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Growth and polyhydroxybutyrate production by *Ralstonia eutropha* in emulsified plant oil medium

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Abstract Polyhydroxyalkanoates (PHAs) are natural polyesters synthesized by bacteria for carbon and energy storage that also have commercial potential as bioplastics. One promising class of carbon feedstocks for industrial PHA production is plant oils, due to the high carbon content of these compounds. The bacterium *Ralstonia eutropha* accumulates high levels of PHA and can effectively utilize plant oil. Growth experiments that include plant oil, however, are difficult to conduct in a quantitative and reproducible manner due to the heterogeneity of the two-phase medium. In order to overcome this obstacle, a new culture method was developed in which palm oil was emulsified in growth medium using the glycoprotein gum arabic as the emulsifying agent. Gum arabic did not influence *R. eutropha* growth and could not be used as a nutrient source by the bacteria. *R. eutropha* was grown in the emulsified oil medium and PHA production was measured over time. Additionally, an extraction method was developed to monitor oil consumption. The new method described in this study allows quantitative, reproducible *R. eutropha* experiments to be performed with plant oils. The method may also prove useful for studying growth of different bacteria on plant oils and other hydrophobic carbon sources.

Keywords *Ralstonia eutropha* · Polyhydroxyalkanoate · Polyhydroxybutyrate · Plant oil · Palm oil

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Introduction

Plant oils are important agricultural products that are harvested from a variety of crops worldwide, including soybean, rapeseed, and oil palm. These oils primarily consist of triacylglycerols (TAGs), in which three fatty acids are attached to a glycerol backbone. The types and distribution of fatty acids present in oil vary depending on the plant species (Srivastava and Prasad 2000). Plant oils have traditionally been used in the food industry, but they can also be chemically processed into other products such as fuels, fine chemicals, and polymers (Hill 2000; Srivastava and Prasad 2000; Meier et al. 2007). Recent studies have investigated the feasibility of using bacteria to biologically convert plant oils and fatty acids into fuels (Kalscheuer et al. 2006), surfactants (Rahman et al. 2002; Kim et al. 2006), and polyhydroxyalkanoate (PHA) bioplastics (Akiyama et al.

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2003). In order to utilize plant oils, bacteria secrete lipases, which catalyze the release of fatty acids from TAGs (Jaeger et al. 1999). The fatty acids are then transported into the cell, where they are catabolized via the β-oxidation cycle (DiRusso et al. 1999). Some bacteria also synthesize surfactants that may increase the surface area and bioavailability of hydrophobic carbon sources, allowing for more efficient growth on these compounds (Rosenberg and Ron 1999).

Our group is interested in studying production of PHA from plant oils by the bacterium *Ralstonia eutropha* H16. PHAs are natural polyesters that can be used as biodegradable bioplastics (Anderson and Dawes 1990), and *R. eutropha* is the model organism for studying PHA synthesis and accumulation (Pohlmann et al. 2006). Wild-type *R. eutropha* is known to make the homopolymer polyhydroxybutyrate (PHB) from plant oils, but the strain can be engineered to make other types of PHA (Fukui and Doi 1998). While there are several reports in the literature of PHA production from plant oils with *R. eutropha* (Fukui and Doi 1998; Kahar et al. 2004; Loo et al. 2005; Mifune et al. 2008), when we began our experiments, we experienced difficulties using plant oil as a carbon source for bacterial fermentations. Addition of insoluble plant oil to aqueous growth medium creates a heterogeneous mixture, in which the low-density oil is concentrated at the top of the vessel. Oil can be dispersed in a fermentor by rapid stirring, but aeration forces the oil back to the top of the fermentor, where it collects on the walls of the vessel. Build up of oil on the vessel walls makes this material unavailable to the cells and leads to errors when attempting to measure oil consumption and product yield. Another issue caused by the heterogeneity of the medium is that taking representative samples early in plant oil cultures is impossible, as there is no way to determine an appropriate depth for the sampling port. *R. eutropha* will eventually grow in a medium in which plant oil is the sole carbon source, and as the cells grow the oil becomes emulsified over time. We found, however, that lag times in these cultures were long and could vary considerably between experiments. In order to conduct quantitative and reproducible experiments, we therefore developed a method for growing *R. eutropha* cultures in which the plant oil is emulsified using a chemical emulsifying agent. These emulsified oil cultures can be grown in both shake flasks and fermentors.

