Project Report

Title of Project: Production of Omega-3 Fatty Acid-Rich Algae from Animal Protein Hydrolysate

Research Organization: Virginia Polytechnic Institute and State University;
USDA ARS, Eastern Regional Research Center

Principal Investigator: Zhiyou Wen
Department of Biological System Engineering (0303),
204 Seitz Hall, Virginia Tech,
Blacksburg, VA 24061
Phone: 540 231 9356; Fax: 540 231 3199;
E-Mail: wenz@vt.edu

Co-Investigator: Rafael Garcia
USDA-ARS, Eastern Regional Research Center
600 East Mermaid Lane
Wyndmoor, PA 19038
Phone: 215 836 3743; Fax: 215 233 6795;
E-Mail: Rafael.Garcia@ars.usda.gov

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EXECUTIVE SUMMARY

a. The problem that was studied:

The rendering industry desires new commercial outlets for its protein meal products. The overall goal of this project was to utilize hydrolysates of rendered protein as less expensive substrates for the production of high-value omega-3 fatty acids in algal culture.

Demand for omega-3 fatty acids is increasing as evidence of their beneficial effects in preventing cardiovascular diseases, cancers, schizophrenia, and Alzheimer's mounts. The traditional source of these fatty acids, wild caught fish, cannot sustainably keep up with this growing demand. Development of a mass algal culture process for producing omega-3 fatty acids may be a very attractive alternative, if it can be done inexpensively. Traditional mass algal culture is limited by the high cost of feedstocks such as yeast extract and peptone. The hypothesis investigated in this research was that hydrolysates of rendered protein can serve as a much less expensive substitute feedstocks.

b. Objectives of original proposal and the specific goals of the original project

We proposed four objectives in the original proposal: The objectives include: (1) production of a range of hydrolysates that vary in terms of hydrolysis method, degree of hydrolysis, and raw material source; (2) analysis of these hydrolysates in terms of properties hypothesized to affect algal performance; (3) investigation of algal growth and omega-3 fatty acid production by using protein hydrolysate as a source of nitrogen and micronutrients; and (4) characterization of the algal biomass produced.

c. Results achieved toward each objective of the project:

In Objective 1, we used both alkali- and enzyme-based methods to hydrolyze different animal proteins including meat and bone meal (MBM), blood meal (BM) and feather meal (FM) to create a variety of hydrolysates. The enzymes used in the enzymatic hydrolysis were Alcalase, Flavourzyme, and Versazyme. The distribution of masses of the soluble peptides decreased as the hydrolysis reaction progressed. The average masses of the peptides in the hydrolysate were larger than those present in commercial Bacto Yeast Extract (BYE), but within the range of sizes found in other commercial complex substrate such as Bacto Typtone. Most experimental hydrolysates had greater crude protein content than yeast extract. Little fat or ash from rendered protein ends up in the hydrolysate, but both alkali and enzymatic hydrolysis agents contribute significantly to the ash content of the hydrolysate.

In Objective 2, the amino acid content of the different hydrolysate samples were analyzed. All batches of hydrolysate differed in their amino acid composition from the raw rendered protein, with alkali-hydrolyzed samples having greater difference. In all alkaline batches, the relative concentrations of arginine, serine, and threonine decreased progressively with increasing reaction time. Alkali treatment of protein also resulted in the formation of some unusual cross-linked amino acids including lysinoalanine and lanthionine. In the enzyme hydrolyzed batches, all alkali- and Versazyme-hydrolyzed samples had an increased concentration of proline, hydroxyproline, glycine, and hydroxylysine suggesting a preferential hydrolysis of collagen; Alcalase- and Flavourzyme-hydrolyzed samples were not altered in this manner.

In Objective 3, animal protein hydrolysates prepared under Objective 1 were used for growing algae which can produce high levels of omega-3 fatty acids. We chose two algal species, *Schizochytrium limacinum* as a DHA producer and *Pythium irregular* as an EPA producer. Both the alkali- and enzyme-hydrolysates were tested for supporting the growth and omega-3 fatty acids production of *S. limacinum* and *P. irregulare*. The growth performance of these two algal species on the different proteins hydrolysates is shown in the table below

Algal growth performance on different protein hydrolysates

	Algal species	
	S. limacinum	*P. irregulare*
Alkali-based hydrolysate	-	+ + +
Enzyme-based hydrolysate	+	+ + + +

Note: -: no growth; +: poor; + + +: good; + + + +: excellent

As shown in the above table, the protein hydrolysate resulted in a better growth performance for *P. irregular* than *S. limacinum*. *P. irregular* had an excellent growth performance when growing on enzyme-based hydrolysate. Among three protein sources (MBM, FM, and BM), MBM and FM supported a better growth performance than BM. Using MBM and FM as a substrate, the EPA content and production yield of *P. irregular* achieved 15mg/g dry biomass, and 80 mg/L, respectively.

In Objective 4, the algal biomass obtained from enzyme-based hydrolysate was characterized. The biomass contains approximately 15% lipid, 36% protein, and 40% carbohydrate, with 9% ash. In addition to EPA (C20:5, n3), the algae also contain palmitic acid (C16:0), palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2) and arachidonic acid (C20:4) as major fatty acids. For the amino acid composition, the algae contain all essential amino acids. We further performed a through elemental analysis to identify the potential heavy metal contamination of the algal biomass, if any. No heavy metals such as mercury were found, indicating the algal biomass is suitable for use as food grade products.

d. Perspective

In summary, we produced both alkali- and enzyme-hydrolysates and characterized these samples. Then, we used the different hydrolysates to test their capabilities of supporting DHA production by *S. limacinum* and EPA production by *P. irregulare*. The results show that it is feasible to use enzyme-hydrolysates for the culture of *P. irregulare* to produce EPA. The protein hydrolysate has a great advantage in term of its cost compared with the substrate commonly used by industry (e.g. yeast extract). The algal biomass obtained from animal protein hydrolysate has a "high quality" in terms of its fatty acid composition, amino acid composition, and freedom from heavy metal contamination. In order to fully realize the potential benefits of omega-3 fatty acid production from animal protein sources, future work is needed to thoroughly optimize culture conditions and develop high cell density culture techniques.

TECHNICAL SUMMARY

SECTION I: ANIMAL PROTEIN HYDROLYSIS

Materials and Methods

Raw animal protein materials and hydrolysis reagents

Ruminant MBM and flash dried cattle blood meal were obtained from Darling International (Irving, TX); hydrolyzed feather meal was obtained from Carolina By-Products (Winchester, VA). The hydrolytic agents included Bell Mine hydrated lime, high calcium (Tannin Corp., Peabody, MA), Versazyme (BioResource International, Morrisville, NC), Alcalase and Flavourzyme (Novozymes, Bagsvaerd, Denmark).

Protein hydrolysate production

The protein materials were partially defatted by hexane extraction prior to hydrolysis. Hydrolysis was conducted in a thermostated reaction vessel with constant stirring adequate to prevent the settling of the substrate in the vessel. All reactions consisted of 9.1% (w/w) solid substrate in water. Alkaline hydrolysates were produced in 6 L batches, with 0.1 g CaOH/g substrate, at 85 °C. Individual batches were hydrolyzed for 4, 8 or 16 hours. Enzymatic hydrolysates were produced in 4 L batches at 50 °C. Individual batches used one of three sets of conditions detailed in Table 1. During enzymatic hydrolysis, pH was monitored continuously and maintained through the addition of 8M NaOH.

