

A Biorefinery Approach for Sustainable Utilization of Newfoundland aquaculture Industry's Waste Resources

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A BIOREFINERY APPROACH FOR SUSTAINABLE UTILIZATION OF NEWFOUNDLAND AQUACULTURE INDUSTRY'S WASTE RESOURCES

(FOCUS ON PROTEINS AND AMINO ACIDS EXTRACTION)

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EXECUTIVE SUMMARY

The objective of this study is to produce protein from salmon processing waste using optimized protocols and later hydrolyze proteins to obtain amino acids. Traditional hydrolysis of amino acids uses harsh chemicals such as acid or alkali to break proteins and in the process, destroy certain amino acids such as tryptophan, cysteine cannot be determined and threonine, serine and tyrosine are partially hydrolyzed. To perform this research, salmon processing waste was obtained from the local processing facilities as head, gut and frames. Each part was individually minced and some percentage of waste were mixed with each other in equal composition to obtain blended waste product. All the samples were vacuum sealed in plastic bags and stored at -80°C until further analysis. The minced salmon processing waste was subjected to enzymatic hydrolysis process to obtain protein hydrolysate which was later spray dried to obtain protein powder. Protein powder obtained from salmon processing waste was subjected to hydrolysis to obtain amino acids. In this study, the proteins are hydrolyzed using two different enzymes: Alcalase and Corolase to produce amino acids and the results are compared against the traditional acid hydrolysis processes. The acid hydrolysis process was carried out using 6N HCl at 110°C for 24 h. The enzymatic hydrolysis was carried out for 16 and 24 h using both Alcalase and Corolase at 55 and 50°C and pH 8 and 6.5, respectively. The results from this study indicated all the amino acids from the salmon processing waste using enzymatic hydrolysis were identified with comparable or better recoveries compared to acid hydrolysis process. Tryptophan was not identified during this study.

1. INTRODUCTION

The Newfoundland and Labrador fisheries and aquaculture industry in Canada is one of world's major exporters of seafood and marine products. Newfoundland and Labrador produced 28,600 tonnes of salmonids worth \$276 million in 2016. A large portion of the salmon (80%) landed in Canada is processed. Atlantic salmon remove the fillet portion of the salmon, but leave the rest (head, skin, frame and trimmings: 45-50% of the body weight) as waste or for low-value uses. Aquaculture firms dedicate a great deal of funds and resources to grow the entire salmon and when this non-fillet portion is not used effectively, this part of the investment is lost. Moreover, additional costs can be incurred in efforts to handle the resource and find a suitable means of disposal (e.g. transportation costs, tipping fees for dumping). In Newfoundland and Labrador, the problem of lost financial potential of unutilized salmon resources is worsened by the logistical and environmental issues posed by limited disposal options (composting, mink feed, rendering and landfilling). These salmon resources are a rich source of essential high value nutraceuticals and pharmaceuticals including: collagen, gelatin, protein, amino acids, bioactive peptides, omega-3 fatty acids, glycosaminoglycans, oil, calcium and enzymes. Salmon non fillet portion typically comprises of 15-19% proteins and 22-25% fat, 53-60% moisture in addition to minerals. Salmon frames and heads contain significant amounts of highly nutritious, easily digestible muscle proteins, nutritionally superior compared to plant proteins and have a better balance of the dietary essential amino acids compared to all other animal protein sources. Salmon waste resources has potential to produce approximately 1900-2400 tonnes of protein/amino acids worth \$9.5-12 billion annually in Newfoundland and Labrador. Therefore, salmon unutilized resources could be a great potential source for pharmaceutical quality protein and amino acids rather than use them to produce low quality products or dispose them as waste. These factors have necessitated the need for the development of biorefinery approach for production of various high value pharmaceuticals from unutilized salmon resources thereby maximizing the sustainability and economic viability of the industry.