When developing this method we investigated several surfactants commonly found in biotechnology laboratories: sodium dodecyl sulfate (SDS), Tween 80 (polyoxyethylene (20) sorbitan monooleate), and Triton X-100 (4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol). We also tested gum arabic (GA), a natural glycoprotein synthesized by the acacia tree (Qi et al. 1991; Goodrum et al. 2000). GA is used as a thickener, emulsifier, and stabilizer in the food processing industry, and is classified by the United States Food and Drug Administration as Generally Recognized as Safe (U.S. Code of Federal Regulations, 21CFR184.1330). Surfactants have been shown to interact with microbes in many ways, and can either inhibit or stimulate growth depending on the structure of the surfactant and the species of bacteria (Volkering et al. 1997). When evaluating our chosen surfactants for use with *R. eutropha*, we therefore examined their toxicity, ability to be metabolized, and influence on PHB production. We found that GA was the most effective emulsifier for growth of *R. eutropha* on plant oil and we believe this method could also be a useful experimental tool for studying growth of other bacteria on oils.

Materials and methods

Bacterial strain and cultivation conditions

All experiments were performed with *R. eutropha* H16 (ATCC 17699). The rich medium used in this study was dextrose free tryptic soy broth (TSB) medium (Becton Dickinson, Sparks, MD). We used a phosphate-buffered minimal medium (pH 6.8) that has previously been described (Budde et al. 2010). Various carbon sources, emulsifying agents, and nitrogen sources were added to the minimal medium as described in the text. All media contained 10 μg/ml gentamicin sulfate. Chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless noted otherwise. *R. eutropha* was always grown aerobically at 30 °C. For shake flask experiments 50-ml media was used in 250-mL baffled flasks, and the shaker was set to 200 rpm.

The inhibitory effects of the emulsifiers were studied by preparing TSB using 90% of the normal volume of water. Concentrated stocks of the emulsifying agents were prepared and autoclaved. The emulsifiers were then added to the TSB to the desired concentration, along with the amount of sterile water necessary to bring the TSB to its normal concentration. The abilities of the emulsifying agents to act as sole carbon sources were studied by adding compounds from sterile stocks to minimal medium, along with 0.1% ammonium chloride as the nitrogen source. As gum arabic (GA) is a glycoprotein, it contains amino acids and could therefore potentially serve as a nitrogen source. This was studied using minimal medium containing 2% fructose, 0.3% GA, and no NH_4Cl. Optical densities at 600 nm (OD_{600}) were measured using a Spectronic Genesys 20 spectrophotometer (Rochester, NY).

We observed that when GA was autoclaved in the presence of the salts found in the minimal medium, the liquid became slightly cloudy. We believed this was due to

some of the protein denaturing and coming out of solution. To determine how this precipitant influenced cell growth and PHB synthesis, minimal medium cultures containing 0.05% NH_4Cl were made. Sets of cultures were then prepared that contained one of the following combinations: (1) 2% fructose and no GA, (2) 2% fructose and 0.3% GA, or (3) no fructose and 0.3% GA. Additionally, flasks were prepared that contained medium with no fructose and 0.3% GA that were not inoculated with *R. eutropha*, in order to determine the amount of material that precipitated over time in the absence of cells. Fructose was used as the carbon source in these experiments instead of plant oil, because this made it easier to examine the effects of GA without the added complication of comparing emulsified versus non-emulsified oil.

Preparation of emulsified oil medium

The oil medium used in this study was designed to stimulate PHB accumulation by *R. eutropha*. A low level of NH_4Cl was supplied to the cells and excess carbon was provided in the form of palm oil (Wilderness Family Naturals, Silver Bay, MN). After nitrogen in the medium was exhausted, cell division ceased and PHB was stored by the cells. We found that in minimal medium with 0.05% NH_4Cl, 1% palm oil provided sufficient carbon, while medium with 0.1% NH_4Cl required 2% palm oil. One percent oil needed 0.3% GA for efficient emulsification, while 2% oil could be emulsified with 0.5% GA. GA is available from Sigma–Aldrich (Cat. No. G9752).

To prepare the medium, a 10× solution of GA was prepared in water. GA dissolves slowly at room temperature, so the solution was stirred rapidly to speed dissolution. The GA solution was then centrifuged (10,500×*g*) to separate out insoluble particles. Water, clarified GA solution, and palm oil were combined, along with the sodium phosphate and K_2SO_4 needed for the minimal medium. The mixture was emulsified by homogenizing with a Sorvall Omni-Mixer for 1 min. The amount of water added before emulsification will depend on the particular apparatus used to make the emulsion. We found that concentrated oil emulsions could be made, and then diluted as necessary. We discovered that homogenizers were the most efficient tools for emulsification, but sonicators could also be used. After emulsifying the oil, the medium was autoclaved, cooled, and $MgSO_4$, $CaCl_2$, trace elements, and gentamicin were all added from sterile stocks to the proper concentrations (Budde et al. 2010).