Alkaline hydrolysis reactions were terminated by sparging with CO_2 until the pH dropped to 9, followed by neutralization with sulfuric acid. Enzymatic reactions were terminated by raising the reaction temperature to 90 °C for 10 minutes. Residual solid material was removed by centrifugation followed by filtration through a filtration capsule (Millipore, Billerica, MA) with 0.45µm pores. The remaining hydrolysate was dehydrated using a Büchi (Flawil, Switzerland) B-191 Mini Spray Drier.

Proximate analysis

Moisture content determinations were performed by dehydrating 1 g samples at 70 °C, under approximately 100-kPa (gauge) vacuum, to constant mass, which typically took 24 hours. Ash determination was conducted according to ASTM D 2617-96 (1996) which involves overnight incineration of 2 g samples in ceramic crucibles in a 600 °C muffle furnace. Nitrogen content was determined in 3-4 replicates using an automated analyzer (model FP-2000, Leco Corporation, St. Joseph, Mich.); crude protein is reported as percent nitrogen × 6.25.

Peptide mass analysis

Each sample was run through two different size exclusion columns using a Waters 2695 Separation Module and isocratic conditions. A Superdex Peptide 10/300 GL column (GE Healthcare, Piscataway, NJ) was used to analyze the lower end of the molar mass range, and a

BioSep-SEC-S 3000 column (Phenomenex, Torrance, CA) was used to analyze the higher end of the molar mass range. The eluting solvent for the Superdex column was 50 mM aqueous HCl; that for the BioSep column was 5 mM aqueous HCl containing 10% (v/v) acetonitrile. A Varian (Palo Alto, CA) 380-LC evaporative light scattering detector was used to quantify the results.

Each column was calibrated against a wide range of standards. A calibration kit containing aprotinin, cytochrome C, carbonic anydrase, albumin, and bradykinin (Sigma, St. Louis, MO), as well as separate kit containing throglobulin, γ-globulin, ovalbumin, myoglobin, and vitamin B_{12} (Biorad, Richmond, CA) were used in calibration. To extend the calibration to lower molar masses, these were supplemented with a Ser-Gly dipeptide and a pentapeptide (Peptide 6A), obtained from Bachem Americas (Torrance, CA). Data from the two columns was combined using calculations described earlier (Garcia and Phillips, 2009).

Amino acid analysis

Samples were hydrolyzed in triplicate using a PicoTag workstation (Waters Corp., Milford, MA) according to the manufacturer's directions. Hydrolyzed samples were filtered, dried under vacuum, and derivatized with AccQFluor reagent (Waters) following the manufacturer's directions. Chromatography was performed using procedures described as "mixture 1" by van van Wandelen and Cohen (1997), with α-aminobutyric acid as an internal standard. Separate analyses were performed for cyst(e)ine, using the method described by Finley (1985) to quantitatively oxidize cysteine and cystine to cysteic acid prior to hydrolysis; these samples were then analyzed in the same manner as the other samples.

Results and Discussion

Conversion of raw animal proteins into small, soluble peptides

Microorganisms which do not secrete proteolytic enzymes cannot utilize the large size peptides and proteins in their growth medium, unless they can be converted into samller molecules and transported into their cytoplasm. Transportation across the cell membrane, into the cytoplasm, largely depends on these molecules being soluble and of low molar mass. In this respect, rendered proteins are poor candidates for meeting a microorganism's amino acid requirements, because these proteins tend to be large (Garcia and Phillips, 2009) and poorly soluble (Garcia et al., 2006). On the other hand, the amino acids in Bacto Yeast Extract (BYE), a common commercial complex nutrient source for growth media, are small, soluble and easy for most microorganisms to utilize; the number-average molar mass (M_n) of peptides in BYE is 113 Da (Table 2), indicating that it consists largely of free amino acids, i.e., single amino acids not linked to other amino acids.

Both alkali and enzymatic hydrolysis increase the solubility and reduce the molar mass of rendered proteins (Figure 1). By either hydrolysis method, most of the peptides solubilized, represented by M_n, are small, even from very early in the reaction. The distribution of masses of the solubilized peptides, however, decreases as the reaction progresses, which is shown by the weight-average molar mass (M_w) approaching the value of M_n.

The average masses of the peptides comprising the hydrolysates in the present research were invariably larger than those present in BYE. For example, eight hour treatment with Versazyme reduced the protein in either MBM or feather meal to peptides with a M_n of 459 Da; treatment of feather meal or MBM with Alcalase and Flavourzyme produced smaller peptides, with M_n of 264 and 285, respectively. While the peptides in the experimental hydrolysates were larger than those in BYE, they are within the range of sizes found in other commercial complex nutrient sources; Bacto Typtone, a widely used commercial peptone has a M_n of 331 Da. BYE and the experimental hydrolysates also differ in the range of their molar mass distributions. Examination of the distributions (Figure 2) shows that while BYE has very few peptides larger than 400 Da, both alkaline and enzymatic experimental hydrolysates have significant amounts of peptides of over 1000 Da.

Proximate analysis of protein hydrolysate

The experimental hydrolysates were very high in crude protein (Table 3), as they are composed largely of peptides and amino acids extracted from the rendered protein material used to make them. Most experimental hydrolysates had greater crude protein content than yeast extract. Little fat or ash from rendered protein ends up in the hydrolysate, but both alkali and enzymatic hydrolysis agents contribute significantly to the ash content of the hydrolysate. The ash contents of the enzymatic batches ranged from 8.5 to 15.1 % (d.b.), while those of the alkali-hydrolyzed batches were considerably higher, ranging from 14.8 to 27.2% (d.b.). In this respect, the enzymatically-hydrolyzed batches are more similar to commercial yeast extract; the yeast extract used in the present research was 11.9 % (d.b) ash.

Analysis of amino acid composition

All batches of hydrolysate differed in their amino acid composition from the rendered protein they had been made from (Table 4), though the differences for alkali-hydrolyzed batches were much greater, in general. Differences in relative amino acid composition between a rendered protein and a hydrolysate of that protein can be the result of at least two different mechanisms. Either the process of hydrolysis is destroying or creating certain amino acids, or the hydrolysis is hydrolyzing some components of the rendered protein and failing to hydrolyze other components.

In all alkaline batches the relative concentrations of arginine, serine and threonine decreased progressively with increasing reaction time (Figure 3), suggesting that these amino acids are labile in under the conditions used in the reaction. This finding is consistent with the results of an earlier study involving the alkali digestion of cattle hair (Coward-Kelly et al., 2006). Enzymatic hydrolysis had little effect on the relative concentration of these amino acids.

In addition to breaking down some amino acids, alkali treatment of protein is known to result in the formation of some unusual cross linked amino acids including lysinoalanine and lanthionine (Friedman, 1999). Lysinoalanine was absent from all samples of rendered protein as well as all samples of enzymatic hydrolysates and yeast extract. Lysinoalanine was, however, present in every alkali-hydrolyzed sample tested. The highest relative concentrations of lysinoalanine were found in alkali-hydrolyzed blood meal; this may be the result of blood meal's relatively high

concentration of lysine, a precursor to lysinoalanine. Lanthionine was absent from all rendered proteins and yeast extract; it was detected in most alkali- and enzymatic -hydrolyzed feather meal samples and a one type of enzymatically-hydrolyzed MBM. The mechanism for lanthionine formation in enzymatically-hydrolyzed batches is unclear.

