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Lipid-Protein Extraction from Wastewater Cultivated Algae for Animal Feed: Optimization and Characterization

Tyler Marlar, Michael Hansen, Ashik Sathish, and Ronald Sims Ph.D

Abstract— The algal lipid-protein extraction procedure (ALPEP) isolates and extracts valued proteins and fatty acids, known as algal lipid-protein (ALP), from microalgal biofilms. The objective of this study was to determine optimal process conditions of the ALPEP to maximize the ALP production. The ALP was characterized by ash-free dry weight, total protein and fatty acid content. Optimal reaction conditions for the ALPEP were 60° C, 30 minutes, and 1.0 g NaOH/g dry algal mass. The ALPEP recovers 58% crude protein (g ALP protein/g total algal protein) and 60% total fatty acids (g ALP fatty acid/g total algal fatty acid). The ALP consists of 29% protein (g ALP protein/g total ALP) and 4.6% fatty acid (g ALP fatty acid/g total ALP) and is a sustainable alternative to traditional bovine supplements thereby creating a sustainable closed-loop between microalgal remediation of agricultural wastewater and animal nutrition.

Index Terms— Agricultural Feed, Algal Bioprocess, Bioprocess Optimization, Wastewater Remediation

1 INTRODUCTION

Microalgae possess several advantages as a source of renewable biomass for proteins, lipids, and biofuels. These advantages include high growth rate, global presence, and relatively small requirements [1], [2], [3]. While the need to reduce global dependence on fossil fuel and crude oil based products has motivated the development of microalgal technologies for the production of renewable fuels, chemicals, and bioproducts [4], [5] an alternative to converting extracted algal lipids to biodiesel is the utilization of the lipid-protein fraction as a feed supplement for agricultural applications. Global interest has increased in the utilization of algal biomass as a natural supplement in animal feeds [6], [7]. Sustainable, industrial-sized cultivation and processing of microalgae could provide for feed, fuel, and environmental challenges throughout the world.

Current hurdles to processing and transforming algal biomass into bioproducts have included challenges related to the extraction of algal lipids, harvesting of algal biomass, dewatering of microalgal biomass, and production and isolation of products [2], [3], [8]. These challenges specifically include the use of high concentrations of caustic materials at high temperatures [5]. Associated with these challenges are a multitude of safety concerns and the high cost of energy and chemical input. Therefore, the feasibility of successfully scaling up many of these methods has not been demonstrated at an industrial scale.

The goal of the research reported here was to develop a procedure, known as the Algae Lipid-Protein Extraction Procedure (ALPEP), to address the challenges identified above for the purpose of extracting protein and lipids from algae cultivated on dairy wastewater to serve as a feed supplement for livestock and aquaculture. The objectives included using low: (1) concentrations of chemicals, (2) reaction times, and (3) reaction temperature, which are conditions that impact scale-up, cost, and safety.

2. MATERIALS AND METHODS

2.1 Algal Biomass Cultivation and Collection

Algal biomass was harvested from a rotating algal biofilm reactor (RABR) at Utah State University's Caine Dairy Farm. The RABR was used to cultivate microalgae utilizing the nitrogen and phosphorus present in the dairy wastewater, and harvesting was accomplished by mechanical scraping [9]. The wet algal biomass was stored at -20^0 C. Prior to use, the wet algal biomass was dried using a Labconco Freezone 6 Lyophilizer operating at 0.1 mbar and -52^0 C. The dry algal biomass was immediately stored at -80^0 C after removal from the lyophilizer.

2.2 Glassware Preparation

All reactions were performed in glass tubes. Glassware was sequentially soaked in deionized (DI) water for 24 hours, a 1 M solution of sodium hydroxide (NaOH) for a minimum of eight hours, and a 1 M solution of hydrochloric acid (HCl) for a minimum of twelve hours. The glassware was then rinsed with DI water, placed in an oven at 105^0 C for a minimum of two hours, rinsed with acetone, and finally placed in a desiccator until use.