Fermentation conditions

Emulsified palm oil fermentations were carried out using an Infors Sixfors multiple fermentor system that utilized 500-ml flat-bottomed vessels (Bottmingen, Switzerland). *R. eutropha* was grown overnight in TSB, and these cultures were used to inoculate 50 ml minimal medium flask precultures with 2% fructose and 0.1% NH_4Cl. Cells from the minimal medium precultures were used to inoculate each fermentor to an initial OD_{600} of 0.1. Each vessel contained 400 ml of emulsified palm oil medium with 2% palm oil, 0.5% GA, and 0.1% NH_4Cl. The temperature of each fermentor was kept constant at 30 °C. The pH of each culture was maintained at 6.8±0.1 through controlled addition of 2 M sodium hydroxide. Stirring was provided by two six-blade Rushton impellers at speeds of 500–900 rpm. Air was supplied at 1 vvm and the dissolved oxygen concentration was maintained above 40% through controlled addition of pure oxygen.

Analytical methods

The cell dry weight (CDW) and PHB content of samples from fructose cultures were measured as described previously (Budde et al. 2010). Residual cell dry weight (RCDW) was calculated as the total CDW minus the mass of PHB. CDW of samples from oil fermentations was measured by taking 10-ml samples in 15-mL preweighed plastic tubes, centrifuging, washing with 5 mL cold water plus 2 mL cold hexane, centrifuging, resuspending the cells in 2 mL cold water, freezing at −80 °C, and lyophilizing. The hexane was included in the washing step to remove unused oil. The PHB content of cells was measured by transferring freeze-dried biomass to screw top glass test tubes, and reacting the samples with methanol and sulfuric acid in the presence of chloroform to convert the PHB monomers into methyl 3-hydroxybutyrate (Brandl et al. 1988). The amount of methyl 3-hydroxybutyrate was quantified using an Agilent 6850 gas chromatograph (Santa Clara, CA) equipped with a flame ionization detector and a DB-Wax column (Agilent, 30 m×0.32 mm×0.5 μm). The carrier gas was hydrogen (3.0 ml/min) and the temperature program was 80 °C for 5 min, ramp to 220 °C at 20 °C/min, and hold at 220 °C for 5 min. A calibration curve for the instrument was generated by treating known amounts of pure PHB as described above and measuring the resulting peak areas on the chromatograms. Fatty acids in lipid samples were quantified using the same methanolysis procedure, with pure fatty acids used as standards.

A previous report described a method for measuring plant oil concentrations in which oil was extracted from 2 ml of medium using hexane (Kahar et al. 2004). We tested this method with emulsified palm oil medium and could not achieve quantitative oil recovery. We therefore developed a new method for measuring the concentration of oil and other lipids in emulsified oil medium. For each sample, 10-ml medium was taken in a 15-ml plastic test

tube and centrifuged 10 min using a swinging bucket rotor. (In this method all centrifugations were performed at room temperature and 7,200×*g*.) The supernatant was transferred to a 50-ml plastic test tube, and the pellet was washed with 5 ml water and centrifuged again to recover oil that had been associated with the cell pellet. This supernatant was then combined with the supernatant from the previous centrifugation (15 ml total). Twenty milliliters of a 2:1 (*v*/*v*) mixture of chloroform and methanol were added to the tube, and the sample was vortexed for 1 min. The sample was then centrifuged and 5 ml of the organic phase (i.e., the bottom phase) was transferred to a preweighed glass test tube. Solvent was removed by incubating the test tube in a heat block at 40 °C, then transferring the test tube to a vacuum oven at 80 °C. After drying, samples were weighed to determine the mass of recovered lipid. The lipid concentration in the medium was calculated using Eq. (1).

$$\text{Lipid concentration} = \text{Mass recovered}\left(\frac{V_{\text{organic}}}{V_{\text{transferred}}}\right)\left(\frac{1}{V_{\text{sample}}}\right) \quad (1)$$

In this equation, V_{organic} is the volume of the organic phase after the extraction. Because the solvent mixture includes methanol, not all of the solvent remains in the organic phase after contact with the aqueous medium. We measured V_{organic} to be 13.3 ml when using chloroform/methanol. $V_{\text{transferred}}$ was 5 ml and V_{sample} was 10 ml (the volume of medium taken from the culture).

The ammonium concentration in fermentation samples was determined from cell free supernatants using an enzymatic ammonium assay kit from Sigma–Aldrich (Cat. No. AA0100). The assay was carried out following the manufacturer's instructions.