All alkali- and Versazyme-hydrolyzed samples had increased concentrations of proline, hydroxyproline, glycine, and hydroxylysine; Alcalase- and Flavourzyme-hydrolyzed samples were not altered in this manner. Collagen is the only significant source of hydroxyproline and hydroxylysine and it is unusually rich in glycine and proline, so we hypothesize that the increased concentration of these amino acids in the hydrolysates is due to preferential hydrolysis of collagen in the rendered protein. Although neither blood nor feathers have significant collagen content, discussions with renderers regarding industrial practice reveals that in many cases neither blood meal nor feather meal is made exclusively from blood or feathers, respectively. In particular plants, some whole birds may be included in feather meal production, and blood meal may contain some MBM, due to the use of shared conveyance, processing and storage equipment (personal communications).

Conclusion:

Rendered animal proteins can be effectively hydrolyzed into peptides through either alkali- and enzyme-based methods. The average mass of the peptides in the hydrolysates were larger than those present in commercial Bacto Yeast Extract, but within the range of sizes found in other commercial complex nutrient sources such as Bacto Typtone. The amino acid composition of the hydrolysate, particularly from the alkali-based hydrolysis, was different from that in the raw proteins, indicating the hydrolysis process changes the amino acid profile.

SECTION II: ALGAL CULTURE ON PROTEIN HYDROLYSATE

Materials and Methods:

Algal species

Omega-3 fatty acids include docosahexaenoic acid (DHA, 22:6, n-3) and eicosapentaenoic acid (EPA, 20:5, n-3). We investigated the production of both of the two fatty acids from animal protein hydrolysate. The DHA producer was *Schizochytrium limacinum*; the EPA producer was *Pythium irregulare*. Both species were purchased from America Type Cell Collection (ATCC). We selected these species based previous reports that high levels of DHA and EPA were found in the organisms (Pyle, et al, 2008; Cheng et al., 1999).

Algal growth medium and culture conditions

The algae were maintained in the culture medium as recommend by ATCC. The medium composition for *S. limacinum* was 5 g/L glucose, 1.0 g/L yeast extract and 1.0 g/L peptone dissolved in artificial seawater. The artificial seawater consisted of (per liter) 18 g NaCl, 2.44 g $MgSO_4$, 0.6 g KCl, 1.0 g $NaNO_3$, 0.3g $CaCl_2 \cdot 2H_2O$, 0.05 g KH_2PO_4, 1 g Tris buffer (Sigma Co.),

0.027 g NH_4Cl, 15.0 x 10^{-8} g vitamin B_{12}, 10 mL/L PI metal solution, and 3 mL/L chelated iron solution (Starr and Zeikus, 1993). The medium compositions for *P. irregulare* were 20 g/L glucose and 10 g/L yeast extract. The pH for these media was adjusted to around 7.5-8.0 before autoclaving at 121 ℃ for 15 min.

The cells were grown in 250-mL Erlenmeyer flasks each containing 50 mL of medium and incubated at 20 °C in an orbital shaker set to 170 rpm. In the studies of omega-3 fatty acids production from animal protein hydrolysate, yeast extract was replaced as the nutrient source by protein hydrolysate prepared from Section I. All the other components were the same as those used in the subculture. For each experimental condition, three replicates were used, and the standard deviations were calculated.

Analyses of cell dry weight

To determine cell dry weight, 5 mL cell suspension sample was transferred to a pre-weighed centrifuge tube and centrifuged at 3444 g for 5 min. The cell pellet was then washed twice with distilled water, and then dried at 80 °C to constant weight.

Proximate Analysis

Algal cells were harvested and freeze-dried for proximate analysis. The lipids from the freeze-dried biomass were extracted and quantified by the Bligh and Dyer method (Bligh and Dyer, 1959). The protein content was estimated by summation of each amino acid. The ash content was determined by heating the sample at 550 °C overnight and weighing the remaining matter. The carbohydrate was then calculated by subtraction.

Fatty acid analysis:

Algal cells were freeze-dried for fatty acid analysis. Fatty acid methyl esters (FAME) were prepared from dried algal biomass according to the protocol developed by Indarti et al. (2005). In short, the method involved a 4 mL mixture of methanol, concentrated sulfuric acid, and chloroform (1.7:0.3:2.0, v/v/v) being added into a tube containing ~20 mg of dried cell biomass and 1 mg heptadecanoic acid (C17:0) as an internal standard. The tubes were heated in a water bath at 90 ºC for 40 min, and then later cooled down to room temperature, at which point 1 mL of distilled water was added. The liquid in the tubes were thoroughly mixed through a vortex for 1 min, and then settled for separation of the two phases. The lower phase containing the FAME was transferred to a clean vial and dried with anhydrous Na_2SO_4. One-half mL of dried solution was transferred into a vial and analyzed using gas chromatography.

A Shimadzu 2010 gas chromatograph (Shimadzu Scientific Instruments, Inc. Columbia, MD) was used for FAME analysis. The GC was equipped with a flame-ionization detector and a SGE Sol Gel-Wax™ capillary column (30m×0.25mm×0.25um). The injector was kept at 250 °C, with an injection volume of 1 µl by split injection mode (ratio: 10:1). The profile of the column temperature was as follows: 80 °C for 0.5 min; raised to 175 °C at 30 °C/min; raised to 260 °C at 5 °C/min; maintained for 6 min; raised to 280 °C at 30 °C/min; maintained for 1 min. Helium was used as the carrier gas. The detector temperature was kept at 300 °C. The fatty acids of the algae

sample were identified by comparing the retention times with those of standard fatty acids (Sigma, MO). To quantify the fatty acids, first, the response factor of each fatty acid was determined by GC-running the FAMEs of the fatty acid and the internal standard at equal amount, and comparing the peak area of the fatty acid to that of the internal standard (C17:0). The fatty acids of the algal sample were then quantified by comparing their peak area with that of the internal standard (C17:0).

Amino acid analysis

Freeze-dried algal biomass was first de-fatted with hexane to reduce interference. The de-fatted biomass was then prepared and analyzed as described in the Section I of this report.

Elemental analysis

Elemental composition of the algal biomass was determined by an inductively coupled plasma semi-quantitative scan of 69 elements according to EPA method SW- 846 6010B (SuperScan 69 performed by Prochem Analytical Inc., Elliston, VA). The EPA method SW-846 7471A was further used to detect any trace amount of mercury possibly contained in the biomass; the detection limit for this measurement was 0.025 ppm (25 ppb).

Results and Discussion:

Feasibilities of producing omega-3 fatty acids by the selected algal strains

We first studied the feasibility of using animal protein hydrolysates for growing the algae *Schizochytrium limacinum* and *Pythium irregulare* and their omega-3 fatty acids production levels. Both the alkali- and enzyme-hydrolysates were tested. The results are reported as follows.

(i) Alkali-hydrolysate as a feedstock for supporting DHA-producer *S. limacinum*. When the alga *S. limacinum* was incubated in media prepared with alkali-hydrolysates, there was no cell growth observed. The results indicate that alkali-hydrolysates of animal proteins are not a suitable replacement for yeast extract in the culture of *S. limacinum* to produce DHA.

(ii) Enzyme-hydrolysate as a feedstock for supporting DHA-producer *S. limacinum*. When the enzyme-hydrolysate was used for growing *S. limacinum*, the growth performance improved compared with that growing on alkali-hydrolysate, however, the cell growth was still very poor, as a little increase of biomass was obtained after 5-6 days of culture (data not shown). The biomass obtained was much less than that from yeast extract culture. Overall, the results indicate that enzyme-hydrolysate was not a suitable replacement for yeast extract in the culture of *S. limacinum*.