2.3 Optimization Conditions and Algal Processing

Optimization of the lipid and protein fraction recoveries was performed by varying the reaction time (30, 60, and 120 minutes), reaction temperature (50, 60, and 70 ^{0} C), and concentration of NaOH (2.0, 1.5, 1.0, 0.5, and 0.25 g NaOH/g dry algal biomass). Every combination was then processed through the Algae Lipid-Protein Extraction Procedure (ALPEP) in triplicate. The starting biomass was 400 mg. Four mL of variable

concentration of NaOH was added to the biomass. Each sample was heated at the specified reaction temperature for the duration of the reaction time. Following the base hydrolysis, samples were centrifuged at 1380 xg for two minutes. The supernatant was collected in a clean tube. The solid fraction was washed with 2 mL of DI water and centrifuged under the same conditions. This supernatant was added to the previously collected supernatant and the solid fraction was discarded. A consistent amount of 3.5 mL of 2 M sulfuric acid (H_2SO_4) was used to precipitate the lipid and protein fraction of each sample. Centrifuging again at 1380 xg for two minutes yielded solid and liquid phases. The liquid phase was discarded and the solid fraction was washed twice by adding 2 mL of DI water, resuspending the solids via vortex, centrifuging at 1380 xg for two minutes, and discarding the liquid phase. After washing, the solids were dried on the lyophilizer using the previously stated conditions, Section 2.1.

2.4 Analytical Methods

The ALP mass yield, ALP total crude protein recovery, and total fatty acid recovery of each sample was measured using the following procedures. Samples of unprocessed dried biofilm algae were analyzed to establish a control group. Approximately 25 mg of each precipitate was used for each procedure.

2.4.1 ALP Mass Yield

Ash-free dry weight was measured according to APHA standards [10]. The ALP samples were placed in previously fired aluminum weigh boats and then placed in an oven at 550 ^{0} C measuring the mass every hour. When constant mass was reached, samples were cooled to room temperature in a desiccator. The recorded mass of each precipitate sample was adjusted by subtracting the amount of ash in each sample. The ash-free ALP mass yield was calculated by dividing the mass of the ALP by the initial algal biomass (g ALP/g biomass).

2.4.2 ALP Total Crude Protein Recovery

Total nitrogen in each sample was determined according to AOAC official methods [11] utilizing a LECO FP-528. Total nitrogen was converted to total protein by multiplying the measured nitrogen by a conversion factor of 6.25 [5], [12]. Total crude protein recovery was determined by dividing the total protein measured in each sample by the total protein measured in unprocessed dry biofilm algae (g ALP protein/g total algal protein).

Since the algae used during this experiment was cultivated on dairy waste, it is possible that inorganic nitrogen was present in solution and is present in the dry biomass. No attempts were made at determining if total nitrogen measured for the control group contained inorganic nitrogen. Since the same source of dry biofilm algae was used throughout the study and the stock was rigorously mixed, it can be assumed that if inorganic nitrogen was present a constant amount is present in each sample. Therefore, relative changes in crude protein recovery between samples should reflect changes in organic nitrogen recovery and this method can still be used to compare and quantify protein recoveries.

2.4.3 ALP Total Fatty Acid Recovery

Fatty Acid Methyl Ester (FAME) analysis was used to measure total fatty acid content in each sample. Similar to protein analysis, unprocessed dry biofilm algae were used as reference for total fatty acids available in the algae. One mL of 5% H_2SO_4 in methanol was used to catalyze the transesterification of fatty acids to fatty acid methyl esters. Reactions were performed at 90 ^{0} C for 45 minutes and 5 mL of hexane was used to extract the FAMEs. FAMEs were measured using an Agilent 7890B gas chromatographer with instrument conditions as described [5]. However, a 1:10 split ratio was employed instead of the splitless method described [5]. Total fatty acid recovery was determined by dividing the total extracted fatty acids of each sample by the total extracted fatty acids in unprocessed dry algae (g ALP fatty acid/g total algal fatty acid).

2.5 Statistical Analysis and Optimal Conditions

Collected data, described in Section 2.4, were evaluated optimal conditions by implementing a 3 x 3 x 5 randomized factorial experiment and analyzed using SAS statistical software version 9.4 [5]. The factors correspond to the optimization conditions defined in Section 2.3. A three-way ANOVA analysis of the data was performed for ALP mass yield, total crude protein recovery, and total fatty-acid recovery after checking for model assumptions of normal residual distribution and constant variance. An alpha value of 0.05 was used to determine significant differences between all the factor combinations tested using the generated Tukey adjusted p-values. The best performing group for ALP mass yield, total crude protein recovery, and total fatty-acid recovery were compared to determine the best performing conditions. Optimal conditions were defined as the highest recovering conditions with the lowest energy and chemical input.