Glycerol was detected using a HPLC assay with an Aminex HPX-87H column (Bio-Rad, Hercules, CA) that was previously used in our lab to measure fructose concentrations (Lawrence et al. 2005). A 100-μL injection was used instead of 25 μL in order to detect low concentrations of glycerol. Glycerol had a retention time of 13.6 min.

The lipid species extracted from fermentation samples were examined qualitatively by thin layer chromatography. Lipid samples were dissolved in chloroform and 30 μg were spotted onto a silica gel TLC plate (250 μm thickness). The mixture of lipid standards spotted on the plate contained a triacylglycerol (TAG, 1,2-distearoyl-3-oleoyl-*rac*-glycerol; 10 μg), diacylglycerol (DAG, 1,2-dipalmitoyl-*rac*-glycerol; 20 μg), monoacylglycerol (MAG, 1-palmitoyl-*rac*-glycerol; 20 μg), and free fatty acid (FFA, palmitic acid; 10 μg). The plate was first developed with chloroform/methanol/water (60:35:5, by volume) to 5 cm from the origin. It was then developed with hexane/diethyl ether/acetic acid (70:30:1, by volume) to the top of the plate. Finally, analytes were made visible by spraying the plate with 3% cupric acetate in an 8% phosphoric acid solution and incubating the plate at 200 °C.

Results

Evaluation of potential emulsifying agents

To determine if the potential emulsifying agents inhibited growth of *R. eutropha*, TSB cultures were prepared that included each of the surfactants. The surfactant concentrations used in this experiment were in the ranges needed to generate 1% palm oil emulsions: SDS, 0.05%; Triton X-100, 0.05%; Tween 80, 0.2%; GA, 0.3%. OD_{600} measurements were made over time to monitor cell growth (Fig. 1). We found that growth of the GA cultures was nearly indistinguishable from the TSB-only control cultures. The cultures that included Tween 80 grew at the same rate as the controls through 12 h, but exhibited a higher cell density at the 24-h time point. The Triton X-100 cultures grew more slowly than the controls, but did show significant growth by 24 h. Cultures that contained 0.05% SDS did not grow over the course of the experiment. When the SDS concentration was reduced to 0.01% *R. eutropha* was able to grow, but the cultures still grew more slowly than the TSB-only control (data not shown).

Our goal was to study growth of *R. eutropha* on plant oil; therefore, it was important that the bacteria could not use the surfactants as an alternate carbon source. To test this, we prepared minimal medium cultures in which each surfactant was the sole carbon source. OD_{600} was monitored and compared to controls that contained no carbon

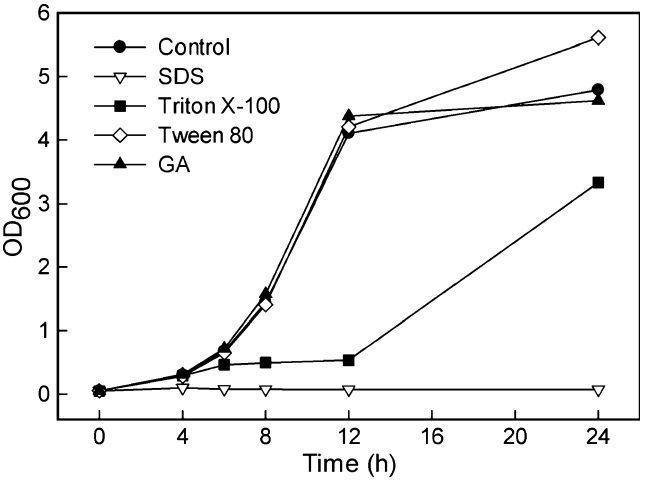

Fig. 1 SDS and Triton X-100 inhibited growth of *R. eutropha* in TSB. GA did not inhibit growth in TSB, while addition of Tween 80 to the cultures led to higher cell densities at the 24-h time point. Values reported are means from triplicate cultures

source (Table 1). The OD_{600} measurements of the SDS and Triton X-100 cultures actually decreased relative to the controls. We observed aggregation of cells in these cultures, which may have caused the drops in optical density. The GA cultures showed slightly higher OD_{600} values than the controls at both time points, but OD_{600} did not increase over time, indicating that no significant growth was taking place. The Tween 80 cultures showed considerably higher optical densities than the controls, and the OD_{600} readings for these cultures increased over time. This demonstrates that Tween 80 can serve as an effective carbon source for *R. eutropha*.