(iii) Alkali-hydrolysate as a feedstock for supporting EPA-producer *P. irregulare*. The alga *P. irregulare* was also grown in medium containing alkali hydrolysates as a feedstock. The results are presented in Figure 4. Compared with *S. limacinum*, the growth performance of *P. irregulare* was much better. Among the three rendered protein types, feather meal hydrolysates

supported the best cell growth. However, the EPA contents from all three meals were similar. Combining the cell biomass and EPA cellular content effects, we found that the high EPA production was obtained from feather meal. Based on the above results, we conclude that alkali hydrolysates of animal proteins can serve as a feedstocks for growing the fungus *P. irregulare* to produce EPA, although the final EPA production level is still lower than the yeast extract culture (Figure 4).

(iv) Enzyme-hydrolysate as a feedstock for supporting EPA-producer *P. irregulare*. The enzyme hydrolysates were also used as a feedstock for the fungus *P. irregulare* to produce EPA. The results are shown in Figure 5. The fungus grew very well on meat and bone meal and feather meal hydrolysates, but the growth was poor on blood meal hydrolysates. Although the EPA cellular content from blood meal hydrolysates was a little higher, the final EPA concentration in the culture solution was still low in the blood meal culture, due to the low cell biomass (Figure 5). The final EPA production from different meat and bone meal, and feather meal hydrolysates had a similar trends. It was also found that the EPA production levels from the meat and bone meal and feather meal were still somewhat lower than the yeast extract culture, however, this difference was not as significant as those in the alkali-hydrolysate cultures (Figures 4 and 5). With the further process optimization, the production level from these two enzyme-hydrolysates can be increased.

Kinetics of cell growth and EPA production of P. irregulare on enzyme hydrolysate

Since feasibility studies have shown that enzyme hydrolysate was a good feedstock for the alga *P. irregular* to produce EPA, the cell growth kinetics and EPA production level was further studied. *P. irregulare* was grown in medium containing 20 g/L glucose and 10 g/L protein hydrolysate (MBM hydrolysate). The maximum biomass value reached to 6.91 g/L at day 6 when glucose was almost consumed completely. As shown in Table 6. The specific growth rate was 0.512 d^{-1} and the growth yield coefficient based on glucose was 0.34 g/g. The EPA content, yield and productivity were 13.98 mg/g, 96.64 mg/L, and 16.10 mg/L-day, respectively. All these data were comparable with the culture in which yeast extract was used (Athalye et al., 2009).

Algal biomass composition

The previous study showed that enzymatic protein hydrolysate resulted in the best growth performance for the alga *P. irregulare* and the EPA production level was relatively high. To evaluate the "quality" of algal biomass in terms of its potential use as human food or animal feed additives, we characterized the chemical compositions of the algal biomass growing on enzyme hydrolyzed MBM.

Table 7 shows the proximate analysis of the biomass. The lipid, protein and carbohydrate were the three major components of the biomass. The non-protein nitrogen content of the biomass was 2.31% (Table 7). The fatty acid profile of the algal biomass is presented in Tables 8. The major fatty acids were C16:0 and C18s with EPA accounting for 12% of TFA. In addition to EPA, the fungus also contained small amounts of C14:0 and C20:4 (arachidonic acid, AA). The amino acid composition shows that the algal biomass was rich in asparagine + aspartic acid and

glutamine + glutamic acid (more than 10% of total protein each) followed by leucine and lysine (Table 9).

We further performed a through elemental analysis to identify the potential heavy metal contamination of the algal biomass, if any. We used an inductively coupled plasma semi-quantitative scan of 69 elements according to EPA method SW- 846 6010B (SuperScan 69 performed by Prochem Analytical Inc., Elliston, VA) to quantify 69 element. The elements and their detection limits are listed in Table 10. The elements detected in the algal biomass are shown in Table 11. No heavy metals such as mercury were found, indicating the algal biomass is suitable for use as food grade products.

Conclusion

Enzyme-hydrolyzed animal proteins can support a good growth performance of the alga *P. irregulare* for producing high levels of EPA. Among the three protein sources, MBM and feather meal resulted in better growth performance than blood meal. The algal biomass contained about 15% lipid, 36% protein, and 40% carbohydrate, with 9% ash. In addition to EPA, the biomass was also rich in the essential amino acids lysine, arginine and leucine, relative to many common feedstuffs. Elemental analysis by Inductively Coupled Plasma showed that aluminum, calcium, copper, iron, magnesium, manganese phosphorus, potassium, silicon, sodium, and sulfur, and zinc were present in the biomass, while no heavy metals (such as mercury and lead) were detected. The results show that it is feasible to use animal protein hydrolysate as a feedstock for producing algal biomass that can serve as EPA fortified food or feed.

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Appendix: Figures and tables

Table 1. Conditions used in different enzymatic hydrolysis treatments.

Treatment	pH	Amount of enzyme	Duration
Versazyme	7.5	8 mg/g substrate	8 hours
Alcalase	8.5	0.4 AU/ g substrate	4 hours
Alcalase + Flavourzyme	8.5/7.0	0.4 AU/ g substrate/50 LAPU/g subs	4 hours with Alcalase only, followed by 4 hours with Flavourzyme added

Table 2. Molar mass distribution of selected hydrolysates and yeast extract. M_n and M_w are two different measures of the average molar mass and PD is a measure of the range of molecular weights in a sample. Samples labeled 'alkaline' are 16-hour alkali hydrolyzed batches, and those labeled 'enzymatic' are 8-hour Versazyme hydrolyzed batches.

	hydrolysis	M_n	M_w	PD
MBM	alkaline	295	2090	7.1
	enzymatic	459	2284	5.0
feather meal	alkaline	433	1939	4.5
	enzymatic	459	1955	4.3
blood meal	alkaline	545	2008	3.7
	enzymatic	194	669	3.5
yeast extract	autolysis	113	915	8.1

Table 3. Proximate analysis of representative hydrolysate batches and yeast extract. Values reported are ± 1 standard deviation. Moisture and ash analyses were repeated 3 times, crude protein analysis was repeated 3-4 times. Use of the conventional 6.25 factor in determining crude protein results in some proximate compositions of >100%.

	hydrolysis	% moisture	% crude protein (d.b.)	%ash (d.b.)
MBM	alkaline	2.9 ± 0.2	75.9 ± 2.2	22.9 ± 0.5
	enzymatic	2.3 ±0.1	77.7 ± 0.3	14.9 ± 0.5
feather meal	alkaline	2.5 ± .02	85.0 ± 0.6	20.5 ± 0.1
	enzymatic	1.4 ± 0.1	88.4 ± 2.3	11.1 ± < 0.1
blood meal	alkaline	2.2 ± 0.3	69.3 ± 0.9	14.8 ± 0.2
	enzymatic	2.3 ± 0.1	95.0 ± 0.7	8.5 ± 0.1
yeast extract	autolysis	4.7 ± < 0.1	61.2 ± 0.7	11.9 ± 0.1

Table 4. Relative amino acid composition of selected hydrolysate batches. Batches labeled 'alkaline' are those that were hydrolyzed for 16 hours; batches labeled 'enzymatic' are those treated with Alcalase and Flavourzyme. Values are the average of 2-3 repetitions ± 1 standard deviation. "<dl" means that concentration of that component was below the detection limit for the analytical method used.