2.6 Scale-Up Validation and Nutritional Information

To confirm observed ALP mass yield, total crude protein recovery, and total fatty acid recovery of optimization results and to demonstrate the validity of extraction of wet algae, 100 g wet algae trials from the same source previously indicated were performed. A sub-sample was lyophilized at the previously stated conditions, Section 2.1, to determine an approximate dry weight of algae. All solids were assumed to be algae. The wet algae were then processed through the ALPEP at the determined optimal conditions. The ALP was collected and ash-free dry weight and total fatty acid recoveries were determined as previously described. However, total crude protein was determined based on the complete amino acid profile provided by the University of Missouri's Agricultural Experiment Station Chemical Laboratories [13]. Total crude protein recovery was determined by dividing the total mass of all amino acids in the ALPEP processed algae by the total mass of all amino acids in an

unprocessed algal sample (g ALP amino acids/g total algal amino acids). Data were analyzed in triplicate.

3. Results and Discussion

3.1. Optimal Conditions

The ALP was characterized by the mass/dry algae mass, recovery of crude protein, and the recovery of total fatty acids. These values were measured because they provide information regarding how much lipid-protein precipitate can be produced from algal biomass and basic nutrition information for feed applications. These results are presented in Fig. 1.

3.1.1. ALP Mass Yield

The concentration of NaOH has a significant effect on the quantity of the algal lipid-protein (ALP) extracted. The highest average mass of ALP was extracted with a NaOH concentration of 1.0 g NaOH/g dry algal biomass, while the lowest concentration of NaOH extracted the lowest mass of lipid-protein. All total crude protein and fatty acid recoveries were calculated on an ash-free dry weight adjusted values, presented in Fig. 1a. This information is useful in designing a scaled-up process, allowing for the prediction of the quantity of algal biomass to produce a certain quantity of ALP. Full statistical analysis for ALP mass is given in SI Fig. 1.

3.1.2. ALP Total Crude Protein and Total Fatty Acid Analysis

Recovery values were calculated by dividing extracted mass of total crude protein and total fatty acids by the respective mass in unprocessed biofilm algae. The recoveries for total crude protein and total fatty acids are presented in Fig. 1b and Fig. 1c, respectively. Full statistical analysis for total crude protein and total fatty acid recoveries are given in SI Fig.s 2-3. Conditions that favor a high recovery of total fatty acids favor a low recovery of crude protein. The highest total crude protein recovery was observed with reaction conditions of 50 ^{0} C, 30 minutes, and 1.5 g of NaOH/g of dry algae biomass; the highest total fatty acid recovery reaction condition was 70 ^{0} C, 120 minutes, and 1.5 g of NaOH/g of dry algae biomass.

A possible explanation for loss of protein recovery under conditions that favor fatty acid recovery is algal proteins could be sensitive to the high temperatures, long reaction times, and high NaOH concentrations associated with high total fatty acid extraction. Algal proteins could be denaturing and precipitating during the base hydrolysis under these conditions which implies that the algal proteins could be in the discarded solids portion after the first centrifugation step. Protein analysis of these solids could be used to determine if algal proteins are associating with these solids.