Because GA is a glycoprotein, it also has the potential to serve as a nitrogen source for *R. eutropha*. This was investigated using minimal medium cultures with 2% fructose and 0.3% GA as the sole potential nitrogen source, along with controls that contained 2% fructose and no GA. Triplicate cultures were inoculated to an initial OD_{600} of 0.1. After 48 h of incubation, the mean OD_{600} of the controls was 0.280±0.008, while the mean OD_{600} of the GA cultures was 0.326±0.006. The increase in OD_{600} of the controls can be attributed to the changes in cell morphology caused by storage of PHB (Wilde 1962). The GA cultures exhibited a slightly greater increase in OD_{600}, but the fact that there was little difference between the GA cultures and the controls indicates that GA does not serve as an effective nitrogen source for *R. eutropha*.

Growth of *R. eutropha* in fructose minimal medium with gum arabic

Based on our investigation of the effects of the different surfactants on *R. eutropha*, we determined that GA was the best emulsifying agent to use in plant oil cultures. In order to gain further insight into the influence of GA on cell growth and PHB production, as well as to determine how precipitated GA could influence measurements, we grew *R. eutropha* in fructose minimal medium in the presence or absence of 0.3% GA (Fig. 2). The RCDW values with and without GA in the medium were almost identical, showing that this compound does not significantly influence cell growth. GA does slightly reduce PHB accumulation, however, as cells grown in medium with GA made 13% less PHB over 72 h than cells not exposed to GA. Despite this reduction, *R. eutropha* grown in the presence of GA still stored significant polymer (79% of CDW). When GA was the only potential carbon source, the maximum CDW observed was <0.1 g/L, which supports our previous finding that GA cannot serve as a carbon source for *R. eutropha*. The PHB content of cells from cultures lacking fructose decreased steadily from 12 to 72 h (Fig. 2b), indicating that the cells mobilized stored PHB in the absence of an exogenous carbon source. The PHB initially present in these cells was accumulated during the precultures.

Table 1 Minimal medium cultures with 0.1% NH_4Cl and surfactants as the sole carbon sources were inoculated with *R. eutropha* to an initial OD_{600} of 0.1

Surfactant	Concentration	OD_{600}	
		10 h	24 h
Control	N.A.	0.104±0.001	0.101±0.002
SDS	0.05%	0.071±0.002	0.062±0.002
Triton X-100	0.05%	0.091±0.002	0.078±0.001
Tween 80	0.2%	0.727±0.027	1.229±0.009
GA	0.3%	0.125±0.009	0.123±0.011

The cultures were incubated 24 h and OD_{600} values were measured

Values reported are means from three independent cultures±SD

The control cultures contained no carbon source

A positive control with 2% fructose reached OD_{600}>10 after 24 h

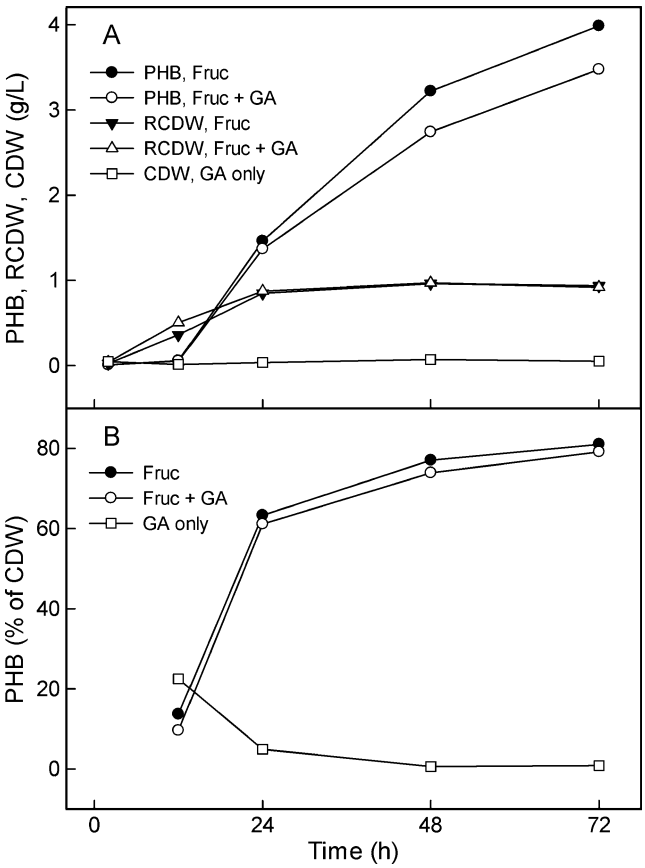

Fig. 2 *R. eutropha* was grown in fructose minimal medium with GA (*open symbols*) and without GA (*closed symbols*). Growth and PHB production were measured (**a**), as was PHB content as a percentage of CDW (**b**). Control cultures with GA and no fructose (*open squares*) showed that GA could not be used as a carbon source and that cells metabolized stored PHB in these cultures. Values reported are means from triplicate cultures

A set of flasks was also prepared with 0.3% GA medium that was not inoculated with *R. eutropha*, in order to determine how much GA comes out of solution during a typical experiment. This was a concern because significant precipitation could make CDW and PHB content measurements inaccurate. No mass of precipitated material could be measured at the 2- and 12-h time points. We did detect precipitant after 24 h, which increased in concentration from 24 to 72 h and reached a maximum of 0.046 g/L (data not shown). Comparing this value to the measurements from the *R. eutropha* cultures with fructose and GA, we found that the precipitated GA accounted for approximately 1% of the total CDW and 5% of the RCDW at the 72-h time point.