One of the major and critical content in the salmon waste is protein. Traditionally, these protein sources are used as low value feed material in the animal feed industry and the surplus and unused materials have been dumped in the landfills or oceans. However, the protein present

in the fish is made up of two major types of protein including: structural protein (70-80%) and sarcoplasmic proteins (20-30%). In addition to that, the fish protein also contains 2-3% of insoluble connective tissue proteins. The fish protein can be converted into many higher-grade forms including: amino acids, bioactive peptides, collagen and gelatin. Fish protein as such can be purified and used as protein supplement in many dietary preparations. However, further processing by breaking down fish protein as amino acids can be used as flavor enhancer, food and feed supplement, pharmaceutical grade applications and sweetener, all extracted from natural fish resources not synthetically produced like current production methods (Banan-Mwine Daliri, Lee, and Oh 2017). Collagen and gelatin are two significant forms of proteins present in fish waste. Fish skin collagen is used various food, cosmetic and biomedical industries. The collagen extracted from the fish waste is also free from the bovine spongiform encephalopathy (BSE) or mad cow disease compared to extracted from animal sources (Ghaly et al. 2013).

Amino acids are building blocks of proteins which are currently produced using bacterial fermentation, chemical synthesis and enzymatic extraction procedure. Currently, manufactures are using fermentation and enzymatic catalysis to produce amino acids on a larger scale. However, few amino acids such as cysteine and phenylalanine are produced by chemical synthesis. The cost effectiveness of fermentation processes depends upon various factors including: carbon source, fermentation yield, purification and the yield of the overall process. Various plant based raw materials such as soybean, wheat, corn and sugarcane are widely used for the fermentation of amino acids. However, the cost of plant based raw materials coupled with the ban for the used bone and meat meal in the EU poultry sector has increased the stress on plant based raw materials and the companies are considering various other options to balance the demand (Van Horne 2018). The enzyme membrane technology is currently used in small scale industries to produce amino acids using enzymatic synthesis. However, enzymatic synthesis also requires starting material and an enzyme to produce amino acids. Acylase is the most commonly used enzyme for amino acid production. For example, L-methionine is produced via enzymatic synthesis using a chemical compound N-acetyl-L-methionine as starting material and acylase as the catalyst (Ghaly et al. 2013).

Protein hydrolysis using various fish protein sources as a starting material could be a possible solution to offset the dependency on plant based raw materials. About 80% of the fish processing by-

products in the fisheries are dumped as waste or small amounts are used for silage. Around 20% of the fish processing by-products contains proteins which are an excellent source of raw material for amino acid extraction. Traditionally, the amino acid extractions are carried out using acid hydrolysis methods, in which the protein raw material is heated at 110°C for 24 h in the presence of 6N HCl. However, during this process, amino acids such as Tryptophan is destroyed, cysteine cannot be determined and threonine, serine and tyrosine are partially hydrolyzed. To tackle this issue, the amino acids were hydrolyzed in the presence of organic acids such as Trifluoroacetic acid to reach the hydrophobic regions of proteins. Mixture of hydrochloric acid with trifluoroacetic acid in the ratio of 1:1 or 2:1, resulted in 100% recovery of all amino acids. Various other modes of hydrolysis including: alkaline hydrolysis using sodium hydroxide, potassium hydroxide or with barium hydroxide and microwave assisted acid hydrolysis were experimented. Using alkali hydrolysis processes resulted in complete identification of tryptophan, however, serine, threonine, arginine and cysteine are destroyed and all other amino acids are racemized. Using microwave technique, the hydrolysis was completed within 4 min and gave results comparable to that of conventional hydrolysis with higher losses of serine and threonine and a higher percentage of racemization of amino acids.

Enzymatic hydrolysis of proteins to release amino acids have very high potential, since, it allows quantification of asparagine and glutamine and other sensitive residues, which are normally destroyed by acid and alkali hydrolysis, and does not cause any racemization during digestion. However, the widespread large-scale applications have been limited due to the specificity of proteases with different peptide bonds. Furthermore, the hydrolysis procedure involves using several proteases in combination for a longer period and must be maintained at the right temperature and pH to achieve results comparable to the acid hydrolysis. In addition, the cost of the proteases used in the process significantly increases the cost of the final products.

The main objective of this current study, is to evaluate, utilizing enzymatic hydrolysis procedure using Alcalase and Corolase enzyme to recover amino acids from the fish protein and compare it against the conventional acid hydrolysis processes.

2. EXPERIMENTAL PROCEDURE

2.1. Raw Material Collection and Storage

The salmon by-products including: heads, guts and frames were collected from the local Newfoundland processors and was shipped in ice. The by-products were immediately minced using a Hobart mincer and stored in vacuum sealed bags at -80°C until further analysis. Equal portions of all three head, guts and frames were taken and minced together to obtain a blended salmon by-product. The minced product is shown in Figure 1.



Figure 1: Minced salmon by-products.

