1 **UTILIZATION OF PAPAYA WASTE AND OIL PRODUCTION BY *CHLORELLA PROTOTHECOIDES***

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10 **ABSTRACT**

11 Microalgae derived oils have outstanding potential for use in biodiesel production. *Chlorella*
12 *protothecoides* has been shown to accumulate lipids up to 60% of its cellular dry weight with
13 glucose supplementation under heterotrophic growth conditions. To reduce production costs,
14 alternative carbon feedstocks have been evaluated and show promise as low-cost alternatives.
15 Regional agricultural residues, such as off-quality fruits and vegetables that cannot be used as
16 human food or animal feed, as well as inedible plant byproducts, represent an abundant and
17 underutilized resource as a feedstock for supporting microbial production of biofuels. Here, we
18 present the finding that *C. protothecoides* isolates are capable of robust cell growth and oil
19 production in growth medium comprised of pH-adjusted puree of culled, waste papaya fruit
20 without any additional growth supplements. Optimization of culture medium and growth
21 conditions were used for lab-scale strain characterization and demonstration of the potential

for scale-up to an industrial bioprocess. The most rapid cell doubling time observed was 6.6 hours and the maximum oil production rate observed was 1.9 g/L/day.

KEYWORDS:

Chlorella protothecoides, Lipids, Biofuels, Agricultural Waste, Zero-Waste

Abbreviations Used:

TAGs – triacylglycerols

DCW – dry cell weight

FAME – Fatty acid methyl ester

1. INTRODUCTION

Microalgae are promising candidates for industrial production of biodiesel due to their rapid growth and high lipid storage capacity [1, 2]. The cells of *Chlorella protothecoides*, although microscopic at about 2-10 µm in diameter, store relatively massive quantities of lipids, primarily in the form of TAGs (triacylglycerols). FAME (fatty acid methyl ester) analysis, of *C. protothecoides* TAG-derived biodiesel indicates it has suitable fuel properties [3, 4]. Oil content on a DCW (dry cell weight) basis have been reported as high as 57.8% [5] and production up to 8,000 L in bioreactors has been demonstrated [6]. While *C. protothecoides* is a microscopic plant fully capable of fixing inorganic carbon through photosynthesis (photoauxotrophy), it has been found to saturate at much higher cell densities and store much more lipid when grown heterotrophically by supplementation with various carbon sources.

However, carbon sources contribute a considerable cost to the production of algal bio-oil. Most commonly, heterotrophic growth media is supplemented with purified glucose, and this supplementation contributes the single largest cost for industrial production. Li et al. calculated the cost of glucose to be 45% of the total production cost for their 8,000 L demonstration experiment [6]. To combat the high production cost of heterotrophic algal growth using purified glucose, a number of alternative carbon feedstocks have been evaluated for their potential use. These fall into three classes: (1) byproducts of the industrial production of a commodity such as waste glycerol from transesterification of TAGs into biodiesel [7, 8]; (2) enzymatic hydrolysates of complex carbohydrates from corn [9], sorghum [10], cassava [11, 12] rice straw [13], Jerusalem artichoke [14]; and (3) in the case of molasses [15, 16], an industrial byproduct that requires enzymatic hydrolysis prior to use in culture medium.

Increasing effort to improve agricultural efficiency and reducing or utilizing waste has poised microbial fermentation as a top candidate to benefit from the excess energy, in the form of stored carbon that is otherwise unused. In our zero-waste model, off-quality or excess agricultural products are diverted to an energy production system where the carbohydrates and other nutrients are scavenged by microorganisms (e.g., algae including *C. protothecoides*) and converted to high value products, such as oil for biodiesel production and high protein animal feeds. Agricultural producers benefit from an additional revenue stream for off-grade or overripe products, and the local economy additionally benefits from locally produced and diversified sources of biofuel and animal feed.