Quantification of oil utilization

We found that a published method for measuring oil concentrations in bacterial growth media that relied on hexane extraction was not quantitative when applied to our emulsified oil medium (Kahar et al. 2004). We therefore tested extraction of palm oil from 10 mL samples using hexane, chloroform, and a 2:1 mixture of chloroform and methanol. (Chloroform/methanol mixtures have long been used to extract lipids from biological samples (Iverson et al. 2001).) As bacteria grow and oil is broken down, other lipids will appear in the culture medium, including MAGs, DAGs, and FFAs. To accurately measure the total amount of lipid present in a medium, an extraction procedure must also recover these polar lipids. To test the extraction of polar lipids, we also measured recovery of oleic acid and a 1:1 mixture of oleic acid and palm oil from GA emulsions. Results of these experiments are shown in Table 2.

Both chloroform and chloroform/methanol recovered greater than 90% of the lipids from each standard. We chose to use chloroform/methanol for measuring lipid concentrations in experimental samples, as it yielded slightly better results with the 2% lipid emulsions. Hexane could not be used to quantitatively recover palm oil or oleic acid. We found that hexane could quantitatively extract emulsified palm oil if sodium hydroxide was added to the samples to break the emulsions (data not shown). Even with this sodium hydroxide treatment, however, oleic acid could not be recovered efficiently. We also tested control standards that contained either 0.3% or 0.5% GA and no lipid. The mass recovered from these samples was negligible, indicating that GA is not extracted by the solvents used in this study (data not shown).

When cells are present in a sample, some of the lipids in the medium are associated with the cell pellet. We attempted to estimate how much lipid was associated with the cells by washing the pellet with hexane after the water washing steps. (Chloroform/methanol could not be used for this step because the chloroform extracts PHB from biomass.) We determined that if the hexane wash is included in the lipid recovery procedure, the observed lipid concentration increases by 1–4% (data not shown). Given the small impact this has on the measurement, we determined that this step does not need to be included in the standard method, but could be added if higher precision is required.

We also discovered that the use of chemical antifoams in fermentation interferes with the lipid recovery method and should be avoided. Both polypropylene glycol and silicone oil are extracted by the chloroform/methanol method, which will artificially increase the measured lipid concentration in the medium. In our experience, foam formation in emulsified oil cultures was not a significant issue at the cell densities used in this study.

Emulsified oil fermentations

Fermentations were carried out in medium containing 20 g/L palm oil emulsified with 0.5% GA, and cell growth and oil utilization were measured (Fig. 3). We observed a measureable increase in CDW by the 9-h time point. This demonstrates that our method leads to cultures with short lag phases, and that we were able to take accurate measurements early in the experiment. By 12 h, all the ammonium in the medium was depleted (data not shown),

Table 2 Palm oil (PO), oleic acid (OA), and a 1:1 mixture of the two were extracted from minimal medium standards in which the lipids were emulsified with gum arabic (GA)

Solvent	Mass of lipid recovered (%)					
	1% lipid+0.3% GA			2% lipid+0.5% GA		
	PO	OA	PO/OA	PO	OA	PO/OA
Hexane	031.4±1.1	062.1±4.5	050.9±2.7	23.5±0.8	47.6±1.8	42.0±1.7
Chloroform	100.5±1.7	099.8±1.2	101.6±1.6	97.2±4.7	90.6±3.5	96.0±2.1
Chloroform/methanol	102.2±1.2	103.5±2.1	100.2±1.7	98.7±2.7	98.1±0.6	98.0±2.7

Ten-milliliter samples were taken from each standard and 20 ml of each solvent was used for extraction

All extractions were performed four times, and the reported values represent the means±SD

