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# Nonfeed Application of Rendered Animal Proteins for Microbial Production of Eicosapentaenoic Acid by the Fungus *Pythium irregulare*

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**ABSTRACT:** Rendered animal proteins are well suited for animal nutrition applications, but the market is maturing, and there is a need to develop new uses for these products. The objective of this study is to explore the possibility of using animal proteins as a nutrient source for microbial production of omega-3 polyunsaturated fatty acids by the microalga *Schizochytrium limacinum* and the fungus *Pythium irregulare*. To be absorbed by the microorganisms, the proteins needed to be hydrolyzed into small peptides and free amino acids. The utility of the protein hydrolysates for microorganisms depended on the hydrolysis method used and the type of microorganism. The enzymatic hydrolysates supported better cell growth performance than the alkali hydrolysates did. *P. irregulare* displayed better overall growth performance on the experimental hydrolysates compared to *S. limacinum*. When *P. irregulare* was grown in medium containing 10 g/L enzymatic hydrolysate derived from meat and bone meal or feather meal, the performance of cell growth, lipid synthesis, and omega-3 fatty acid production was comparable to the that of culture using commercial yeast extract. The fungal biomass derived from the animal proteins had 26−29% lipid, 32−34% protein, 34−39% carbohydrate, and <2% ash content. The results show that it is possible to develop a nonfeed application for rendered animal protein by hydrolysis of the protein and feeding to industrial microorganisms which can produce omega-3 fatty acids for making omega-3-fortified foods or feeds.

**KEYWORDS:** rendered protein, yeast extract, *Pythium irregulare*, lipid, omega-3 fatty acid, meat and bone meal, feather meal, blood meal

## INTRODUCTION

In meat processing, the low-value tissues (bone, offal, feather, and blood) from farm animal carcasses are typically rendered into dry meals that can be used as protein components in animal feeds or pet foods; these meals include meat and bone meal (MBM), feather meal (FM), and blood meal (BM). The traditional market for these products is maturing and, in some cases, threatening to shrink due to the concern of bovine spongiform encephalopathy caused by specific risk materials (SRMs) contained in the rendered protein products.[1] As a result, there is a need to develop a new outlet for large quantities of rendered animal proteins.

Research has been attempted to increase the nonfeed usage of the rendered animal proteins. For example, Garcia et al.[2] investigated the hydrolysis of MBM, FM, and BM to increase the solubility of the raw protein and prepare small peptides with potential nutritional value for microorganism growth. The authors used enzyme-hydrolyzed MBM as a medium ingredient for fermentation of biopolymer-producing *Escherichia coli*.[3] The non-nutritional characteristics of the protein hydrolysates, such as hygroscopicity, chromicity, autoclave stability, viscosity, and foaming, were also reported; it is indicated that rendered proteins hydrolysate can be used as a low-cost substitute for commercial peptone.[4] All of these results demonstrate an opportunity for exploring animal proteins as a low-cost nutrition source for microorganism fermentation. This is particularly attractive for industrial fermentation focusing on commodity and moderate-value products, as this type of fermentation process is characterized by a larger volume and being of moderate product value and more sensitive to the cost of growth medium ingredients.[5]

Omega-3 polyunsaturated fatty acids, including eicosapentaenoic acid (EPA, C20:5, n-3) and docosahexaenoic acid (DHA, C22:6, n-3), have been shown to possess many health-promoting properties such as prevention of human cardiovascular disease, cancer, schizophrenia, and Alzheimer's disease[6] and, thus, can be used for making various omega-3-fortified food products. In the aquaculture industry, omega-3 fatty acids are essential nutrients for cultured marine fish. Currently, fish oil is still the major commercial source of omega-3 fatty acids, although it has various limitations such as odor/taste problems, heavy metal contamination, and limited supply. Microbial fermentative production of omega-3 fatty acids for moderate-value products such as fish and animal feeds is still not economical due to the high production cost. Indeed, commercial production of omega-3 fatty acid through microbial fermentation is only available in products with high profit margins (e.g., infant formula).

In previous studies, we have developed algal and fungal fermentation processes to produce omega-3 fatty acids.[7,8] The microalga *Schizochytrium limacinum* (DHA producer) and the fungus *Pythium irregulare* (EPA producer) can use crude glycerol from the biodiesel industry as a carbon source, so the growth medium cost can be significantly reduced. However, these two microorganisms also need a large amount of peptone and yeast extract as a source of nitrogen, vitamins, and trace elements for

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their growth; the inclusion of commercial peptone and yeast extract represents another significant portion of the medium cost.[7−9] This cost could be reduced if yeast extract and peptone were replaced by relatively less expensive rendered protein meals, which can provide the required nitrogen, vitamins, and minerals. The objective of this work is to test the feasibility of using these low-cost animal protein meals as a substitute for commercial peptone and yeast extract in the fermentation of *S. limacinum* and *P. irregulare*.