The first feedstock we have evaluated is puree from culled papaya fruits, and we have found that this crude agricultural residue supports high cell density and high lipid accumulation

in strains of *C. protothecoides*. As a snapshot, commercial papaya production on Hawaii Island is an \$8.2M industry producing in excess of 25M pounds [17]; however, an estimated 35%-50% of the total crop is off-quality fruit that are unsalable and are wasted. We present here quantitative data on the growth and oil production of *C. protothecoides* on growth medium comprised of solely diluted waste papaya as a demonstration on the feasibility of zero-waste agriculture in Hawaii and abroad. Lab scale demonstration and validation of microbial industrial processes are essential precursors for large scale, commercial production. The ultimate goal of our zero-waste model is to decrease the dependency of Hawaii on the importation of fossil fuel and animal feeds, while improving the profitability of Hawaii's agriculture by creating value from its waste streams.

2. MATERIALS AND METHODS

Organisms and Culture Maintenance

Algal strains were acquired from the University of Texas Culture Collection. UTEX strains B25, B31, 249, 256, and 264, all *Chlorella protothecoides*, were included in this study. Algal purification of strain B25 required sequentially streaking single colony isolates onto water agar medium until isolates free of bacterial contamination were obtained. Algal colonies from the original B31 stock grew preferentially over the bacterial contaminant on papaya heterotrophic growth medium, and a bacteria-free isolate was maintained. Strains 249, 256, and 264 were supplied as axenic cultures from UTEX. P1 agar medium, a modification of Bristol medium [18] supplemented with 12.5% clarified juice from Hawaii Rainbow Papaya and 1.5% agar and adjusted to pH 6.5 with NaOH was used for the maintenance of all strains. Inoculum cultures were prepared by inoculation of a single colony into 50 ml liquid P1 medium in Erlenmeyer

flasks, and shaken at 200 RPM, 25°C, for 4-5 days until cell density saturated. Inoculum cultures were diluted between 1:1,000 and 1:10,000 for inoculation of each experiment. Supplemental lighting was not used; flasks were exposed to ambient laboratory lighting, agar plates (for strain maintenance) were kept in a dark 25°C incubator.

Experimental Growth Medium and Culturing

Papaya puree was prepared from culled fruits obtained from a local papaya packing house (Hilo, HI). Fruits were peeled, seeds were removed, and a puree was prepared by passing the fruits through a household centrifugal juicer (L'EQUIP, St. George, UT). Diluted papaya puree, 10-100% (w/v) for the dilution series experiment (Fig. 1), and 35% (w/v) for all other experiments, with or without pH adjustment, was used as the culture medium for all experiments without the addition of any other components (e.g. Bristol medium salts). For the pH adjustment study, HCl was used to lower the pH to 3 or 4; NaOH was used for all other adjustments. Media was aliquotted at 125 ml in 250 ml Erlenmeyer flasks, the opening was covered by silicone sponge filters (Chemglass, Vineland, NJ), and steam sterilized for 45 minutes at 121°C. The growth cycle for each experiment was two weeks and cultures were shaken on rotary platforms as above. All experimental samples were repeated in triplicate.

Culture Monitoring and Biomass Yield Measurement

Intermittent sampling with cell enumeration by hemocytometer counting throughout the culture cycle was used to measure the rate of cell division during the exponential growth phase. At the completion of the culture cycle, cell density was again measured by hemocytometer counting, and subsamples were prepared for dry weight analysis. Aliquots of 50 ml culture were harvested by centrifugation at 4,800 x g for 20 minutes; the supernatant

containing spent media soluble solids and pellet consisting of cells and insoluble papaya solids were separately dried in foil weigh boats at 105°C overnight prior to weighing. To measure insoluble materials in the uninoculated papaya puree, aliquots of sterilized media were likewise centrifuged and dried.

Oil analysis

Aliquots of culture derived from 6.25 g (wet mass) papaya puree (e.g. 25 ml from culture in 25% papaya medium) were harvested by centrifugation at 4,800 x g for 20 minutes in 15 ml conical bottom centrifuge tubes (BD Biosciences, San Jose, CA). To each pellet, 7.5 g of 0.4-0.6 mm zirconium oxide grinding media (Glenn Mills Inc., Clifton, NJ) was added, and the total aqueous volume was adjusted to 10 ml with H₂O. Samples were homogenized at a speed of 6.5 M/s for 60 seconds in a FastPrep-24 instrument (MP Biomedicals, Santa Ana, CA). Subsequently, two hexane extractions of 5 ml each were performed by homogenizing the emulsion in the FastPrep instrument as above, and centrifugation at 4,800 x g for 30 minutes prior to pipetting off the organic phase. Solvent was evaporated overnight in a fume hood and mass of the oil extracted was measured. Fatty acid profile was determined by New Jersey Feed Laboratory, Inc. (Trenton, NJ).