2.2. Enzymatic Extraction of Oil

The salmon by-products were subjected to enzymatic hydrolysis using alcalase enzyme to extract oil (Ramakrishnan et al., 2013). In this procedure, 200 g of salmon head, frame and blend were weighed in a mason jar and mixed with 200 ml of 1M pH 8 phosphate buffer (1:1) and placed in a pre-heated incubator shaker at 55°C for 15 min. Once the sample temperature has reached 55°C , 0.5%(v/w) Alcalase enzyme was measured and added to the sample. The mason jars were then flushed with nitrogen and the lids were tightly closed and placed in incubator shaker at 150 rpm for 2 h. After 2h, the samples were heated to 90°C for 10 min to inactivate enzyme. The samples were then cooled to room temperature and then centrifuged at 10,000 rpm for 20 min. The centrifugation of samples resulted in four different layers including: salmon oil, light-lipid

layer, protein hydrolysate and sludge. At first, the salmon oil layer was carefully pipetted out in a separate centrifuge, final weight of salmon oil was measured and stored at -80°C until further analysis. The protein hydrolysate was separated from the other two layers via vacuum filtration and then it was spray dried using a spray drier (Buchi, USA) at an inlet temperature of 190°C and an outlet temperature of 100°C and flow rate of 1-2 ml/min. The spray dried protein was stored at -20°C until further analysis.

2.3. Protein Hydrolysis

2.3.1. Chemical Hydrolysis

The chemical hydrolysis of proteins was carried out using the conventional method where 10 mg of protein was reacted with 1 ml 6N HCl at 110°C for 24 h. The resulting solution was centrifuged at 13,000 rpm for 15 min. The supernatant was collected and stored in glass GC vial until further analysis.

2.3.2. Enzymatic Hydrolysis

The enzymatic hydrolysis was carried out to extract amino acids from the proteins derived from the salmon by-products. 4 g of dried salmon protein powder was measured in a 250 ml Erlenmeyer flask and 100 ml of distilled water was added to make it a 4% solution. The solution was then placed in an incubator shaker at 55°C , 150 rpm for 15 min. Once the temperature reached 55°C , the pH of the solution was adjusted using 1N NaOH to 8 and at 0.5% concentration of enzyme Alcalase and Corolase was added to the solution. The hydrolysis was then carried out for 16 and 24 h. The samples were then heated at 90°C for 10 min to deactivate the enzymes. The samples were cooled down to room temperature and stored at -20°C until further analysis. The sample procedure was followed for both the enzymes individually and in combination on all the salmon protein samples.

2.4. Experimental Analysis

2.4.1. Chemical Analysis

2.4.1.1. Moisture Content

Moisture content was determined using AOAC Official Method 938.08. In this procedure pre-numbered aluminum pans were dried in drying oven at 105°C for one hour and then cooled in a desiccator for 20 min. The pans were then weighed to the nearest 0.001 g and the values were noted. Salmon by-product samples (head, gut, frames and blend) were each homogenized and around 5-10 g of each sample was weighed in triplicates to the nearest 0.001 g in the pre-weighed aluminum pans. The samples were then dried at 105°C for 24 h. Later, the samples were transferred to the desiccator and cooled to room temperature before weighing the samples for final dried weight. The moisture content of all the samples were calculated by the following Equation 1:

$$M(\%) = \frac{W_1 - W_2}{W_1 - W_0} * 100 \quad (1)$$

Where,

M = Percent Moisture content

W₁ = weight of dish and sample before drying

W₂ = weight of dish and sample after drying

W₀ = weight of empty dish

2.4.1.2. Crude Protein Content

The total crude protein content of fish samples was determined by AOAC Official Method 940.25 using the Kjeldahl nitrogen method (N X 6.25) (AOAC, 2000). 0.5 g of dried salmon by-product samples were weighed to the nearest 0.001 g on weighing paper. The weighed samples were transferred to digestion tubes and two copper sulfate tablets were added to each tube. To the samples, 20 ml concentrated sulfuric acid was added inside a fume hood. The digestion containing the samples was placed on a heating block and water circulated manifold was placed on the digestion tubes to neutralize sulfuric acid fumes. The heating block was heated until 380°C and once the temperature reaches 380°C, the samples were further digested for 75 min. After 75 min, the digestion tubes were lifted from the heating block to check for complete sample digestion. The complete sample digestion was identified by the clear greenish-blue liquid with no residues left in the tubes. Once this stage was achieved, the heat was turned off and the digestion

tubes were suspended in a rack over the heating block. After complete cooling, the water was turned off the water circulating manifold was removed from the tubes. The tubes were then set aside for distillation.