## MATERIALS AND METHODS

**Raw Animal Protein Materials and Hydrolysis Reagent.** Ruminant meat and bone meal (MBM) and flash-dried cattle blood meal (BM) were obtained from Darling International (Irving, TX); feather meal (FM) 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), and Alcalase 2.4 L and Flavourzyme (Novozymes, Bagsvaerd, Denmark). Commercial Bacto Yeast Extract and Bacto Proteose Peptone were obtained from DIFCO Laboratories (Detroit, MI) and Becton, Dickinson and Co. (Sparks, MD), respectively.

**Protein Hydrolysate Preparation.** The preparation of animal protein hydrolysate was described previously.[2] In brief, the protein meals were defatted by hexane extraction followed by alkaline or enzymatic hydrolysis. The durations of alkaline hydrolysis were 4, 8, and 16 h, respectively (Table 1). For enzymatic treatment, three different enzymes were involved, and conditions are shown in Table 2. The hydrolysis reactions were terminated using the protocols described previously,[2] then the residual solid was removed from the liquid, and the remaining hydrolysate was dehydrated using a Büchi B-191 mini spray-dryer (Flawil, Switzerland).

**Table 1. Conditions Used for Alkali Hydrolysis and Hydrolysate IDs**[a]

| raw animal protein | time (h) | hydrolysate ID |
|---|---|---|
| meat and bone meal (MBM) | 4 | MA4 |
| | 8 | MA8 |
| | 16 | MA16 |
| feather meal (FM) | 4 | FA4 |
| | 8 | FA8 |
| | 16 | FA16 |
| blood meal (BM) | 4 | BA4 |
| | 8 | BA8 |
| | 16 | BA16 |

[a] Hydrolysis was conducted in saturated calcium hydroxide solution at 85 °C.

**Microorganisms, Medium, and Culture Conditions.** The microalga *S. limacinum* SR-21 (ATCC MYA-1381) and the fungus *P. irregulare* (ATCC 10951) were used. The two species were maintained in culture medium as recommended 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 of NaCl, 2.44 g of $MgSO_4 \cdot 7H_2O$, 0.6 g of KCl, 1.0 g of $NaNO_3$, 0.3 g of $CaCl_2 \cdot 2H_2O$, 0.05 g of $KH_2PO_4$, 1.0 g of Tris buffer (Sigma Co.), 0.027 g of $NH_4Cl$, $15.0 \times 10^{-8}$ g of vitamin $B_{12}$, 3 mL of chelated iron solution, and 10 mL of trace element solution including boron, cobalt, manganese, zinc, and molybdenum.[10] The medium for *P. irregulare* consisted of 20 g/L glucose and 5 g/L yeast extract. The pH for both of the two subculture media was adjusted to around 7.5−8.0 before autoclaving at 121 °C for 15 min. The cells were grown in 250 mL Erlenmeyer flasks each containing 50 mL of medium and incubated at 25 °C in an orbital shaker set to 180 rpm. In the investigation of cell growth on animal protein hydrolysates, 30 g/L glucose was used, whereas yeast extract and peptone were replaced by protein hydrolysates prepared from either alkaline or enzymatic hydrolysis. All other components were the same as those in the subculture media.

**Analyses.** *Cell Dry Weight and Glucose Concentration.* The cell dry weight of *S. limacinum* and *P. irregulare* was determined using the procedure reported previously.[7,8] Glucose concentrations were determined using the dinitrosalicylic acid (DNS) method.[11]

*Proximate Analysis.* The freeze-dried algal/fungal biomass was subjected to proximate analysis. The lipids of the algal biomass were extracted and quantified according to the Folch method.[12] The crude protein content was estimated by measuring the total Kjeldahl nitrogen (TKN) and multiplying by the conversion factor of 6.25. The carbohydrate was estimated by subtracting lipid and protein contents from the dry biomass.

*Fatty Acid Analysis.* The procedure for fatty acid methyl esters (FAME) preparation was the same as previously reported.[7] The fatty acid profile was analyzed by a Varian GC-450 gas chromatograph equipped with a flame ionization detector and a SGE SolGel-Wax capillary column (30 m × 0.25 mm × 0.25 $\mu$m). The fatty acids were identified by comparing the retention times with those of standard fatty acids (Nu-Chek Prep Inc., Elysian, MN) and quantified by comparing their peak area with that of the internal standard (C17:0).[13]

**Table 2. Conditions Used for Enzymatic Hydrolysis and Hydrolysate IDs**

| raw animal protein | enzyme treatment | pH | time (h) | hydrolysate ID |
|---|---|---|---|---|
| meat and bone meal (MBM) | Alcalase + Flavourzyme; 0.4 AU/g substrate, 50 LAPU/g substrate | 8.5/7.0 | 4 each | M ALC FLA |
| | Alcalase; 0.4 AU/g substrate | 8.5 | 4 | M ALC |
| | Versazyme; 8 mg/g substrate | 7.5 | 8 | MV |
| feather meal (FM) | Alcalase + Flavourzyme; 0.4 AU/g substrate, 50 LAPU/g substrate | 8.5/7.0 | 4 each | F ALC FLA |
| | Alcalase; 0.4 AU/g substrate | 8.5 | 4 | F ALC |
| | Versazyme; 8 mg/g substrate | 7.5 | 8 | FV |
| blood meal (BM) | Alcalase + Flavourzyme; 0.4 AU/g substrate, 50 LAPU/g substrate | 8.5/7.0 | 4 each | B ALC FLA |
| | Alcalase; 0.4 AU/g substrate | 8.5 | 4 | B ALC |
| | Versazyme; 8 mg/g substrate | 7.5 | 8 | BV |