Raw material	**Meat & bone meal**			**Hydrolyzed feather meal**			**Blood meal**			**Yeast extract**
Hydrolysis method	unhydrolyzed	alkaline	enzymatic	unhydrolyzed	alkaline	enzymatic	unhydrolyzed	alkaline	enzymatic	autolysis
Amino acid	(parts per thousand)									
asx*	78.7 ± 1.3	71.9 ± 0.1	82.7 ± 0.5	82.4 ± 0.6	80.2 ± 4.0	80.8 ± 0.4	100.3 ± 0.9	121.2 ± 1.4	101.9 ± 0.2	99.0 ± 0.8
ser	34.2 ± 0.9	14.0 ± 0.4	35.4 ± 0.3	46.1 ± 0.5	19.4 ± 0.5	44.6 ± 0.4	46.2 ± 0.4	20.0 ± 0.5	47.5 ± 0.9	43.2 ± 0.1
glx*	141.8 ± 2.4	141.3 ± 0.5	147.5 ± 0.8	140.8 ± 0.9	144.0 ± 6.0	144.4 ± 2.5	89.4 ± 0.7	92.1 ± 0.8	90.3 ± 0.2	189.6 ± 1.8
gly	107.7 ± 2.3	168.7 ± 0.4	105.8 ± 0.5	84.9 ± 0.2	140.9 ± 4.7	86.1 ± 1.2	37.8 ± 0.1	74.5 ± 0.8	38.6 ± 0.1	44.9 ± 0.5
his	25.5 ± 0.6	24.8 ± 0.6	27.4 ± 0.4	26.4 ± 0.3	23.4 ± 0.3	21.6 ± 0.9	63.4 ± 0.5	63.7 ± 0.7	65.0 ± 0.3	21.3 ± 0.4
NH3	17.9 ± 2.3	14.6 ± 0.9	14.3 ± 0.2	18.3 ± 0.5	15.8 ± 1.0	14.8 ± 0.7	13.3 ± 0.6	11.4 ± 1.5	11.7 ± 0.2	23.3 ± 0.1
arg	74.0 ± 6.6	42.8 ± 0.1	67.3 ± 0.3	74.6 ± 0.1	46.6 ± 1.2	70.0 ± 0.3	41.2 ± 0.1	24.5 ± 0.2	41.2 ± 0.1	54.5 ± 0.7
thr	30.6 ± 0.7	9.4 ± 0.2	32.8 ± 0.2	36.8 ± 0.3	11.0 ± 0.4	37.1 ± 0.2	43.9 ± 0.4	10.6 ± 0.2	46.2 ± 0.4	44.9 ± 0.4
ala	68.3 ± 1.2	87.1 ± 0.7	70.2 ± 0.6	62.1 ± 0.2	76.4 ± 1.8	64.1 ± 1.6	76.8 ± 1.2	100.4 ± 1.1	75.3 ± 1.0	66.4 ± 0.6
pro	80.1 ± 1.5	110.8 ± 3.6	78.3 ± 2.7	66.3 ± 0.2	93.9 ± 3.4	68.0 ± 2.4	36.0 ± 0.5	41.2 ± 1.8	38.4 ± 1.5	41.0 ± 2.0
tyr	34.2 ± 0.8	20.8 ± 0.1	32.3 ± 0.3	36.5 ± 0.5	29.8 ± 1.0	25.4 ± 1.0	31.8 ± 0.3	36.4 ± 0.4	31.8 ± 0.4	25.6 ± 0.3
val	50.2 ± 0.7	39.2 ± 0.7	50.2 ± 0.3	53.4 ± 0.7	46.9 ± 0.3	55.0 ± 0.5	88.5 ± 1.0	81.7 ± 1.1	88.5 ± 1.1	61.4 ± 1.8
ile	36.8 ± 0.9	25.1 ± 0.5	35.2 ± 0.2	44.6 ± 0.4	35.8 ± 0.2	44.2 ± 0.5	5.8 ± 0.1	6.9 ± 0.6	6.2 ± 0.1	53.3 ± 1.8
leu	72.1 ± 0.8	67.8 ± 1.2	68.0 ± 0.1	78.2 ± 0.8	76.6 ± 0.1	75.8 ± 0.9	128.8 ± 0.7	109.3 ± 1.4	123.9 ± 1.5	77.4 ± 2.1
lys	51.5 ± 0.3	35.4 ± 0.3	49.8 ± 0.1	63.8 ± 0.8	40.1 ± 1.8	58.2 ± 1.1	96.4 ± 1.3	88.6 ± 0.2	96.8 ± 0.2	80.1 ± 1.4
phe	40.2 ± 0.5	31.2 ± 0.6	37.6 ± 0.2	44.4 ± 0.3	38.9 ± 0.7	39.5 ± 0.9	78.1 ± 0.5	60.9 ± 0.7	75.5 ± 0.9	44.4 ± 1.3
hyl	2.7 ± 0.2	7.2 ± 0.6	4.2 ± 0.6	2.3 ± 0.1	5.6 ± 0.2	3.9 ± 0.4	1.1 ± 0.3	1.5 ± 0.2	3.2 ± 0.7	0.5 ± 0.1
lal	< dl	3.3 ± 0.5	< dl	< dl	10.3 ± 0.0	< dl	< dl	25.7 ± 1.0	< dl	< dl
lan	< dl	< dl	< dl	< dl	< dl	15.2 ± 4.5	< dl	< dl	< dl	< dl
hyp	21.5 ± 0.4	72.2 ± 0.4	44.1 ± 0.1	13.5 ± 0.1	47.9 ± 1.1	27.7 ± 0.8	0.4 ± 0.0	2.8 ± 0.0	0.5 ± 0.0	< dl
cys**	8.1 ± 2.8	< dl	3.2 ± 1.2	5.5 ± 2.5	2.0	6.4 ± 2.7	5.9 ± 2.4	1.5	4.2 ± 1.9	5.6 ± 0.4
met**	24.1 ± 3.9	12.1 ± 0.3	13.6 ± 0.5	18.9 ± 4.8	15.5 ± 1.9	17.1 ± 6.1	15.1 ± 4.6	26.1 ± 0.7	13.4 ± 0.6	23.7 ± 11.9

*glx = glu + gln ; asx = asp + asn

**cys and met were oxidized quatitatively and measured as cysteic acid and methionine sulfone, respectively.

Table 5. Sample ID codes for the alkali- and enzyme-hydrolysates

	ID code	description
Alkali hydrolysates	MA4	meat and bone meal, alkaline hydrolysis, 4 hours
	MA8	meat and bone meal, alkaline hydrolysis, 8 hours
	MA16	meat and bone meal, alkaline hydrolysis,16 hours
	FA4	feather meal, alkaline hydrolysis, 4 hours
	FA8	feather meal, alkaline hydrolysis, 8 hours
	FA16	feather meal, alkaline hydrolysis,16 hours
	BA4	blood meal, alkaline hydrolysis, 4 hours
	BA8	blood meal, alkaline hydrolysis, 8 hours
	BA16	blood meal, alkaline hydrolysis,16 hours
Enzyme hydrolysates	BV	blood meal, Versazyme hydrolysis,
	MV	meat and bone meal, Versazyme hydrolysis,
	FV	feather meal, Versazyme hydrolysis,
	B ALC	blood meal, Alcalase hydrolysis,
	M ALC	meat and bone meal, Alcalase hydrolysis,
	F ALC	feather meal, Alcalase hydrolysis,
	B ALC FLA	blood meal, Alcalase and Flavourzyme hydrolysis,
	M ALC FLA	meat and bone meal, Alcalase + Flavourzyme hydrolysis,
	F ALC FLA	feather meal, Alcalase and Flavourzyme hydrolysis,

Table 6. The cell growth and EPA production parameters of *P. irregulare* [a]

Parameters	**Unit**	**Value**
Max. cell dry wt, X_{max}	g/L	6.91 ± 0.43
Specific growth rate	day^{-1}	0.512 ± 0.023
Biomass productivity	g/L-day	1.15 ± 0.09
Growth yield, $Y_{X/S}$	g/g	0.34 ± 0.02
EPA content	mg/g DW	13.98 ± 1.01
EPA yield	mg/L	96.64 ± 7.25
EPA productivity	mg/L-day	16.10 ± 1.21

[a] Data are means of three replicates ± standard deviations

Table 7. Proximate analysis of freeze dried algal biomass [a]

Composition	mass % of dry biomass
Lipid	15.29 ± 0.59
Protein	35.64 ± 1.41
Carbohydrate	40.09 ± 0.74
Total nitrogen	8.02 ± 0.04
Non-protein nitrogen	2.31 ± 0.07
Ash	8.97 ± 0.63

[a] Data are means of three replicates ± standard deviations.