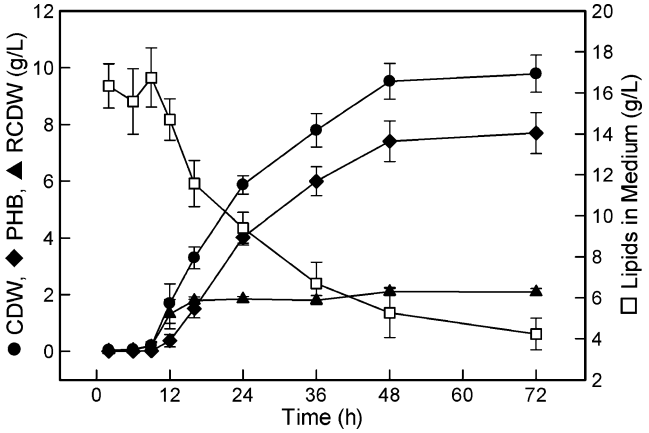

Fig. 3 *R. eutropha* was grown in emulsified palm oil fermentations. Cell growth, PHB production, and oil consumption were measured over time. Oil consumption was measured by extracting lipids from the medium with chloroform/methanol. Values reported are means from six fermentations and error bars represent standard deviations

and by 16 h, the RCDW reached its maximum value. After this point, cell division stopped and further increases in CDW were due to storage of PHB. By 72 h, 79% of CDW consisted of PHB. Six fermentations were carried out in this experiment, and the small error bars in Fig. 3 show that this method allows for reproducible growth of *R. eutropha*.

The initial measured concentration of oil in the medium was 17 g/L, which is lower than the 20 g/L added to each vessel. We attribute this discrepancy to the fact that some oil collected on the walls of the vessels and was therefore removed from the medium. Significant oil consumption by the cells was first observed between the 9- and 12-h time points. Oil consumption continued throughout the experiment, although the rate of consumption decreased over time. Measurement of lipid utilization allows for calculation of product yields. We found that over the course of the entire fermentation, polymer was formed at a yield of 0.61 gPHB/g palm oil. If only the PHB production phase of the culture is considered, the PHB yield is 0.84 g/g palm oil. These values are similar to other yields from plant oils reported in the literature (Kahar et al. 2004).

TLC analysis revealed that over time TAGs in the medium were broken down and the concentrations of FFAs, MAGs, and DAGs increased (Fig. 4). Even at the 72-h time point, however, complete breakdown of the TAGs had not occurred. We determined the fatty acid distributions in the lipid extracts from the fermentation medium with a methanolysis assay (Table 3). The key observation from this experiment was that the proportion of oleic acid in the medium increased over time, suggesting that *R. eutropha* preferentially uses other fatty acids relative to oleic acid. This could be due to the substrate specificity of the *R. eutropha* lipase, or could result from different rates of transport into the cell for the different fatty acids.

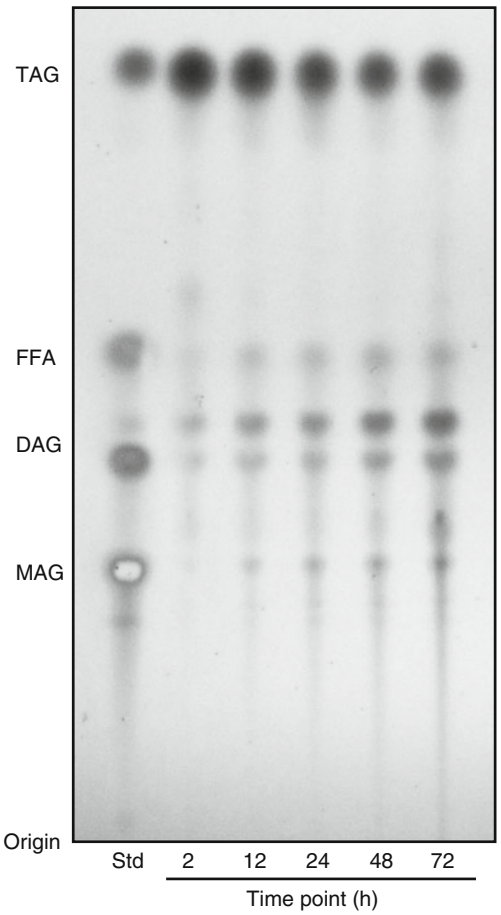

Fig. 4 The types of lipids present in the medium of an emulsified palm oil fermentation were analyzed by TLC. Samples were taken at various times during the culture and lipids were extracted. Thirty micrograms of lipid were loaded for each time point. As the cells grew MAGs, DAGs, and FFAs were released from the palm oil

We also developed an HPLC method for detecting glycerol in culture supernatants. Glycerol would be released if all three fatty acids were cleaved from a TAG molecule. We were unable to detect glycerol in the medium at any time point in this experiment, suggesting that any glycerol that appeared in the medium was rapidly consumed by the cells.