**Table 3. Algal Growth Performance on Different Protein Hydrolysates**[a]

| | species | |
|---|---|---|
| | *S. limacinum* | *P. irregulare* |
| alkali-based hydrolysate | − | + + + |
| enzyme-based hydrolysate | + | + + + + +[b] |

[a] The cell growth performance was defined as follows: −, no growth; +, poor growth (cell has some growth but the cell density was <10% of the control); + + +, good (cell density was >10% but <80% of the control); + + + + +, excellent (cell density was at least 80% of the control). The control was the cell culture using commercial yeast extract and/or peptone. [b] Blood meal resulted in a rating of "good" (+ + +) growth performance.

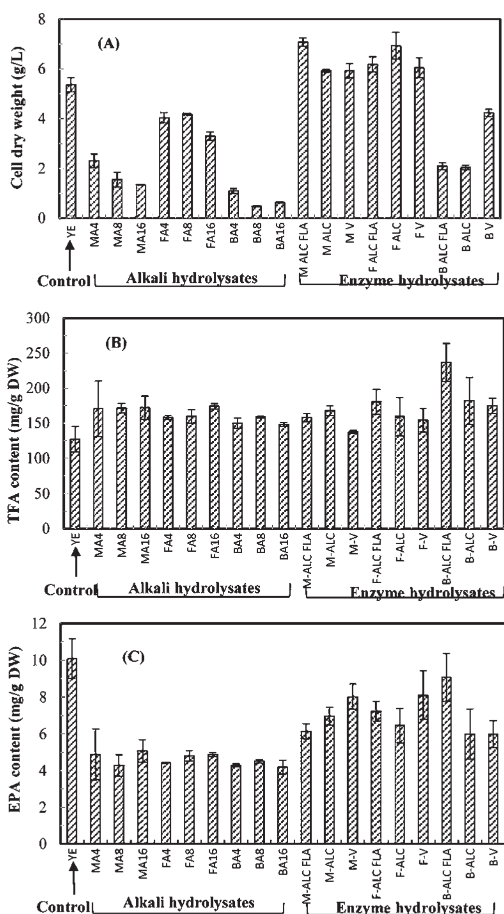


**Figure 1.** Cell growth (A), total fatty acid content (B), and EPA content (C) of *P. irregulare* grown in control (YE, yeast extract), alkali hydrolysates, and enzyme hydrolysates containing medium (refer to Tables 1 and 2 for hydrolysate IDs). Data are the mean of three replicates, and error bars show standard deviations.

**Statistical Analyses.** Each experiment was performed in three replicates with the mean and standard deviation being determined. Cell growth and fatty acid production at different experiment conditions were analyzed by one-way analysis of variance (ANOVA) using JMP software (SAS Inc., Cary, NC). The data to be analyzed were combined into a group. For each group, ANOVA was applied to evaluate the difference between mean values. A probability value of <10% ($P < 0.1$) was defined as statistically significant.

## RESULTS

**Characteristics of Animal Protein Hydrolysates.** The hydrolysates of MBM, FM, and BM prepared under various hydrolysis conditions were characterized for changes of their physical and chemical properties relating to their utility as medium ingredients. Properties including the solubility of organic matter, molar mass distribution, amino acid composition, and proximate analysis have been reported previously.[2] In brief, both alkali and enzyme hydrolyses increased the solubility of organic matters and reduced the molar mass of the rendered proteins. However, the average masses of the peptides in the hydrolysates were still larger than that of the commercial yeast extract. Both alkali and enzyme hydrolysates comprised largely peptides and amino acids extracted from the rendered protein materials.[2] Alkali hydrolysis destroyed some amino acids and created cross-linked amino acids in the hydrolysate, whereas enzyme hydrolysis affected the amino acid composition much less.[2] 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.[2]

**Feasibilities of Utilizing Animal Protein Hydrolysates by *S. limacinum* and *P. irregulare*.** Different protein hydrolysates were tested for their capability of supporting the growth of *S. limacinum* and *P. irregulare*. When *S. limacinum* was incubated in medium containing alkali hydrolysates, no cell growth was observed; when it was grown in enzyme hydrolysate supplemented medium, slight cell growth was observed, but the cell biomass accumulated after 5−6 days was still appreciably less than that from yeast extract culture (Table 3). The growth performance of *P. irregulare* on protein hydrolysate was much better than that of *S. limacinum*, with enzyme hydrolysate resulting in a better growth than alkali hydrolysate (Table 3). These results indicate that neither alkali nor enzyme hydrolysates were suitable substitutes for yeast extract in the culture of *S. limacinum*. Consequently, *P. irregulare* was selected for subsequent studies.