The distillation of the samples was carried out in KjelTec 8000 Foss Distillation unit, in which a program was setup to add 70 ml distilled water, 100 ml 40% NaOH and the samples were distilled for 4 min. The distilled was collected in 250 ml Erlenmeyer flask filled with 25 ml 4% boric acid solution to be obtain a blueish green distillate. Later the distillate was titrated against standardized 0.1 N HCl to a pinkish red endpoint. The volume of HCl consumed during titration was noted and the percentage crude nitrogen was calculated as shown in Equation 2 and the percentage protein was calculated as shown in Equation 3.

$$\%N = \frac{(Vol_{HCl\ sample} - Vol_{HCl\ Blank}) * Normality\ of\ HCl * 14.0067}{Weight\ of\ Sample(mg)} * 100 \quad (2)$$

$$\%Crude\ Protein = \%N * 6.25 \quad (3)$$

2.4.2. Amino acid Analysis

Amino acid analysis was carried out using gas chromatograph-mass spectrometer (ThermoFisher Trace 1300 GC/ISQ-LT MS). The standards were purchased from Sigma-Aldrich and contained 17 amino acids at a concentration of 2.5 μ moles/ml each, except L-Cystine at concentration of 1.25 μ moles/ml (**Table 1**). All amino acids were prepared in 0.1 N HCl. The standards were prepared in various concentrations to construct a calibration curve and it is shown in **Table 2**. Both standards and samples were subjected to derivatization using N-tertbutyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA). During this derivatization process, MTBSTFA forms tertbutyl dimethylsilyl (TBDMS) derivatives when reacted with polar functional groups containing an active hydrogen as shown in **Figure 4**.

Table 1. Amino acid standards

Amino acids	Mol wt (g/mol)	Concentration (μ moles/ml)	Concentration (moles/l)	Concentration (g/l)
L-Alanine	89.09	2.50	0.00250	0.223
Glycine	75.07	2.50	0.00250	0.188
L-Valine	117.20	2.50	0.00250	0.293

L-Leucine	131.20	2.50	0.00250	0.328
Isoleucine	131.20	2.50	0.00250	0.328
L-Proline	115.10	2.50	0.00250	0.288
L-Methionine	149.20	2.50	0.00250	0.373
L-Serine	105.10	2.50	0.00250	0.263
L-Threonine	119.10	2.50	0.00250	0.298
L-Phenylalanine	165.20	2.50	0.00250	0.413
L-Aspartic acid	133.10	2.50	0.00250	0.333
L-Glutamic acid	147.10	2.50	0.00250	0.368
DL-Ornithine	174.20	2.50	0.00250	0.436
L-Lysine	146.20	2.50	0.00250	0.366
L-Histidine	155.20	2.50	0.00250	0.388
L-Tyrosine	181.20	2.50	0.00250	0.453
L-Cystine	240.30	1.25	0.00125	0.300
Total Stock Solution				5.637

Table 2. Amino acid calibration standards.

Stock (µg/µl)	Stock (µl)	stock (µg)	Reaction volume (µl)	Final Concentration (µg/µl)	Concentration (g/l)
5.64	10.00	56.40	410.00	0.14	0.14
5.64	20.00	112.80	420.00	0.27	0.27
5.64	30.00	169.20	430.00	0.39	0.39
5.64	40.00	225.60	440.00	0.51	0.51
5.64	50.00	282.00	450.00	0.63	0.63

During the derivatization process, aliquots of standards as shown in **Table 2** were taken in a 10ml test tube and completely dried at 70°C under nitrogen for five min and 100µl of neat MTBSTFA was added, followed by 100µl of acetonitrile were added. The test tubes were tightly capped and was heated at 100°C for 2h. The sample was then allowed to cool at room temperature and 200µl of acetonitrile was again added to the tubes. The samples were then transferred into GC vials for analysis.

