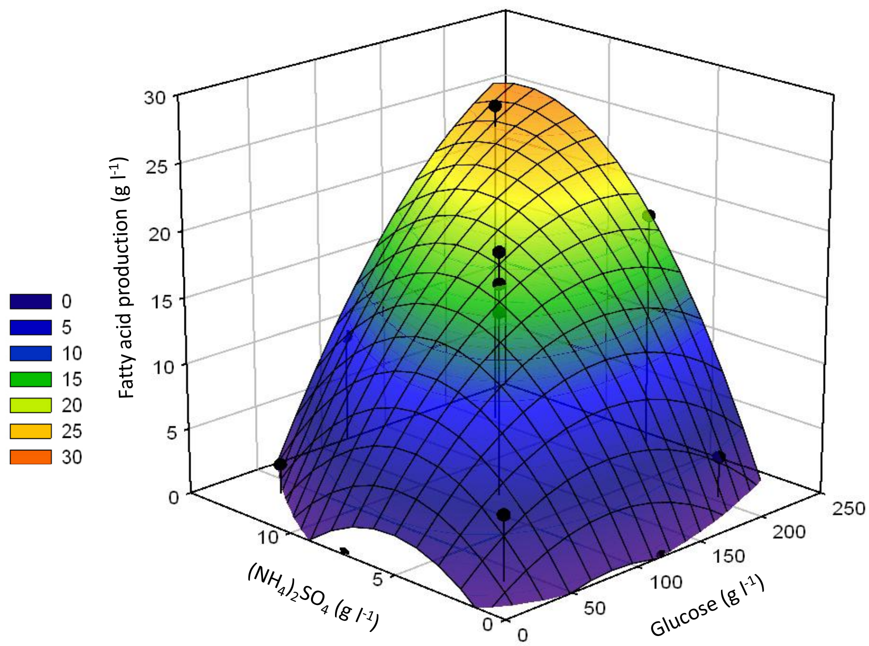

Figure 5: The response surface curve of the effect of glucose and $(NH_4)_2SO_4$ concentrations on fatty acid production by *R. opacus* PD630 in batch-culture fermentations (Curves: predicted value; points: experimental data).

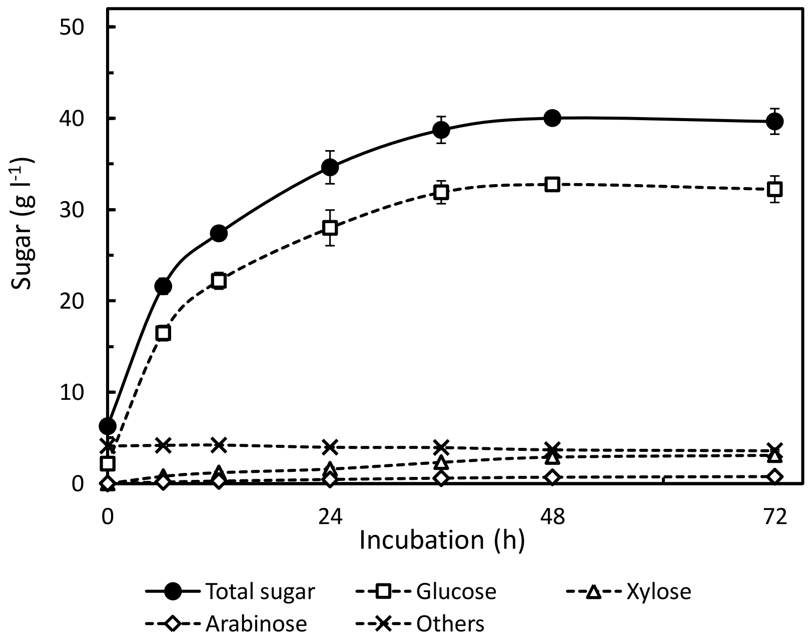

Figure 6: Saccharification of corn silage by commercial enzymes. The homogenized feedstock (67 g l^{-1} of dried material) was adjusted to pH 5.0, and Novozymes (2 ml Viscozyme and 0.5 ml Celluclast) were added into 100 ml of the suspension, and hydrolyzed at 45°C at 200 rpm. The error bars represent the standard deviation of three independent replicates.