Figure 1 shows the quantitative results of the cell growth, total fatty acid content, and EPA content of *P. irregulare* growing in different types of hydrolysates. Overall, cell dry weight obtained from enzyme hydrolysates was significantly ($P < 0.1$) higher than that from alkali hydrolysates. Among various enzyme hydrolysates, MBM- and FM-derived hydrolysates resulted in higher ($P < 0.1$) growth performance than the BM-derived hydrolysates (Figure 1A). Although the cell growth performance from different protein hydrolysates varied widely, the total fatty acid content of the biomass was not statistically different ($P > 0.1$), being comparable to or even higher than of the control (Figure 1B). The EPA content of the protein hydrolysate derived biomass, however, was relatively low compared to the control; the trends of EPA content were similar to that of the cell growth (Figure 1C).

**Effects of Protein Hydrolysate Concentration and C/N Ratio.** The feasibility study shows that protein hydrolysate, particularly the enzyme hydrolysates of MBM and FM, can support good growth and fatty acid accumulation of *P. irregulare*. To further increase the cell growth and EPA productions of *P. irregulare*, the effects of protein hydrolysate concentrations and the C/N ratio were investigated using the MV and FV hydrolysates prepared from the hydrolysis of MBM and FM, respectively.

As shown in Table 4, cell dry weight increased with the MV from 1 to 10 g/L and leveled off from 10 to 15 g/L. Cell dry

Table 4. Effects of MV (MBM Hydrolysate) Concentration and C/N Ratio on Growth and Fatty Acid Composition of *P. irregulare*

| parameter | unit | | | | | | | control[b] |
|---|---|---|---|---|---|---|---|---|
| glucose | g/L | 30 | 30 | 30 | 30 | 30 | 30 | 30 |
| MBM hydrolysate | g/L | 1 | 2 | 3 | 5 | 10 | 15 | 10 (yeast extract) |
| C/N ratio[a] | | 85 | 43 | 28 | 17 | 9 | 6 | 10−12 |
| cell dry wt[c] | g/L | 3.34 ± 0.42 | 5.50 ± 0.41 | 7.97 ± 0.53 | 9.99 ± 0.12 | 13.09 ± 0.16 | 12.85 ± 1.38 | 10.73 ± 1.40 |
| fatty acid composition[c] | | | | | | | | |
| C14:0 | %TFA | 10.95 ± 0.48 | 11.59 ± 1.80 | 11.06 ± 0.78 | 10.98 ± 0.78 | 11.29 ± 2.53 | 11.15 ± 0.59 | 10.20 ± 0.41 |
| C16:0 | %TFA | 28.18 ± 0.83 | 29.19 ± 2.51 | 29.38 ± 0.24 | 28.24 ± 0.36 | 28.39 ± 4.73 | 28.72 ± 1.86 | 28.62 ± 1.92 |
| C16:1 | %TFA | 20.73 ± 0.53 | 20.27 ± 1.82 | 21.24 ± 0.75 | 20.87 ± 0.62 | 18.41 ± 3.16 | 16.67 ± 1.13 | 7.91 ± 1.15 |
| C18:0 | %TFA | 1.01 ± 0.09 | 1.08 ± 0.21 | 1.12 ± 0.30 | 0.91 ± 0.04 | 1.14 ± 0.17 | 1.16 ± 0.09 | 2.92 ± 0.27 |
| C18:1 | %TFA | 24.33 ± 1.15 | 24.51 ± 0.94 | 24.22 ± 0.98 | 24.66 ± 1.17 | 24.64 ± 1.90 | 23.31 ± 2.28 | 16.43 ± 0.73 |
| C18:2 (n-6) | %TFA | 9.65 ± 0.12 | 9.21 ± 1.52 | 9.03 ± 0.71 | 9.56 ± 0.72 | 9.15 ± 1.39 | 10.68 ± 1.66 | 18.07 ± 0.38 |
| C18:3 (n-3) | %TFA | 0.55 ± 0.03 | 0.57 ± 0.11 | 0.50 ± 0.03 | 0.55 ± 0.01 | 0.64 ± 0.09 | 0.75 ± 0.08 | 1.63 ± 0.07 |
| C20:4 (n-6) | %TFA | 2.07 ± 0.47 | 1.61 ± 0.43 | 1.65 ± 0.16 | 2.02 ± 0.22 | 3.04 ± 0.47 | 3.66 ± 0.93 | 6.77 ± 1.09 |
| C20:5 (n-3) | %TFA | 2.53 ± 0.38 | 1.96 ± 0.82 | 1.78 ± 0.11 | 2.23 ± 0.33 | 3.10 ± 0.23 | 3.89 ± 1.15 | 7.44 ± 1.08 |
| TFA content | mg/g DW | 166.03 ± 16.76 | 191.96 ± 2.36 | 207.40 ± 23.80 | 241.97 ± 18.17 | 226.50 ± 14.48 | 197.34 ± 30.85 | 113.48 ± 13.71 |
| EPA content | mg/g DW | 4.19 ± 0.62 | 3.77 ± 1.56 | 4.10 ± 0.92 | 5.69 ± 0.50 | 6.18 ± 0.59 | 7.62 ± 2.36 | 8.44 ± 1.14 |
| EPA yield | mg/L | 13.89 ± 1.75 | 20.46 ± 7.64 | 32.36 ± 5.36 | 56.80 ± 4.81 | 80.99 ± 8.31 | 95.79 ± 18.40 | 90.56 ± 4.16 |