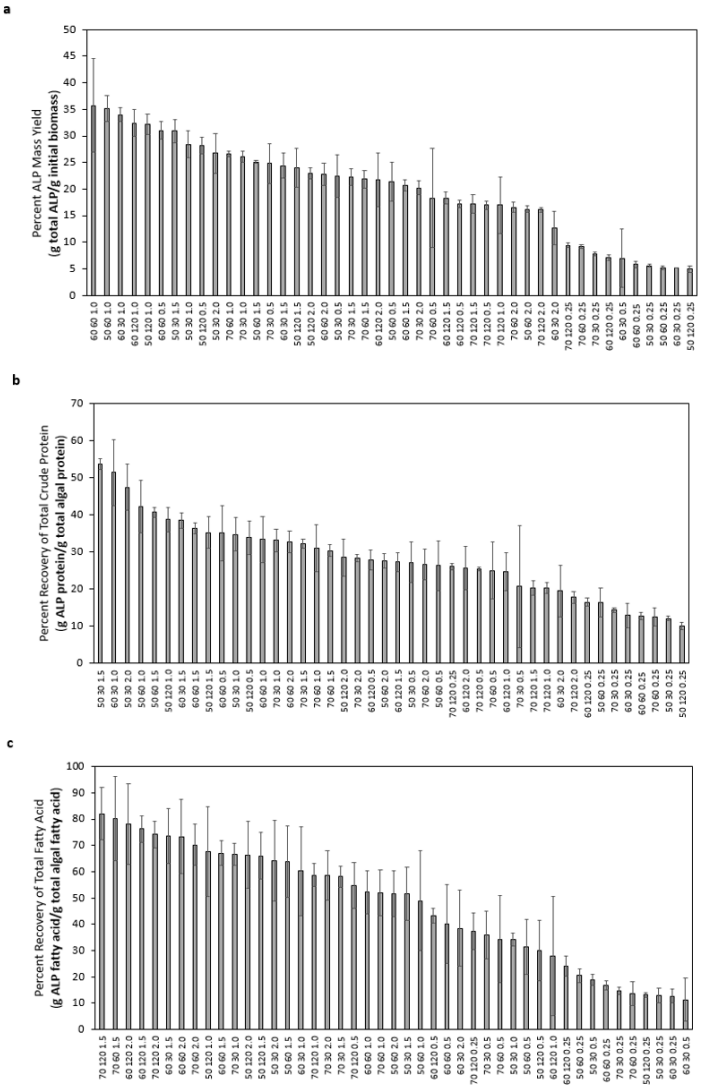

Fig. 1. ALPEP optimization results. a ALP protein mass yield. b ALP total crude protein recovery. c ALP total fatty acid recovery. Conditions are listed top to bottom as NaOH concentration (g NaOH/g dry algal biomass), reaction time (min.), and reaction temperature (□C). Error bars represent one standard deviation.

Total fatty acid recovery appears to be less sensitive to reaction conditions than crude protein recovery. This result could indicate that some of the extracted proteins are lipophilic and could be attached to fatty acids keeping the overall fatty acid recovery relatively constant at these conditions. At the lowest NaOH concentration, 0.25 g NaOH/g dry biofilm algae, minimal crude protein and total fatty acid recoveries are observed. This result suggests that a minimal or threshold amount of energy and chemical input is needed to successfully extract algal fatty acids and proteins. Under low NaOH conditions this threshold is not reached, therefore minimal products are obtained.

3.1.3. Determination of Optimal Conditions

The three highest recoveries for ALP mass, total crude protein, and total fatty acid were compared in Table 1. After accounting

for the energy and chemical input of each condition as well as the observed recoveries of products optimal conditions were determined to be 60 ^{0} C reaction temperature, 30-minute reaction time, and 1.0 g NaOH/g dry algal biomass. The ALP extracted at these conditions yields 0.34 g ALP/g dry biofilm algae, recovers 58% of total crude protein (g ALP protein/g of total algal protein), and 60% of the total fatty acids (g ALP fatty acid/g of total algal fatty acid). The composition of the crude protein (g ALP protein/g total ALP) and total fatty acids (g ALP fatty acid/g total ALP) was determined to be 29% and 3.0%, respectively.

TABLE 1

OPTIMIZATION SUMMARY OF HIGHEST RECOVERY GROUPS

	Reaction Temperature (°C)	Reaction Time (min)	Concentration (g NaOH/ g dry algal biomass)	ALP Mass Yield (g ALP/ g initial biomass)	Crude Protein Recovery (g ALP protein/ g total algal protein)	Total Fatty Acid Recovery (g ALP fatty acid/ g total algal fatty acid)
Protein Mass	60	60	1.0	0.36	33	52
	50	60	1.0	0.35	42	49
	60	30	1.0	0.34	58	60
Crude Protein	50	30	1.5	0.31	54	52
	60	30	1	0.34	51	60
	50	30	2	0.27	47	64
Total Fatty Acid	70	120	1.5	0.17	18	82
	70	60	1.5	0.22	30	80
	60	120	2	0.22	26	78

These conditions only reflect optimal recoveries and not mass fraction compositions. Composition data at each condition for ALP mass, total crude protein, and total fatty acid is given in SI Table 1. The data further demonstrates the flexibility of the ALPEP to produce more total crude protein or more total fatty acids based on the needs for protein or lipids. The intended application for this study was to supplement lactating bovine feed. Therefore, the nutritional properties of the algal lipid-protein precipitate at these conditions was investigated through fatty acid and amino acid analyses.