The GC analysis was carried out using SLB-5ms, 20 m x 0.18 mm column with an internal diameter of 0.18 µm. The inlet temperature was 280°C and was operated at splitless mode. The split flow was maintained at 100 ml/min and splitless time was 0.3 min. The column flow was 0.5 ml/min. The GC oven was maintained at 60°C for 0 min, ramped up to 100°C at 20°C/min and

held for 1 min, ramped up to 290°C at 10°C/min and held for 3 min and ramped up to 340°C and held for 2 min. The MS transfer line temperature was 320° and the ion source temperature was 280°C. The standards were initially scanned using MS in range of 40-639 (m/z). Once the target compounds are identified, the compound data was exported and the samples were analyzed in SIM acquisition mode.

3. RESULTS AND DISCUSSION

The moisture analysis and protein content of the salmon waste products was carried out and the results are shown in Tables 3 and 4. The results indicated the moisture content in the salmon blend, gut, frame and head range between 63-67%. The protein analysis indicated that salmon blend had the highest protein content of 14.00% (on dry basis) was found in blend and the least protein content was found in gut with 11.49% (on dry basis).

Table 3. Moisture content of salmon waste products.

Salmon Waste	Moisture (%)
Blend	64.12
Gut	66.83
Frame	63.20
Head	63.87

Table 4. Protein content of salmon waste products.

Salmon Waste	Protein Wet basis (%)	Protein Dry basis (%)
Blend	38.78	14.00
Gut	34.64	11.49
Frame	35.17	13.03
Head	35.69	12.89

The enzymatic hydrolysis of salmon waste products was carried out on the salmon head, frame and blend. The extracted protein was centrifuged, filtered and spray dried and it was stored at -20°C until further processing. The protein powder extracted from salmon head, frame and blend were then subjected to acid and enzymatic hydrolysis to extract amino acids and the results are shown in Figures 5, 6 & 7.

The acid hydrolysis was carried out using 6N HCl for 24 h at 110°C and the highest amount of amino acids were recovered from head salmon protein, followed by blend salmon protein and frame salmon protein. The results also indicate the most dominant amino acid present in the all the parts after acid hydrolysis was glycine. Methionine, tyrosine and cystine were almost destroyed during acid hydrolysis. The results from this study indicated that the Alcalase and Corolase were able break protein into individual amino acids under proper processing conditions compared to chemical hydrolysis process. In this study, 17 amino acids were identified and quantified using GC-MS. There were 7 non-essential and 10 essential amino acids present in salmon waste protein hydrolysates. Essential amino acid tryptophan was not analyzed in this study.