[a] N content was based on total Kjeldahl nitrogen measured for MV. N content of commercial yeast extract ranged from 10.0 to 12.5% as indicated by the manufacturer. [b] Yeast extract was used. [c] Data are the mean of three replicates ± standard deviations.

Table 5. Effects of FV (FM Hydrolysate) Concentration and C/N Ratio on Growth and Fatty Acid Composition of *P. irregulare*

| parameter | unit | | | | | | | control[b] |
|---|---|---|---|---|---|---|---|---|
| glucose | g/L | 30 | 30 | 30 | 30 | 30 | 30 | 30 |
| FM hydrolysates | g/L | 1 | 2 | 3 | 5 | 10 | 15 | 10 (yeast extract) |
| C/N ratio[a] | | 99 | 50 | 25 | 20 | 10 | 7 | 10−12 |
| cell dry wt[c] | g/L | 4.30 ± 0.47 | 7.21 ± 0.49 | 8.53 ± 0.38 | 9.39 ± 1.19 | 12.51 ± 0.42 | 12.30 ± 1.11 | 10.73 ± 1.40 |
| fatty acid composition[c] | | | | | | | | |
| C14:0 | %TFA | 11.96 ± 0.22 | 12.52 ± 0.45 | 12.19 ± 1.15 | 12.47 ± 0.11 | 13.21 ± 2.25 | 12.68 ± 1.79 | 10.20 ± 0.41 |
| C16:0 | %TFA | 27.12 ± 0.38 | 28.49 ± 1.04 | 27.16 ± 0.78 | 27.64 ± 0.68 | 29.25 ± 4.29 | 27.48 ± 0.50 | 28.62 ± 1.92 |
| C16:1 | %TFA | 23.06 ± 0.34 | 23.23 ± 1.53 | 23.02 ± 1.62 | 21.47 ± 0.05 | 17.17 ± 2.34 | 18.59 ± 0.89 | 7.91 ± 1.15 |
| C18:0 | %TFA | 0.84 ± 0.04 | 0.85 ± 0.14 | 0.85 ± 0.03 | 1.12 ± 0.12 | 1.31 ± 0.42 | 0.89 ± 0.18 | 2.92 ± 0.27 |
| C18:1 | %TFA | 23.51 ± 0.59 | 23.34 ± 1.17 | 24.86 ± 2.26 | 23.73 ± 0.67 | 22.55 ± 3.20 | 23.74 ± 2.97 | 16.43 ± 0.73 |
| C18:2 (n-6) | %TFA | 8.45 ± 0.18 | 7.37 ± 0.34 | 7.56 ± 0.36 | 8.54 ± 0.61 | 10.10 ± 1.73 | 10.40 ± 0.46 | 18.07 ± 0.38 |
| C18:3 (n-3) | %TFA | 0.50 ± 0.03 | 0.45 ± 0.01 | 0.48 ± 0.05 | 0.71 ± 0.17 | 0.60 ± 0.17 | 0.58 ± 0.02 | 1.63 ± 0.07 |
| C20:4 (n-6) | %TFA | 1.82 ± 0.13 | 1.55 ± 0.05 | 1.69 ± 0.22 | 2.37 ± 0.48 | 3.06 ± 0.64 | 2.86 ± 0.11 | 6.77 ± 1.09 |
| C20:5 (n-3) | %TFA | 2.74 ± 0.17 | 2.20 ± 0.05 | 2.19 ± 0.34 | 1.97 ± 0.15 | 2.74 ± 0.26 | 2.78 ± 0.09 | 7.44 ± 1.08 |
| TFA content | mg/g DW | 198.87 ± 13.78 | 207.16 ± 19.70 | 242.78 ± 11.58 | 266.74 ± 23.05 | 268.28 ± 9.59 | 239.63 ± 6.94 | 113.48 ± 13.71 |
| EPA content | mg/g DW | 5.46 ± 0.61 | 4.57 ± 0.55 | 5.31 ± 0.84 | 5.24 ± 0.29 | 6.37 ± 0.58 | 6.66 ± 0.38 | 8.44 ± 1.14 |
| EPA yield | mg/L | 23.27 ± 0.71 | 33.08 ± 5.73 | 45.41 ± 9.03 | 49.00 ± 4.65 | 79.88 ± 9.95 | 81.77 ± 6.69 | 90.56 ± 4.16 |