3.2. Scale-Up Validation

Due to time and energy cost associated with drying algal biomass, a wet algal biofilm was used to validate the ALPEP during scale-up as described in Section 2.6. The wet algal biofilm was approximately 0.093 g dry algae/g of wet algae sludge. This was used to calculate the amount NaOH to process the wet algal biofilm at the determined optimal reaction conditions (1.0 g NaOH/g of dry algal biomass). Mass yield of crude protein, from the scaled-up procedure (100 g), was comparable to small-scale (400 mg) mass yields, resulting in 0.28 ± 0.03 and 0.29 ± 0.05 g ALP protein /g total ALP, respectively. A comparison of the mass fraction of the ALP to other traditional bovine feeds [14] is given in Table 2. Both crude protein and total fatty acids from algal biomass treated at optimal conditions were in ranges similar to traditional feeds. Based on these data, the ALP is most similar to a canola supplement.

TABLE 2

FEED COMPOSITION OF CRUDE PROTEIN AND TOTAL FAT OF ALP COMPARED TO VALUES OF OTHER TRADITIONAL BOVINE FEED [14]

Feed Source	% Crude Protein	% Total Fat
Algal Lipid Protein (ALP)	28	3.0
Blood Meal	92	1.4
Canola	40	2.7
Cotton Seed	46	5.0
Dry Distiller Grains	30	9.5
Soy	49	1.5

3.3. Nutritional Analysis and Comparison

Utilizing the ALP as a feed supplement in agricultural systems effectively creates a sustainable closed-loop system between microalgal cultivation and animal nutrition. Since algae used throughout this study was cultivated from dairy wastewater, application as a feed supplement for lactating bovine was investigated. The composition of essential amino acids and fatty acids in the ALP were compared to ideal feed compositions.

3.3.1 Amino Acid Profile

Amino acids Arg, His, Ile, Leu, Met, Phe, Thr, Tyr, and Val are considered essential in producing nutrient rich milk from lactating bovines [15]. Therefore, the content of these amino acids in the ALP were compared with an ideal feed and are presented in Fig. 2.

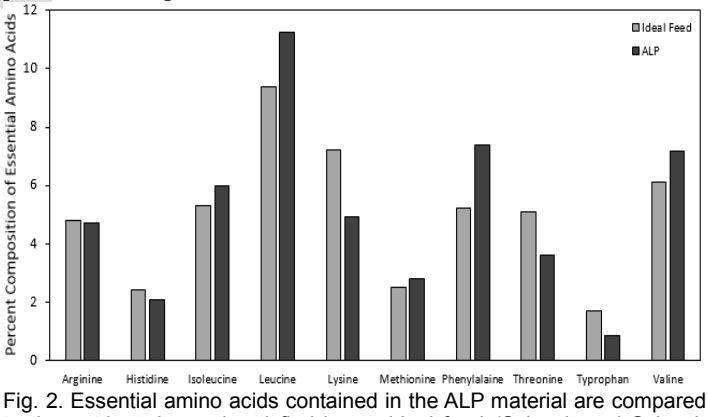

Fig. 2. Essential amino acids contained in the ALP material are compared to the nutrient demand satisfied by an ideal feed (Schwab and Schwab Consulting, 2012). Values obtained are similar to those of an ideal feed. While ideal ratios of lysine to methionine are 3 to 1 in bovine feed, the ALP exhibits a ratio of 2 to 1.