3. RESULTS AND DISCUSSION

Our lab-scale experiments were designed to simplify and optimize culture medium formulation for *C. protothecoides* strains specifically on papaya puree. We found that all essential nutrients for cellular growth and oil production are contained wholly within pH-adjusted papaya puree. This is not altogether a surprising result, given that hydrolyzed molasses was found to produce a suitable culture medium for *C. protothecoides* without

additional medium requirements [15]. Dry weight analysis of our papaya medium indicates that per kg of puree, 118 g of soluble solids and 14 g of insoluble solids are contained. Previous chromatographic analysis of sugar extracts of Hawaii Rainbow papaya found the content to be approximately 101 g total sugar per Kg fresh fruit weight [19]. To maximize the efficiency of our culture system, we analyzed oil production from cell cultures with varying papaya puree concentrations. Because heterotrophic algal production is carried out in fixed volume culture vessels at a high operating cost, yield optimization on a liquid volume basis is crucial. We found that increasing papaya puree from 10 to 35% in 5% intervals resulted in proportionate, incremental gains in oil concentration per volume culture (Fig. 1A). At concentrations beyond 35% and up to 50%, only marginal increases in oil concentration were observed, and at or above 60% papaya puree resulted in diminished or no oil production. Interpretation of this data on a per unit feedstock basis (Fig. 1B) showed that the 35% papaya puree medium oil samples were the highest yielding at just over 20 g/Kg feedstock.

Figure 1. Oil yield of strain UTEX 249 on heterotrophic culture medium containing 10-100% papaya puree. (A) Yield of oil per liquid volume of culture. (B) Oil yield normalized to amount of papaya feedstock contained within culture medium. Error bars represent ± 1 SD from the mean of 3 replicates.

Papaya puree, like many fruit products, has a somewhat variable and acidic pH; we typically observed the pH to be between 4.5 and 5.5 in our freshly prepared product (prior to autoclaving). The only culture medium supplement we observed to affect cell growth was NaOH, which was used to neutralize the acidity of the fruit puree. We evaluated cell growth and oil production in culture medium that was pH-adjusted from pH 3 to 7 prior to sterilization (Table 1). An unanticipated drop in pH of the culture media adjusted to pH 5.00, 6.00 and 7.00 pre-sterilization to 4.93, 5.28, and 5.37, respectively, post-sterilization can be attributed to

additional papaya cellular lysis during the autoclave cycle. Regardless, our results showed that unadjusted media with a post-sterilization pH of 4.52 and acidified media (pH 4.09 and 3.11) did not support algal growth; whereas all media that had been partially neutralized (pH 4.93, 5.28, and 5.37) supported robust growth. Altogether, these results suggest that neutralization beyond pH 5.0 and pH readjustment throughout the culture cycle are unnecessary.

Pre-sterilization pH	Post-sterilization pH	Cell Density (cells/ml)	Oil Yield (g/Kg papaya)
3.00	3.11	$1.7\text{E}+03 \pm 1.2\text{E}+03$	1.1 ± 0.8
4.00	4.09	$7.5\text{E}+03 \pm 6.1\text{E}+03$	3.2 ± 2.3
5.00	4.93	$2.5\text{E}+08 \pm 2.2\text{E}+07$	20.3 ± 0.8
6.00	5.28	$2.4\text{E}+08 \pm 3.7\text{E}+07$	19.7 ± 0.8
7.00	5.37	$2.4\text{E}+08 \pm 1.8\text{E}+07$	19.2 ± 0.0
Unadjusted	4.52	$2.0\text{E}+06 \pm 2.1\text{E}+06$	0.5 ± 0.8

Table 1. Effect of pH on growth of UTEX 249. Culture medium was pH adjusted to prior to sterilization, and pH was measured prior to inoculation and at the end of the culture cycle. Cell density and oil yield are reported as the average of 3 replicates $\pm$ 1 SD. Culture medium contained 25% papaya puree. Our finding that media with a threshold of approximately pH 5 must be maintained is consistent with observations that related species do not thrive in acidified media. Unbuffered photoautotrophic growth media containing ammonium salts as a nitrogen source has been shown to negatively impact growth of *C. vulgaris* due to an acidic pH shift (pH 3-5) caused by assimilation of ammonium [20, 21]. Maintenance of a particular culture pH is automated in a

bioreactor setup; nonetheless it is critical to determine the optimum pH for the organism, as well avoid unnecessary media component expenses (e.g., sodium hydroxide). In the case of *C. protothecoides*, neutralization of the naturally acidic papaya puree to pH 5 is most economical.