Table 3 The fatty acid distributions in lipid samples extracted from emulsified palm oil medium at different time points were determined

Fatty acid		Fatty acid content (wt.%)		
		2 h	48 h	72 h
Myristic acid	(C14:0)	1.05±0.02	0.91±0.09	1.21±0.2
Palmitic acid	(C16:0)	35.5±00.2	29±2	34±6
Stearic acid	(C18:0)	3.72±0.01	04.9±0.6	05.5±0.7
Oleic acid	(C18:1)	47.3±00.1	55.3±0.5	55±2
Linoleic acid	(C18:2)	12.4±00.1	10±1	04±3

The values reported are means from triplicate measurements±SD

Discussion

Plant oils have been projected to be more efficient carbon sources for industrial PHA production than sugars (Akiyama et al. 2003). Our group is most interested in palm oil, a major agricultural product in Southeast Asia with a high oil yield per acre of land (Waltz 2009). While *R. eutropha* is able to grow on non-emulsified palm oil, these cultures exhibit variable lag times and representative samples cannot be taken early in experiments. Oil will eventually become emulsified as the bacteria grow, but the mechanism by which this emulsification occurs is unknown. Some bacteria excrete surfactants (Rosenberg and Ron 1999), but there is no evidence that *R. eutropha* synthesizes these compounds. It is possible that polar lipids released during the breakdown of TAGs (i.e., MAGs, DAGs, and FFAs) could themselves emulsify the oil. Our group recently showed that the putative lipase encoded by gene h16_A1322 (GeneID: 4249488) is essential for robust growth of *R. eutropha* on non-emulsified plant oil (Brigham et al. 2010).

In order to conduct quantitative, reproducible experiments with plant oil as the carbon source, we developed an emulsified oil culture method for *R. eutropha*. Two-phase bacterial cultures have previously been investigated as a method for increasing the rate of biotransformation of compounds with low water solubility (Salter and Kelt 1995; Bühler et al. 2003). There are also many reports in the literature in which either synthetic or natural surfactants are used to study growth of bacteria on hydrophobic carbon sources (Tiehm 1994; Grimberg et al. 1996; Bruheim et al. 1997; Schmid et al. 1998; Willumsen et al. 1998). Most of these studies were concerned with the bioremediation of hydrophobic pollutants. It is known that surfactants can effect bacterial growth in many different ways (Volkering et al. 1997). We therefore evaluated how four different emulsifying agents influenced the growth of *R. eutropha*. Both SDS and Triton X-100 inhibited cell growth at the concentrations tested in this study. The antimicrobial properties of these surfactants had previously been observed in a study of *Bacillus subtilis* (Meyers et al. 2008). Tween 80 did not inhibit growth of *R. eutropha*, but the bacteria were able to use this surfactant as a sole carbon source. Tween molecules contain a fatty acid group. We hypothesize that a *R. eutropha* esterase may cleave the fatty acid from Tween, allowing it to be used as a carbon source by the bacteria. We decided to use GA as the emulsifying agent for our palm oil cultures, because it created stable emulsions and did not significantly affect growth of *R. eutropha*. There were two minor issues with GA: some of the material precipitated when autoclaved, and the presence of GA led to a slight decrease in PHB production (Fig. 2). Neither of these issues had a major impact on the results of *R. eutropha* growth and PHB production experiments.

R. eutropha fermentations with emulsified palm oil demonstrated the effectiveness of this method. Cultures had short lag phases and were highly reproducible (Fig. 3). The cells accumulated high levels of bioplastic, with PHB content of 79% of CDW reached after 72 h. A lipid extraction method was also developed, which allowed us to monitor oil consumption by the cells and the breakdown of oil in the medium (Fig. 4).

We performed emulsified oil fermentations in several bioreactors and learned many lessons on how to best implement this method. Fermentors with fully jacketed vessels work best, because in vessels with partial jackets oil will stick to the portions of the vessel wall that are cooler than the bulk medium. We also found it is preferable to use fermentors in which the stirring shaft enters from the top of the reactor and the bottom of the vessel is fully sealed. When the stirring shaft enters through the bottom of the vessel, there is occasionally leakage of culture broth through the mechanical seal. This may be due to the hydrophobic nature of the emulsified oil medium. Chemical antifoams should not be used with emulsified oil cultures because they interfere with the oil extraction method. If foaming is found to be an issue in an experiment, the use of mechanical foam breakers should be investigated.

We have developed a new method for studying growth of *R. eutropha* on plant oils using GA as an emulsifying agent. The emulsified oil medium can be used in both fermentors and shake flasks. While this method is a useful experimental tool, it is unlikely to have industrial applications due to the cost of gum arabic. Our method may also prove useful for studying growth of other bacteria, and could potentially be applied to other hydrophobic carbon sources.

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