[a] N content used was based on total Kjeldahl nitrogen measured for FV. N content of commercial yeast extract ranged from 10.0 to 12.5% as indicated by the manufacturer. [b] Yeast extract was used. [c] Data are the mean of three replicates ± standard deviation.

weight from the 10−15 g/L MV culture was approximately 20% higher than that of the control. The biomass contained C14:0, C16:0, C16:1, C18:1, and C18:2 as the major fatty acids, and the proportions of those fatty acids among total fatty acid were relatively stable ($P > 0.1$) with the change of C/N ratio. The total fatty acid (TFA) content was also maintained at a relatively constant level ($P > 0.1$) except at 1 g/L MV concentration. Compared to the control, *P. irregulare* grown in MV had a higher ($P < 0.1$) TFA content (mg/g DW) but lower ($P < 0.1$) EPA proportion (%TFA) and EPA content (Table 4). Eventually, MV at high concentration (10−15 g/L) resulted in an EPA yield comparable to that of the control culture (Table 4). Table 5 shows that the trends of cell growth, fatty acid profile, and EPA production of *P. irregulare* growing in FV were similar to those of MV-based culture.

**Growth Kinetics of *P. irregulare* on MV- and FV-Containing Medium.** The above results indicate that 10 g/L MV and FV with 30 g/L glucose led to the best cell growth performance and EPA yield. The kinetics of cell growth, nutrient consumption, and TFA/EPA production of *P. irregulare* were then investigated at this medium composition. The culture with 30 g/L glucose and 10 g/L yeast extract was also included as control. As shown in panels A and B of Figure 2, the trends of biomass concentration and glucose consumption of the protein hydrolysate culture were similar to those of the control. The trends of the TFA contents

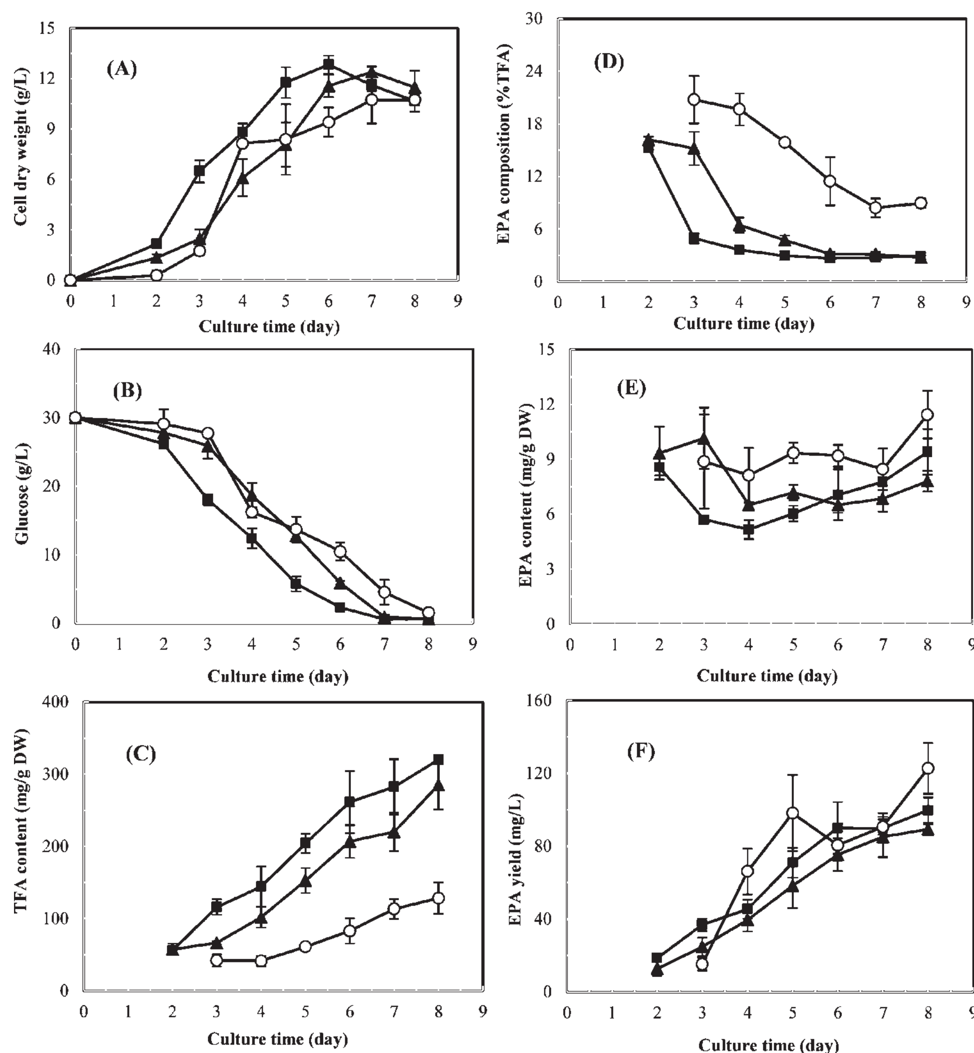


**Figure 2.** Time course of cell dry weight (A), residual glucose concentration (B), TFA content (C), EPA composition (D), EPA content (E), and EPA yield (F) of *P. irregulare* grown in medium supplemented with MV (MBM hydrolysate) (▲), FV (FM hydrolysate) (■), and yeast extract (○) as nutrients (refer to Table 2 for hydrolysate IDs). Data are the mean of three replicates, and error bars show standard deviations.