Our next goal was to determine the growth and oil accumulation rates of our algal culture system. In order to characterize the growth rate, we collected daily samples for cell enumeration so that multiple data points within the exponential growth phase would be produced. Exponential growth was observed over three days during which the cell density increased by three orders of magnitude from 1.3×10^5 to 1.4×10^8 cells/ml (Fig. 2). The calculated doubling time during this phase was 6.6 ± 0.3 hours for three replicate cultures. Whereas the cell density saturated within 198 hours from inoculation, our oil yield time course analysis showed that oil content took an additional 110 hours to plateau. The maximum oil production rate, which occurred from the onset of cell density saturation for the next two and a half days was 1.9 g oil/L/day.

Figure 2. Cell growth and oil accumulation over 2 week culture cycle of UTEX 249 in 35% papaya media.

Markers × and ▲ indicate cell density (cells/ml) and oil yield (g/L), respectively. Error bars represent ± 1 SD from the mean of 3 replicates.

The maximum cell growth and oil production rates under small, fixed-volume batch growth conditions, such as conducted here, are important considerations when considering cultivation of larger volumes and/or the conversion to a fed batch system. Validation of scale up will include monitoring of these two parameters to ensure maximum efficiency is maintained. Our time course experiments highlight the asynchrony of oil accumulation with respect to cell growth (Fig. 2), which is a major complication to converting the culture system to a continuous fed batch process. One possible explanation for this phenomenon is that rapidly dividing cells are dedicating the cellular energy released by catabolism of the culture medium carbohydrates, and it is not until cell division ceases that the prerequisite excess of fatty acid precursors (e.g., acetyl-CoA) accumulate. Nevertheless, in a continuous culture system, introduction of new feedstock will likely result in competition for metabolites between cell reproduction and energy storage, thus compromising the rates of each individual parameter from those we observed in simple batch cultures. Evaluation of pre-pilot scale cultures and potential implementation of continuous fed-batch methodology up to 150 L is anticipated as equipment is acquired by our facility.

The ability of *C. protothecoides* to accumulate lipids to 60% DCW under heterotrophic conditions is remarkable, yet the mechanism underlying the production of such high oil content remains mostly unknown. A screen for genes selectively expressed when cells grown under autotrophic conditions are supplied with excess organic carbon identified an ammonium transport gene, *CpAMT1* as a possible component of a C/N ratio sensory mechanism [22]. In

support of their hypothesis that nitrogen availability regulates lipid accumulation, the authors of this study found that supplementation of various amino acids in the culture medium resulted in diminished oil content. Nitrogen starvation has also been found to induce the storage of TAGs in *Chlorella vulgaris* strain UTEX 29 [23]. Subsequent genomic analysis of *C. protothecoides* Cp0170 revealed the presence of genes encoding three nitrogen transporters (nitrite, ammonium, and amino acid), in addition to the presence of nine hexose-proton symporter genes [24].

Because UTEX 249 showed promise in preliminary trials for tolerating high concentrations of papaya puree (up to 50%), our experiments were designed to optimize culture for this strain. Once a set of optimum conditions were determined, we tested the performance of 4 additional strains from the UTEX collection for oil yield in our culture system (Table 2). UTEX 249 proved to be the best of the 5 strains in terms of oil yield (61% DCW, 21.0 g oil/Kg papaya), and UTEX B25 accumulated a roughly equivalent DCW oil percentage and yielded 21.3 g oil/Kg papaya. Fatty acid profiles for UTEX 249 and UTEX B25 (Table 3) closely mimic those reported for heterotrophically grown *C. protothecoides* using other feedstocks [5, 25].