Table 8. Fatty acid composition and total fatty acid (TFA) content of *P. irregulare*

Fatty acid	Unit	Value
14:0	%TFA	8.23 ± 0.36
16:0	%TFA	26.1 ± 0.79
16:1	%TFA	7.10 ± 0.64
18:0	%TFA	2.68 ± 0.10
18:1	%TFA	17.72 ± 0.95
18:2	%TFA	8.73 ± 0.75
20:4 (ARA)	%TFA	8.7 ± 0.55
20:5 (EPA)	%TFA	9.90 ± 0.42
TFA content	mg/g DW	198.19 ± 7.81

[a.] Data are expressed as mean ± SD of three replicates. DW: cell dry weight.

Table 9. Amino acid content of algal biomass

Amino acid	mg/g dry, defatted biomass***	mass % of protein
asx*	41.50 ± 1.15	10.26 ± 0.10
ser	17.68 ± 0.45	4.37 ± 0.02
glx*	63.61 ± 2.40	15.73 ± 0.36
gly	17.03 ± 0.41	4.21 ± 0.02
his	10.47 ± 0.23	2.59 ± 0.01
NH3	11.52 ± 0.43	2.85 ± 0.07
arg	26.23 ± 0.51	6.49 ± 0.01
thr	19.78 ± 0.46	4.89 ± 0.05
ala	22.74 ± 0.50	5.62 ± 0.02
pro	14.48 ± 0.32	3.58 ± 0.01
tyr	16.46 ± 0.34	4.07 ± 0.04
val	23.40 ± 0.45	5.79 ± 0.05
ile	19.45 ± 0.37	4.81 ± 0.05
leu	30.81 ± 0.59	7.62 ± 0.06
lys	30.77 ± 0.50	7.61 ± 0.03
phe	19.56 ± 0.37	4.84 ± 0.04
cys**	8.41 ± 0.39	2.08 ± 0.14
met**	10.48 ± 0.41	2.59 ± 0.14

* glx = glu + gln ; asx = asp + asn
** cys and met were oxidized quantitatively and measured as cysteic acid and methionine sulfone, respectively.
***trp has not yet be measured
nd=none detected

Table 10. Detectable limits of various elements with ICP analysis.

Element	Detection Limit (ppm)	Element	Detection Limit (ppm)
aluminum	10	nickel	5
antimony	20	niobium	50
arsenic	50	osmium	10
barium	5	palladium	50
berylium	2	phosphorus	50
bismuth	10	platinum	50
boron	10	potassium	100
cadmium	2	praseodymium	50
calcium	50	rhenium	50
cerium	50	rhodium	100
chromium	2	ruthenium	50
cobalt	5	samarium	50
copper	2	scandium	10
dysprosium	10	selenium	50
erbium	10	silicon	10
europium	10	silver	2
gadolinium	20	sodium	50
gallium	20	strontium	10
germanium	50	sulfur	100
gold	20	tantalum	50
hafnium	50	tellurium	100
holminum	50	terbium	50
indium	50	thallium	50
iodine	200	thorium	50
iridium	50	thulium	50
iron	10	tin	10
lanthanum	50	titanium	2
lead	20	tungsten	50
lithium	10	uranium	100
lutetium	10	vanadium	5
magnesium	50	ytterbium	10
manganese	2	ytrtrium	2
mercury*	50	zinc	2
molybdenum	10	zirconium	10
neodymium	20		

Table 11. ICP elemental analysis of algal biomass grown on enzyme hydrolysate (Elements analyzed in Table 10, but not included in this table were not detected in any sample).

Element	Reporting Limit (mg/kg)	Average (ppm)
calcium	50	528 ± 31.11
copper	5	12.55 ± 0.21
iron	10	56.75 ± 3.89
magnesium	50	310 ±1.41
manganese	2	2.95 ± 0.64
phosphorus	50	7795 ± 134.35
potassium	100	19700 ± 1131.37
silicon	10	72.8 ± 5.52
sodium	50	1380 ± 127.28
sulfur	100	3875 ± 190.92
zinc	2	122 ± 2.00