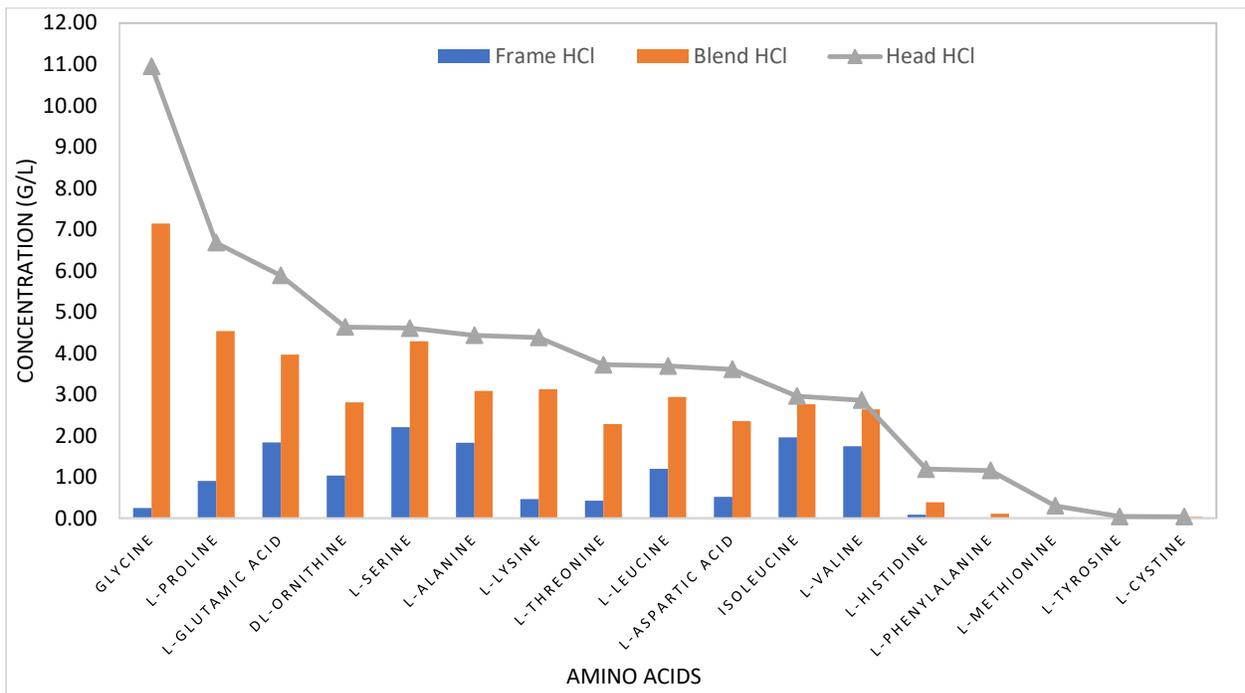


Figure 5. Acid hydrolysis of salmon protein

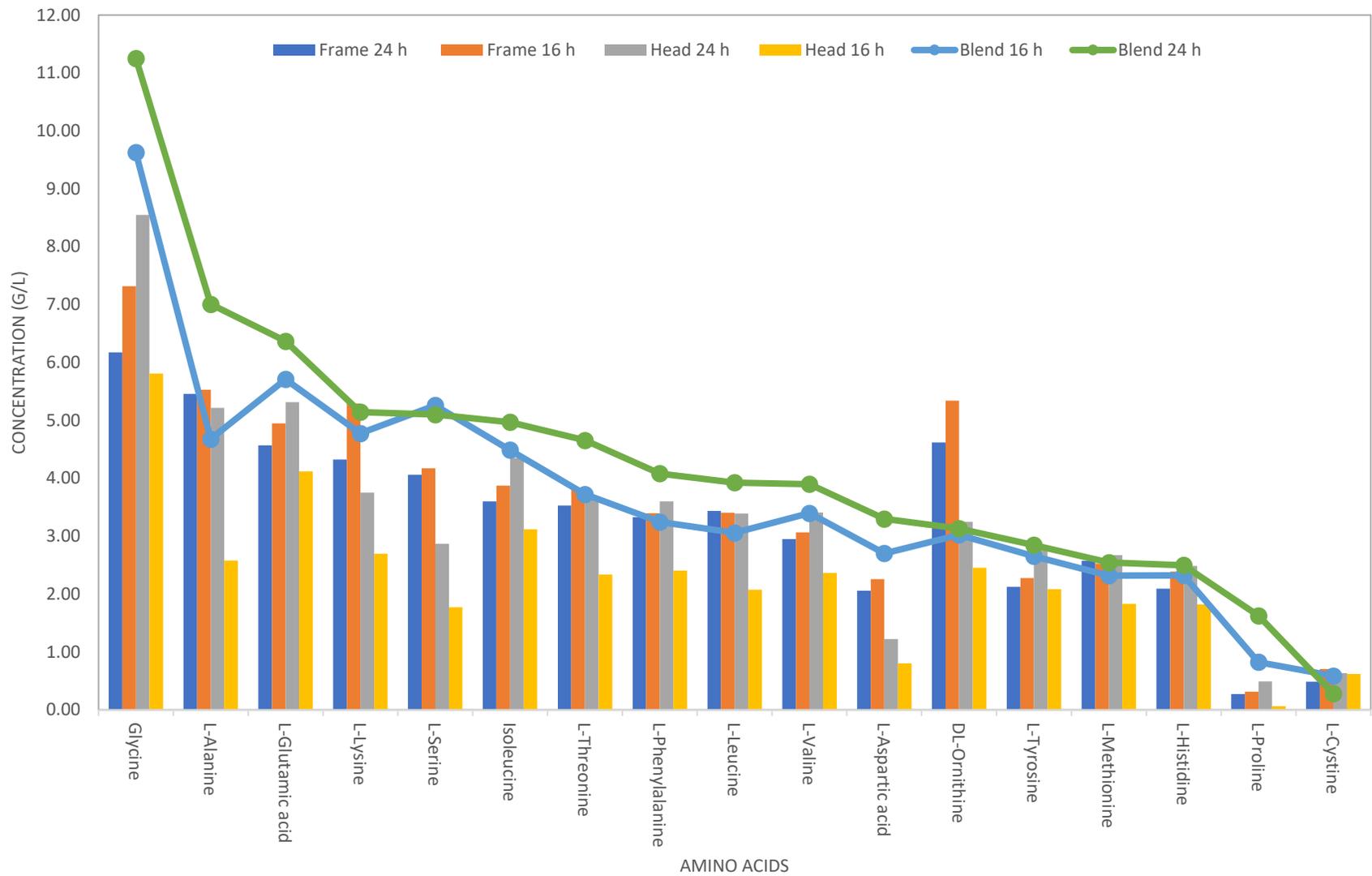


Figure 6. Alcalase hydrolysis of salmon protein.