UTEX 256 and UTEX 264 yielded less oil, 17.4 and 15.6 g/Kg papaya, despite having a higher DCW yield, implicating a higher %DCW yield of other molecules (e.g., protein). UTEX B31 underperformed compared to the other strains, as it had the lowest DCW and % oil yields; the elevated dry culture yield of this strain (93.1 g/Kg papaya compared to 69.6-73.3% for the other strains) can be explained by increased unutilized papaya soluble solids in the cultures of this

228 strain (i.e., UTEX B31 has the least efficient carbohydrate uptake and growth on papaya
229 medium).

Strain	Cell Density (cells/ml)	Dry Culture Yield (g/Kg papaya)	DCW (g/Kg papaya)	Oil Yield (g/Kg papaya)	% Oil (DCW)
UTEX B25	4.8E+08 ± 1.0E+08	70.37 ± 1.02	35.52 ± 0.97	21.31 ± 0.18	60.06 ± 2.12%
UTEX B31	4.0E+08 ± 1.0E+08	93.13 ± 1.70	32.67 ± 1.64	12.54 ± 0.92	38.35 ± 1.35%
UTEX 249	3.7E+08 ± 2.7E+07	69.56 ± 0.64	34.76 ± 0.54	21.04 ± 0.58	60.90 ± 2.69%
UTEX 256	4.9E+08 ± 9.7E+07	73.31 ± 1.12	36.48 ± 0.97	17.43 ± 0.09	47.82 ± 1.55%
UTEX 264	4.2E+08 ± 9.5E+07	71.69 ± 0.18	36.10 ± 0.54	16.64 ± 0.36	46.14 ± 1.63%

230 Table 2. Each column shows the average of 3 replicates ± 1 SD, except oil samples for UTEX 249
231 represent only 2 replicates. Culture medium contained 35% papaya puree.

Fatty Acid	UTEX 249	UTEX B25
Palmitic (16:0)	9.5%	9.5%
Stearic (18:0)	6.3%	6.3%
Oleic (18:1ω9)	63.6%	63.6%
Linoleic (18:2ω6)	15.6%	15.7%
Others	5.0%	5.0%

232 Table 3. Fatty acid profiles

233 As evidenced from our survey of the five UTEX strains, there is substantial variation in
234 biochemical potential between strains previously identified to be the same species. We also
235 found an additional strain from the UTEX collection identified as *C. protothecoides*, UTEX 29,
236 that did not grow in liquid papaya medium and did not de-green on agar medium like the other
237 strains evaluated (data not shown), but as mentioned above, another report has identified this
238 strain as *C. vulgaris*, not *C. protothecoides* [23]. Indeed the taxonomy of Chlorophyte algae, and
239 in particular the designation of species within genus *Chlorella*, has not been thoroughly
240 scrutinized. Phylogenetic analysis of many members by comparison of ribosomal DNA barcode

sequences has revealed the relationship of some members [26], however considerable divergence has been observed in whole genomes of three members of order Trebouxiophyceae, *Chlorella variabilis* NC64A [27], *Coccomyxa subellipsoidea* C-169 [28], and the newly available draft genome for *Chlorella protothecoides* Cp0170 [24]. For example, the *C. protothecoides* Cp0170 genome is less than half the total size and contains almost 30% fewer genes than its most closely sequenced relative, *C. variabilis* NC64A [24].

Because of this remarkable genetic variability among morphologically similar organisms, for the purpose of bioprospecting candidate microorganisms for specialized applications, it is wise to evaluate multiple candidate organisms for optimum feedstock utilization. Here, we have identified *Chlorella protothecoides* strain UTEX 249 as the most robust oil producer on papaya feedstock among five independent accessions, and have optimized culture conditions for efficient utilization of feedstock. Additionally, specialized applications may require particular end product formulations (e.g., fatty acid composition of TAGs) to be suitable for downstream process regulatory (e.g. ASTM) standards. Furthermore, applications for defatted algae meal, such as animal feeds, remain to be investigated or optimized although utilization of this product is another facet of our zero-waste program. Indeed, the development of locally produced animal feed from spent algae meal would have a strong impact on meat production in Hawaii, as it is the overwhelming consensus that the cost of feed importation is the biggest bottle neck to developing those industries in the islands.

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