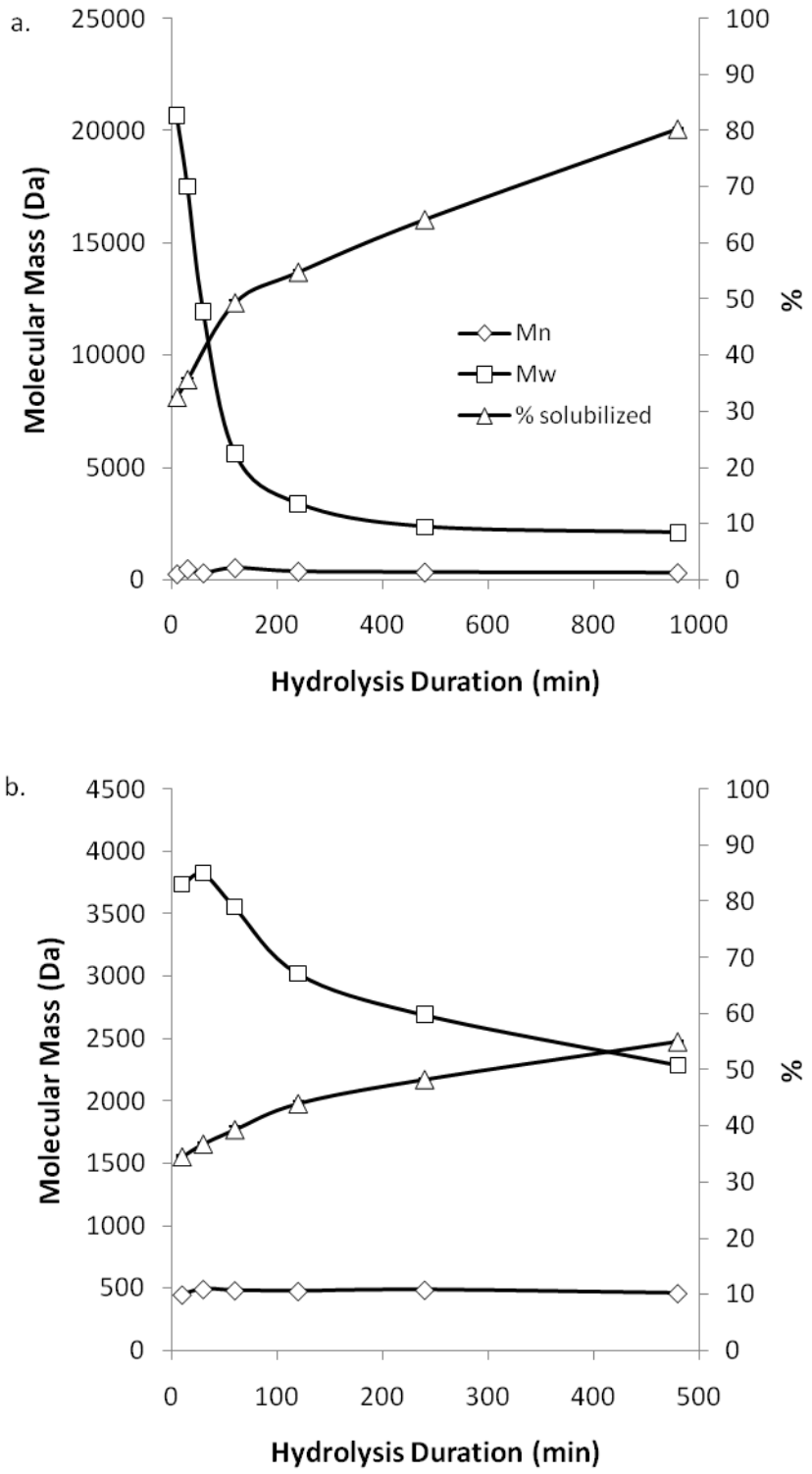

Figure 1. Change in solubility and molar mass throughout the course of the hydrolysis reaction. Results from a) alkali and b) Versazyme hydrolyzed MBM shown. Triangles represent the proportion of the rendered protein material that has been solubilized, diamonds indicate the number average molar mass (M_n) and squares represent the weight-average molar mass (M_w).

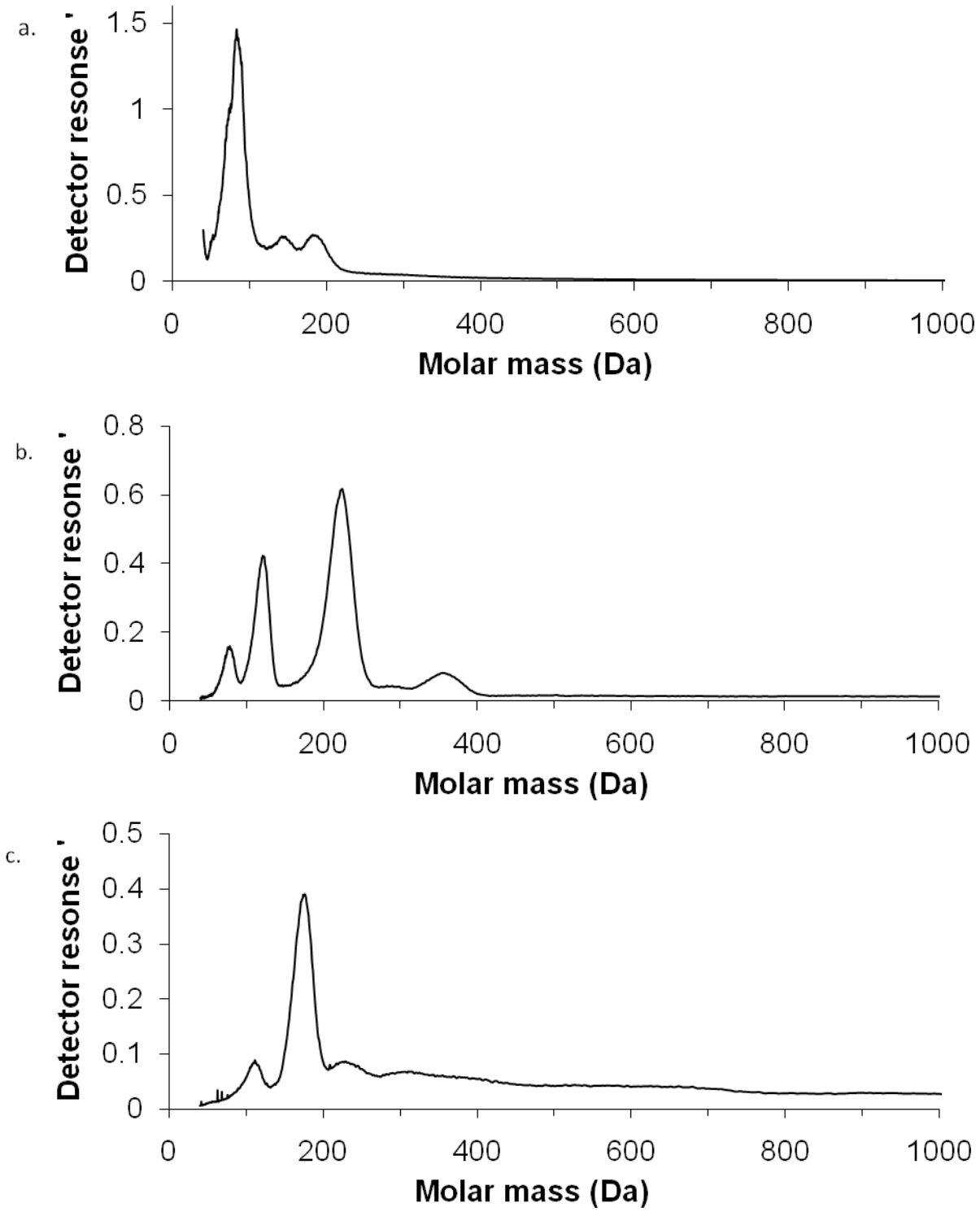

Figure 2. Hydrolysate peptide size distribution analyzed by size exclusion chromatography. Representative data includes a.) commericial Bacto Yeast Extract, b.) MBM hydrolyzed in alkali for 8 hours, and c.) MBM hydrolyzed with Versazyme for 8 hours.

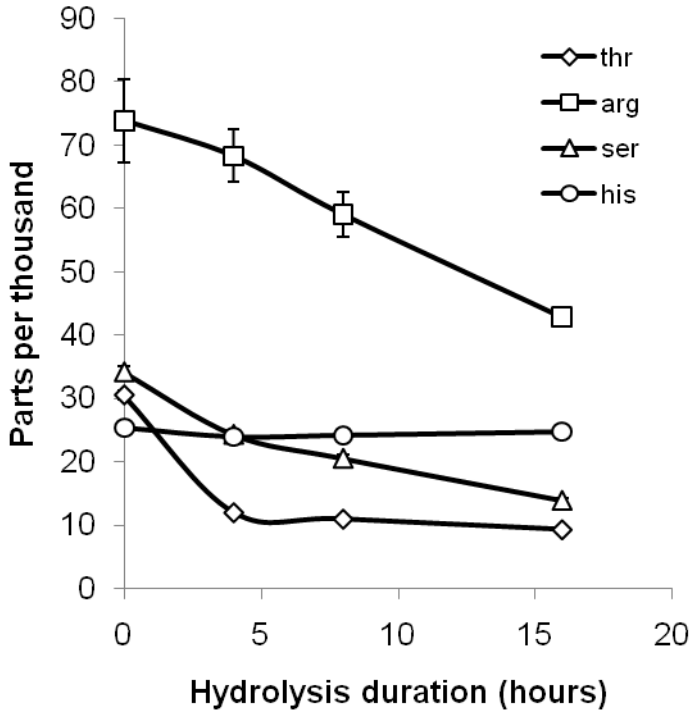

Figure 3. The progressive decrease in the relative concentration of some amino acids during alkaline hydrolysis. Data are from alkali-hydrolyzed MBM batches. Each data point represents the average of 2-3 measurements, and error bars representing one standard deviation are present for each point. Histidine is included for contrast.