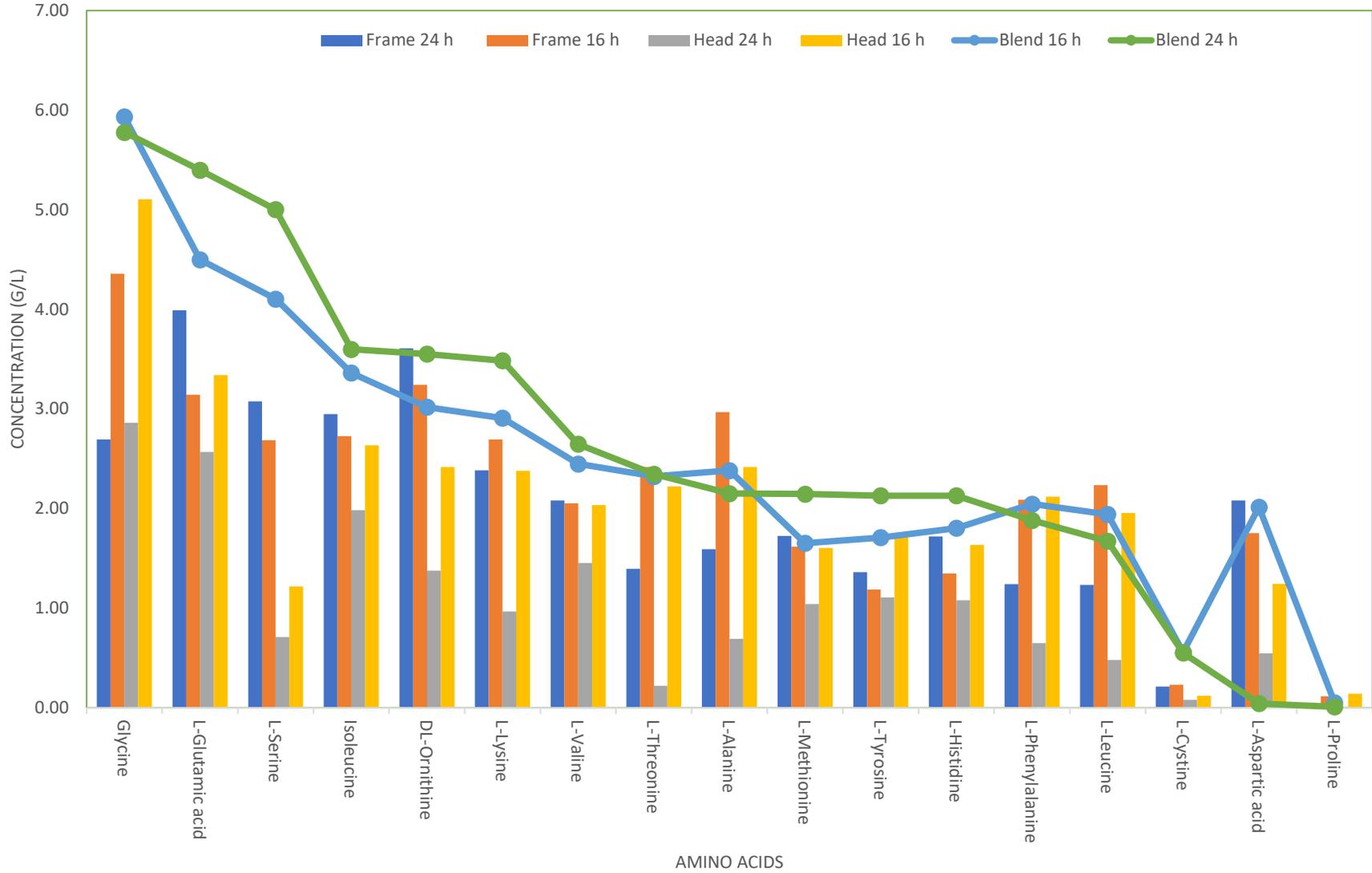


Figure 6. Corolase hydrolysis of salmon protein.