**Table 6. Cell Growth and EPA Production Parameters of *P. irregulare* in Medium Containing 30 g/L Glucose and 10 g/L Different Protein Hydrolysates**

| parameter | unit | protein hydrolysate[a] | | |
|---|---|---|---|---|
| | | yeast extract | MV | FV |
| specific growth rate, $\mu$ | day$^{-1}$ | 0.48 ± 0.07 | 0.55 ± 0.03 | 0.55 ± 0.01 |
| max cell dry wt | g/L | 10.73 ± 1.40 | 12.40 ± 0.33 | 12.83 ± 0.55 |
| biomass productivity | g/L·day | 1.53 ± 0.20 | 1.77 ± 0.05 | 2.14 ± 0.09 |
| growth yield | g/g | 0.42 ± 0.04 | 0.43 ± 0.02 | 0.45 ± 0.03 |
| EPA content | mg/g DW | 8.44 ± 1.14 | 6.85 ± 0.71 | 7.05 ± 1.39 |
| EPA yield | mg/L | 90.56 ± 4.16 | 85.02 ± 11.06 | 89.98 ± 14.18 |
| EPA productivity | mg/L·day | 12.93 ± 0.59 | 12.15 ± 0.10 | 15.00 ± 0.29 |

[a] Refer Table 2 for hydrolysate IDs. Data are the means of three replicates ± standard deviations.

were similar for all three cultures, with the protein hydrolysates consistently having a higher ($P < 0.1$) TFA content than the control (Figure 2C). The EPA composition (%TFA), however, showed the opposite trend as compared to the TFA content (Figure 2D). Figure 2E shows that the EPA content ranged from 5 to 10 mg/g DW, with a slight increase at the end of culture period. The EPA yield, however, monotonically increased with culture time (Figure 2F), indicating a later harvest time is preferred from a concentration point of view. The kinetics parameters of the three cultures are summarized in Table 6. It was found that the protein

**Table 7. Proximate Analysis of Freeze-Dried Fungal Biomass Grown on Different Protein Hydrolysates**

| | protein hydrolysate[a] (% dry biomass) | | |
|---|---|---|---|
| component | yeast extract | MV | FV |
| lipid | 18.27 ± 1.61 | 26.24 ± 1.44 | 29.58 ± 1.67 |
| crude protein | 43.69 ± 9.31 | 32.88 ± 1.38 | 34.13 ± 0.31 |
| carbohydrate | 34.67 ± 1.49 | 39.52 ± 0.55 | 34.71 ± 1.53 |
| ash | 3.36 ± 0.30 | 1.37 ± 0.06 | 1.58 ± 0.13 |

[a] Refer to Table 2 for hydrolysate IDs. Data are the mean of three replicates ± standard deviations.

hydrolysate-based culture resulted in a higher specific growth rate, maximum cell dry weight, and biomass productivity, but lower EPA content, compared to that of control. The EPA yield and productivity of the protein hydrolysate culture, however, were similar ($P > 0.1$) to those of the control (Table 6).

**Characteristics of Fungal Biomass.** The fungal biomass obtained from MV and FV media as well as the control medium (yeast extract) were further characterized for their potential nutrition value. Table 7 shows the proximate analysis of the biomass. The lipid of biomass grown in MV and FV media was significantly ($P < 0.1$) higher than that of the yeast extract culture, whereas the protein content shows an opposite trend ($P < 0.1$). The carbohydrate contents of the three cultures were similar. The fatty acid profiles of the biomass have been described in Table 4 (MV, MBM hydrolysate) and Table 5 (FV, FM hydrolysate). The two protein sources resulted in a similar fatty acid composition. Although the EPA portion in TFA was low, the TFA content of the MV- and FV-derived biomass was much higher than that of yeast extract derived biomass.

## DISCUSSION

Microorganisms that do not secrete proteolytic enzymes typically cannot utilize large peptides and proteins in their growth medium. 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[14] and poorly soluble.[15] On the contrary, amino acids in yeast extract, a common commercial complex nutrient source for growth media, are small, soluble, and easy for most microorganisms to utilize. Therefore, hydrolyzing proteins into smaller peptides and free amino acids should greatly facilitate their utilization by microorganisms.