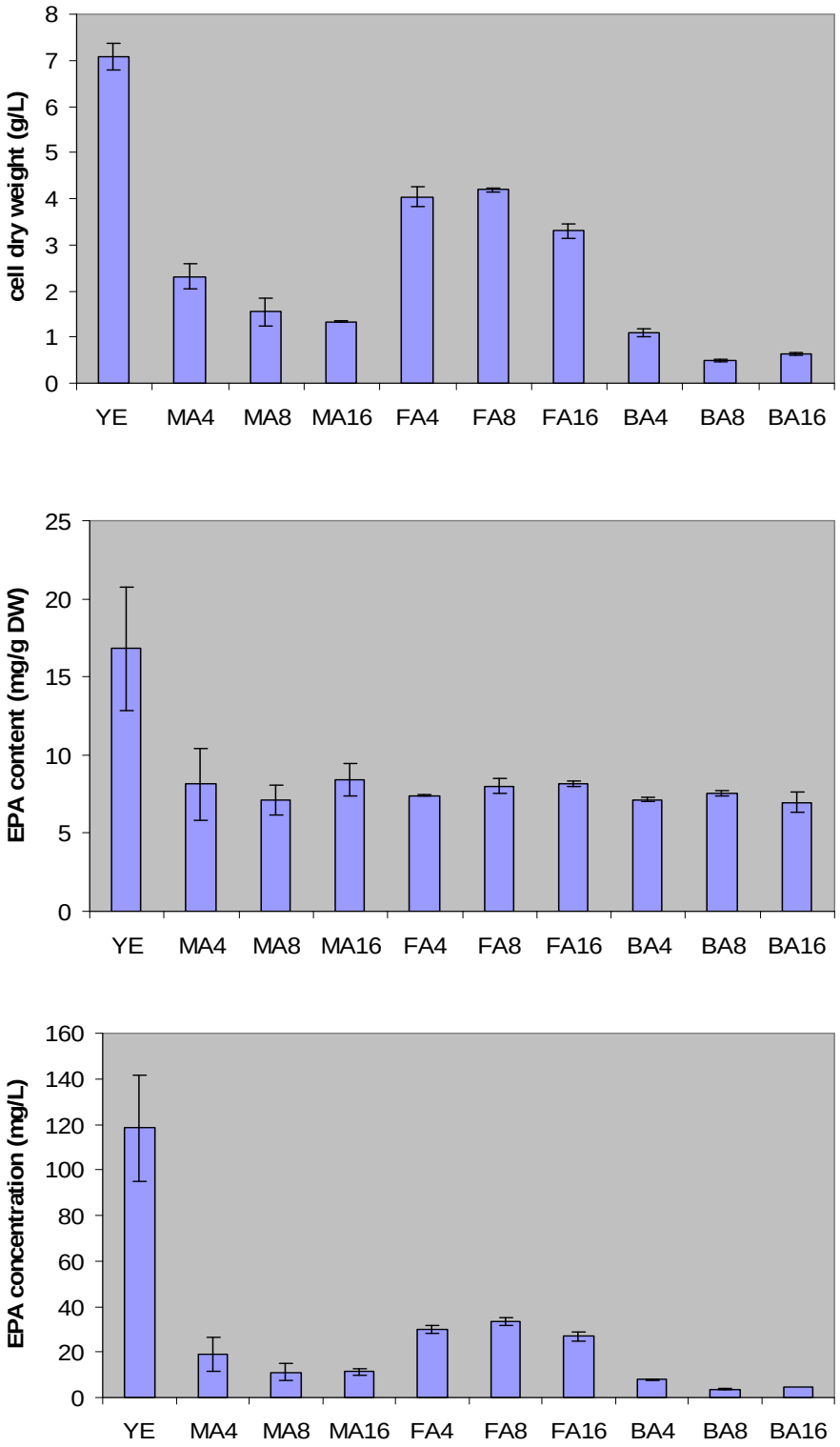

Figure 4. Cell growth, EPA content, and EPA concentration in the culture of *P. irregular* with alkali hydrolysates being used as feed stocks. (see **Table 5** for sample ID description)

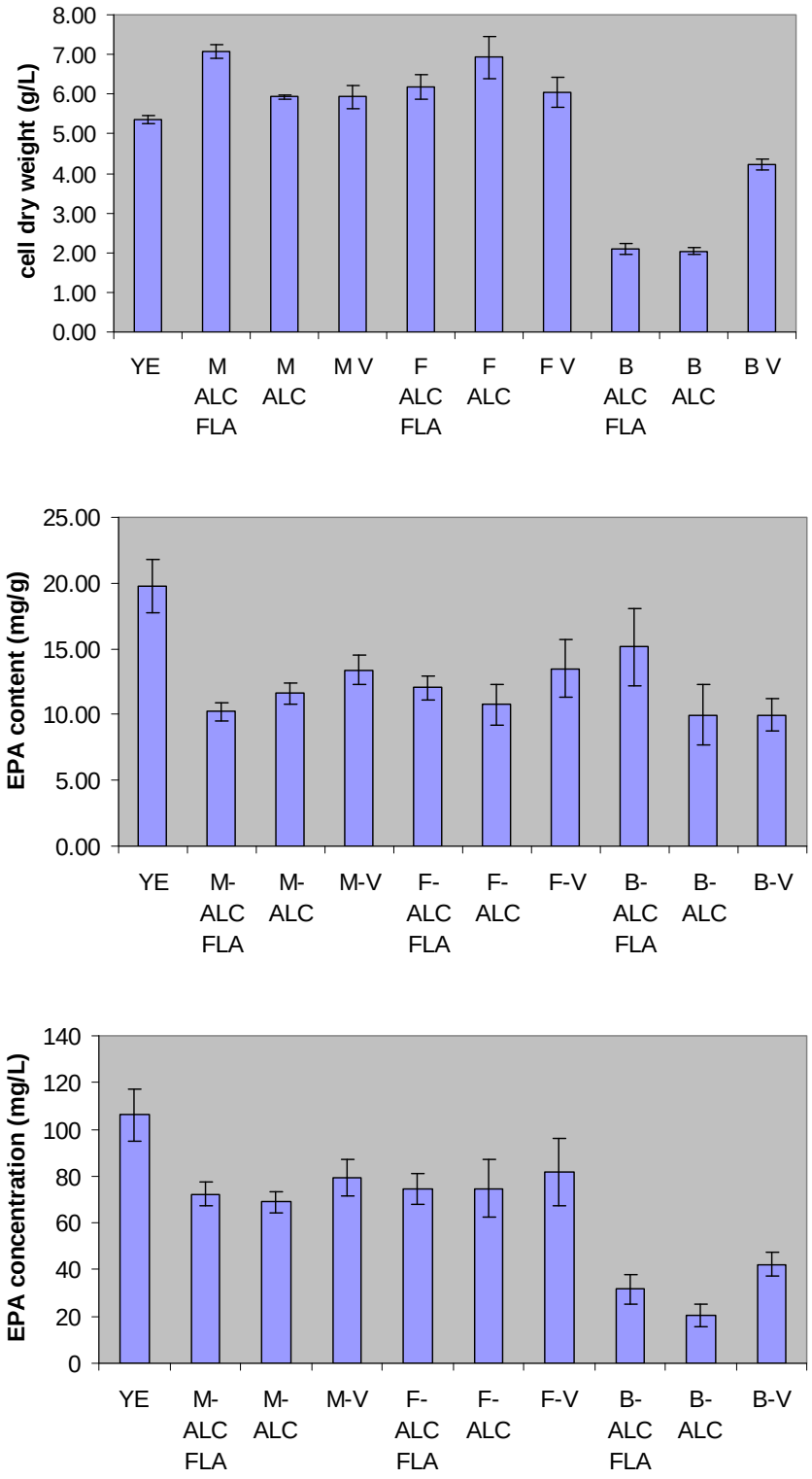

Figure 5. Cell growth, EPA content, and EPA concentration in the fungal culture with various enzymatic hydrolysates being used as feedstocks. (see **Table 5** for sample ID description)