In order to maximize amino acid utilization efficiency during digestion, the ideal feed ratio of lysine to methionine is 3:1 [16]. A ratio of 2:1 was obtained based on values of 4.93 and 2.82 g /g total ALP for lysine and methionine, respectively. Furthermore, the ALP values were within 15% of ideal values for Arg, His, Ile,

and Met while possessing at least 50% of all other amino acids. Therefore, the ALP would be an acceptable protein supplement to traditional feeds. The full amino acid profile is given in SI Fig. 4.

3.3.2. Fatty Acid Profile

The fatty acid profile of the ALP is given in Fig. 3. Linolenate (C18:3) accounts for 19% of total algal fatty acid recovery. Studies indicate that high quantities of supplementary C18:3 fatty acids can lead to increased lactation [17]. The ALP also contains a high recovery of palmitate (C16:0), which accounts for more than 20.8% of extracted fatty acids. C16:0 fatty acids have been correlated with increased lactation and protein content in milk [18].

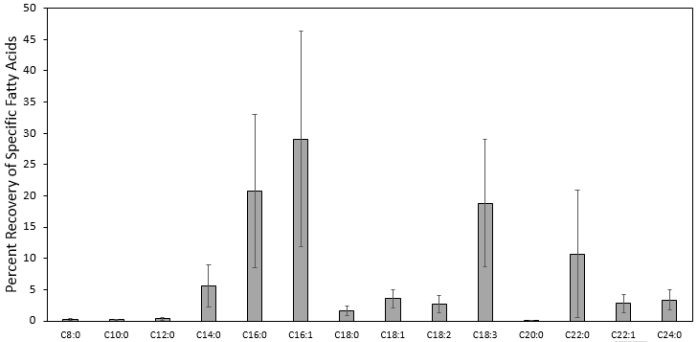

Fig. 3. Fatty acid profile of algal lipid protein precipitate under optimal conditions. ALP is composed of about 22% C18:2 and C18:3 fatty acids. C18:3 fatty acids account for 19% of the total extracted fatty acids. C18:2 fatty acids account for 3% of the total extracted fatty acids. C18:2 and C18:3 fatty acids correspond to omega-6 and omega-3 fatty acids, respectively. Error bars indicate one standard deviation.

3.4 Biochemical Mechanism for ALPEP

Considering all recovery data, the ALPEP is thought to operate as follows: (1) the addition of strong base increases the pH of solution, (2) this increased pH induces an overall negative charge to the zwitterionic amino acid residues composing the various algal proteins, (3) this negative charge allows the proteins to dissolve in aqueous solution, and (4) once separated from the insoluble cell components, the pH is decreased and the proteins become uncharged and precipitate out of solution. Similar chemical transformations occur with the fatty acid components. Under high pH, the hydrogen atom on carboxylic acid group of the fatty acids is removed, creating a sodium salt of the fatty acid. This polar molecule can be dissolved in aqueous solution and co-precipitated with a decrease in pH. Additionally, it is possible that the some of the algal proteins bind to fatty acids which can also influence their solubility and precipitation.

3.5 Future Work

Future work will include scaling up the lipid-protein extraction procedure to produce enough precipitate for live bovine feed trials. A technoeconomic model can also be developed to determine the size of agricultural system, as number of animal units, which would need to be used to make the ALPEP economically effective. Since the dry biofilm algae was derived from dairy wastewater, application as a feedstock supplement would create a sustainable and renewable source of fatty acids and proteins in bovine systems. Therefore, this study demonstrates the feasibility of using the ALPEP to create a closed-loop algal biorefinery.

4. Conclusion

The ALPEP can be applied to produce ALP material, a potential feed-supplement in agricultural systems, creating a closed-loop by recycling wastewater nutrients into valued protein and fatty acids through microalgae cultivation on dairy wastewater. Optimal reaction conditions of the ALPEP are: 60^0 C, 30 minutes, and 1.0 g NaOH/g dry algal biomass. The ALP recovers 58% crude protein (g ALP protein/g total algal protein) and 60% total fatty acids (g ALP fatty acid/g total algal fatty acid). The ALP consists of 29% protein (g ALP protein/g total ALP) and 4.6% fatty acid (g ALP fatty acid/g total ALP), similar to canola supplements.

E-supplementary data of this work can be found in the online version of the paper.

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Corresponding Author: Tyler Marlar – Utah State University, Logan Utah, tyler.marlar@aggiemail.usu.edu.