The enzymatic hydrolysis of salmon protein using both Alcalase and Corolase yielded excellent amino acid recoveries compared to acid hydrolysis. The results from Alcalase protein hydrolysis indicated that the highest amino acid recoveries were obtained from the blend salmon protein hydrolyzed for 24h followed by blend protein hydrolyzed for 16 h. Similar results were found when blend proteins were hydrolyzed using Corolase enzyme in which 24 h of hydrolysis yielded more amino acid recoveries than 16 h process. Increased amino acid recoveries from blend proteins in both hydrolysis processes could be due to the presence of all waste products including: frames, gut and heads. However, on individual basis, frame protein resulted in better amino acid recoveries than head during both Alcalase and Corolase hydrolysis.

Alcalase hydrolysis of salmon frame, blend and head for both 16 and 24 h resulted in higher glycine content, followed by L-Alanine and L-Glutamic acid present in them. The highest essential amino acid present in the Alcalase hydrolyzed salmon waste products was L-Lysine during both 16 and 24 h hydrolysis processes.

Corolase hydrolyzed salmon protein resulted in a slightly different and lower amino acid composition compared to Alcalase hydrolyzed protein. The highest amino acid present in the Corolase hydrolyzed protein was glycine in all the samples except for frames which was hydrolyzed for 24 h had high amounts of L-glutamic acid (3.99 g/L). The highest essential amino acid present in blend and head for both 16 and 24 h hydrolyzed protein was Isoleucine, however, DL-Ornithine was the highest essential amino acid present in frame protein in both reaction times (16 and 24 h hydrolysis). During derivatization process with N-methyl-N-(tert-butyl-dimethylsilyl) trifluoroacetamide (MTBSTFA), arginine is converted into a derivative DL-Ornithine (Smith, Villa, and King 2010).

Traditionally protein is hydrolyzed into amino acids using acid hydrolysis process however, the appearance, solubility, flavor and biochemical safety of the products are greatly affected. Therefore, many researchers and manufacturers have adapted to safe and mild enzymatic hydrolysis process to produce protein hydrolysates (Sun 2011). Even though the protein extracted from fish processing waste can be used for producing various high value bioactive peptides, but under limited supply of raw materials and economic constraints, the protein can

be converted as a functional and nutritional food ingredient after mild enzymatic hydrolysis process. In the present study, the protein extracted from fish waste was broken down into amino acids after several hours of hydrolysis process thereby potentially improving its emulsification, digestibility, foaming and water absorption properties of hydrolyzed protein.

The type of enzymes used play an important role in the in the hydrolysis process of fish protein. There are four different classes of proteases including: serine, thiol, carboxyl and metallo. The enzymes are further classified by their hydrolyzing mechanism into endoproteases and exopeptidases. The endoproteases cleaves peptide bonds within the protein molecules especially at specific residues producing larger peptide chains. The exopeptidases cleave either from N-terminus using aminopeptidases or at C-terminus using carboxypeptidases. Together both endoproteases and exopeptidases can be used in combination for complete degradation of protein into smaller peptides(Kristinsson and Rasco 2000). In this study, the fish protein extracted from salmon waste including: head, frame and blend were hydrolyzed individually using two endoproteases (Alcalase and Corolase). These two endoproteases share similar hydrolytic activity, similar reaction temperature and pH range which enables to be used in combination in the future studies for better hydrolysis and shorter hydrolysis times.

4. CONCLUSION

The salmon processing waste including: head, gut, frame and blend was utilized to extract protein by enzymatic hydrolysis process. The protein hydrolysate was spray dried to obtain protein powder. The protein powder was subjected to acid hydrolysis using 6N HCl and enzymatic hydrolysis using Alcalase and Corolase enzymes at 0.5% enzyme concentration. The study resulted excellent amino acid profile using enzymatic hydrolysis and the results were comparatively similar or better than traditional acid hydrolysis process. The amino acids produced using enzymatic hydrolysis process will not be racemized and the rate of hydrolysis and the time required for the hydrolysis is similar to traditional acid hydrolysis process. Further research and optimization with purification and separation studies can enable us to separate individual amino acids using various chromatographic techniques like column, size or gel filtration chromatography. These amino acids can also be broken down into much smaller size peptides

with active functional properties. All these value-added products can be produced from a waste product from our local salmon processing waste with significant effect on lessening the environmental impact.

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