Protein hydrolysis is usually achieved by using enzyme, alkali, or acid catalysis. Although different protein hydrolysates have been prepared and characterized in previous studies,[2,4] their effectiveness on microorganism growth performance has not been compared. In the present study, the enzyme hydrolysates resulted in better growth performance for *S. limacinum* and *P. irregulare* than alkali hydrolysates (Table 3). The reason may be that some amino acids were destroyed during the alkaline hydrolysis process. For example, compared with the original raw protein materials, the contents of arginine, serine, and threonine in the alkali hydrolysates all decreased, whereas enzyme hydrolysis caused little change in those amino acids.[2] Alkali treatment of proteins also resulted in the formation of unusual cross-linked amino acids such as lysinoalanine and lanthionine, which may not be utilized by microorganisms;[2,16] whereas the cross-linking of amino acids in enzyme hydrolysis was very minor.[2] The high ash content in alkali hydrolysates may also result in poor cell growth. It has been reported that the ash content of the alkali hydrolysate of MBM, FM, and BM ranged from 14.8 to 27.2%, which is much higher than that of enzyme hydrolysate (8.5–15.1%). The majority of the ash originated from reagents used in the hydrolysis, that is, calcium salt, which may inhibit the growth of *S. limacinum* and *P. irregulare*.[2,4] Finally, it can be speculated that the enzyme-hydrolyzed materials were richer in growth-promoting factors; some of those factors (such as B vitamins) are well-known for their sensitivity to the high pH of the alkali hydrolysis process.

In addition to the hydrolysis method, the protein source also influences growth performance. It was found that MBM and FMhydrolysates resulted in better cell growth performance than BM hydrolysate, even if the three proteins were all hydrolyzed by the same type of enzyme (Figure 1). Such a difference in the cell growth may also be caused by any number of factors, including differences in amino acid composition,[2] differences in the content of growth-promoting factors, or the presence of inhibitory substances.

The effects of protein hydrolysates on cell growth performance are also species-specific. Compared with *P. irregulare*, *S. limacinum* is more sensitive to the protein hydrolysate. The reason may be that *S. limacinum* has more complex nutritional requirements, which are not fully met by the animal protein hydrolysates. On the contrary, the growth medium for *P. irregulare* is much simpler, with glucose and yeast extract being the only ingredients. Yeast extract is generally prepared by the autolysis of yeast cells under well-controlled conditions to preserve the naturally occurring B vitamins. The primary function of yeast extract in growth media is to provide organic nitrogen as well as various vitamins and trace elements. The growth performance of *P. irregulare* (Figure 1A) indicates that the animal protein hydrolysates provided a similar function as yeast extract for the growth of *P. irregulare*. However, the total fatty acid and EPA synthesis of *P. irregulare* cells growing on protein hydrolysate medium were different from the yeast extract culture (Figure 1B,C).

Tables 4 and 5 indicate that the concentration of protein hydrolysate and the C/N ratio of the culture medium significantly affected the cell growth and fatty acid compositions of *P. irregulare*; a C/N ratio above approximately 9 was found to change growth limitation from carbon to nitrogen source. It has been reported that nitrogen source starvation usually leads to an increase in lipid content due to metabolism switching from protein synthesis to lipid and carbohydrate synthesis.[17,18] However, the results obtained from this work (Tables 4 and 5) did not show the same trend for *P. irregulare*, indicating the effects of nitrogen starvation on lipid synthesis is species-specific.

In a previous study we have characterized the biomass of *P. irregulare* when using biodiesel-derived crude glycerol and yeast extract as culture medium ingredients.[8] Compared with biomass obtained from glycerol and yeast extract, the biomass obtained from glucose and animal protein hydrolysates contained more lipid but less protein. The results indicate that the composition of the *P. irregulare* biomass highly depends on the nutrient used in the medium. Indeed, the amino acid composition in the growth medium has been reported to greatly influence the lipid accumulation of microorganisms.[19] The result obtained in this work indicates that the amino acid profile of the animal protein hydrolysates is more favorable for lipid production. Future work of a thorough characterization of amino acid profile of the fungal biomass can lead to an in-depth explanation on the applicability and effectiveness of protein hydrolysates.

The above results indicate that rendered animal proteins can be a potential nutrient source for growing microorganisms, providing that the proteins are appropriately hydrolyzed into smaller molecules. Specifically, the enzymatic hydrolysates were found to be able to replace yeast extract as nitrogen source to support growth and EPA production of *P. irregulare*. The growth performance, EPA production, and proximate composition of *P. irregulare* using enzyme protein hydrolysate are comparable to those of the culture using commercial yeast extract as nutrient supply. This work will provide a new alternative for the rendering industry as an outlet for the large amounts of animal proteins produced in their facilities. In the future, more fermentation tests using different industrial microorganisms are planned to determine the breadth of applicability of the protein hydrolysates. The hydrolysis process will also need to be optimized to prevent the loss of growth-promoting factors and improve cost effectiveness. Finally, it should be noted that a thorough characterization of fungal biomass is needed to ensure the biomass is free of mad cow disease concern and, thus, can be used as animal feed supplement.

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