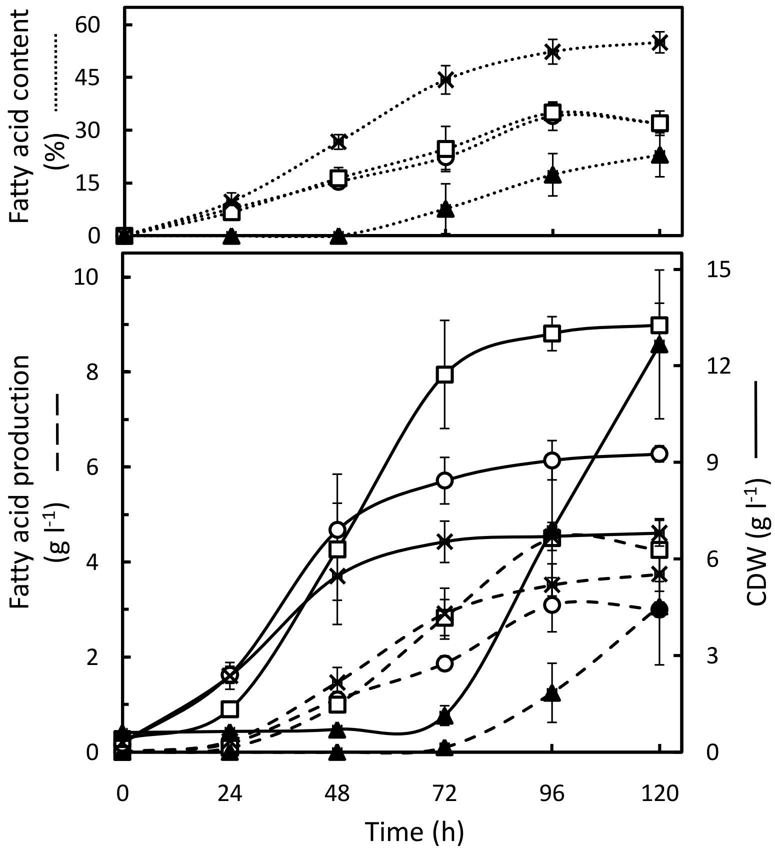

Figure 7: Growth and lipid production of *R. opacus* PD630 on various concentrations of the saccharified corn silage solution in flasks. The strain was inoculated in the saccharified 50% (○), 75% (□) and 100% (▲) solutions and a defined medium (x) containing 18 $g\ l^{-1}$ glucose and 1 $g\ l^{-1}$ $(NH_4)_2SO_4$ at an initial OD_{660} of 0.3. A saccharified stock diluted 1:1 with water is termed "50%," and a saccharified stock diluted 3:1 with water is termed "75%" (see text). The error bars represent the standard deviation of three independent replicates.

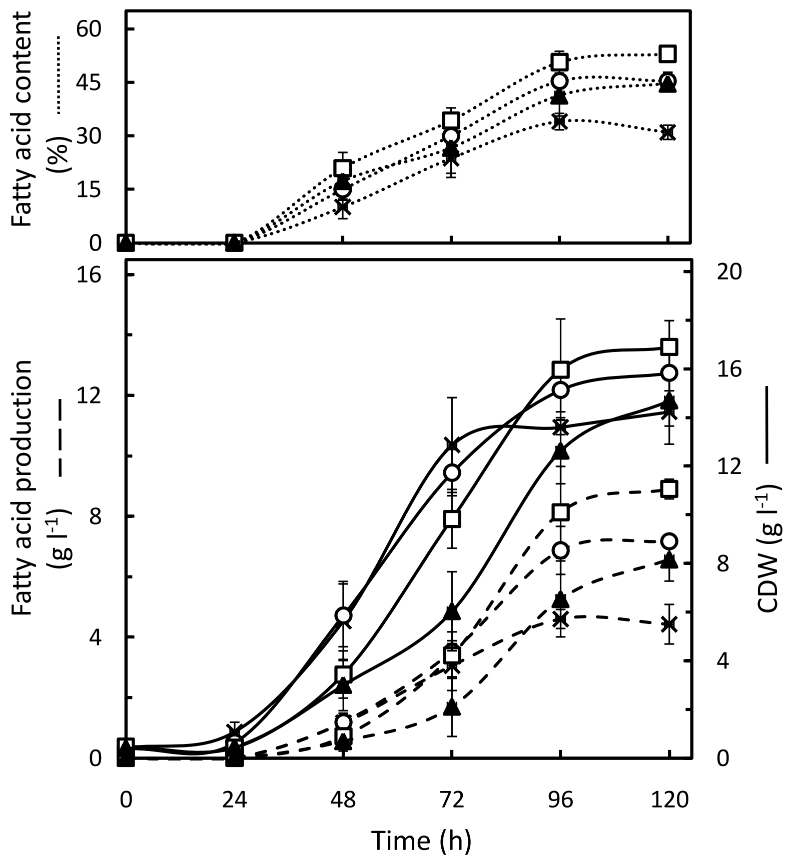

Figure 8: Effect of additional glucose on lipid production by *R. opacus* PD630 grown on the saccharified corn silage solution in flasks. The strain was inoculated in the saccharified 75% solution supplemented with 10 (○), 20 (□) or 30 (▲) g l^{-1} of glucose, or without (x), at an initial OD_{660} of 0.3. The error bars represent the standard deviation of three independent replicates.

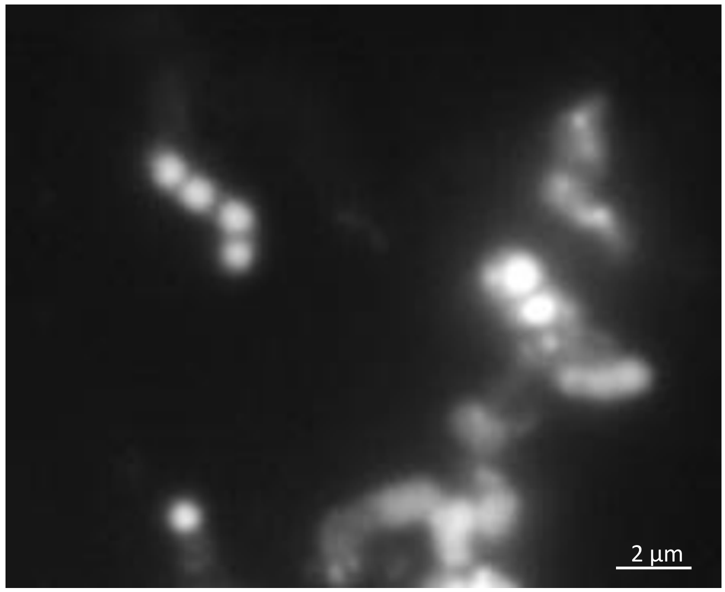

Figure 9: Fluorescent micrograph of *R. opacus* PD630 cells stained with the lipophilic fluorophore Nile Red. Cells were grown in minimal medium containing a saccharified silage solution for 120 h in a flask culture.