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Comparison of Enzymatic Hydrolysis and Chemical Methods for Oil Extraction from Rainbow Trout *(Oncorhynchus Mykiss)* Waste and Its Influence on Omega 3 Fatty Acid Profile

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ABSTRACT

Background: During fish processing operations, significant fish waste is generated, causing environmental problems. This study compared enzymatic hydrolysis and chemical methods for oil extraction from rainbow trout (*Oncorhynchus mykiss*) waste and its influence on omega 3 fatty acid profile.

Methods: Oil extraction efficiency and fatty acids profile from trout fish head were analyzed by comparing enzymatic method (protease from *Bacillus subtilis*) and chemical extraction with hexane (soxhlet method) to produce a valuable product by fish waste. The enzymatic hydrolysis and chemical methods for oil extraction from rainbow trout (*Oncorhynchus mykiss*) waste and its influence on omega 3 fatty acid profile were compared. The contents of fatty acids were analyzed by gas chromatography.

Results: No significant difference was noticed between the enzyme treatments with water (200, 100, and 50 mL) and without additional water regarding oil extraction efficiency. The samples without water were economically more affordable because of their lower volume and reducing energy consumption. The oil extraction efficiency with optimum enzymatic method (150 ppm of concentrated protease, without using water) was significantly lower than soxhlet method. Omega-3 content in the optimum biological method (9%) was significantly higher than that in soxhlet method (5.53%). Fatty acids with high contents of trout head oil in both methods were linoleic acid (18:2) and oleic acid (18:1).

Conclusion: Due to the suitable oil extraction efficiency and higher omega-3 fatty acid content of the enzymatic method compared to chemical solvent (hexane) extraction, enzymatic method was preferred as a safe and environment-friendly extraction technique.

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Introduction

Most industries perform fish processing, including frozen fish, marinated fillets, and canned fish (1). Filleting processing waste is a source rich in omega-3 fatty acids, which is discarded by the factory and includes heads, tails, bones, skin, and viscera (2). These by-products are a good source for having a product as an additional revenue. Due to the presence of poly-unsaturated fatty acids, especially eicosapentaenoic acid (EPA, C20:5, n-3) and docosahexaenoic acid (DHA, C22:6, n-3), which cannot be synthesized by human beings, fish oil is a special oil in comparison to other animal and vegetable (3).

The important roles of these fatty acids in human health were recognized three decades ago (4). Among highly unsaturated fatty acids (HUFAs), DHA (docosahexaenoic, C22:6n-3) has an important impact on retina and brain in human beings, while EPA (eicosapentaenoic, C20:5n-3) has anti-inflammatory and anti-tumoral effects, reduces obesity disorders, and is effective in improving depression symptoms (5). They are also used in prevention and treatment of coronary artery disease, cancer, diabetes, hypertension, and rheumatoid arthritis (6-10). The 2D chemical structures of major omega 3 fatty acids (eicosapentaenoic acid, docosahexaenoic acid and α-Linolenic acid) were showed in Figure 1 (PubChem CID: 56842239).

There are several procedures for marine oil extraction, including supercritical fluid method, solvent extraction, and heating methods (11). These methods are different in oil extraction efficiency, process costs, and inappropriate remains (12). Conventional methods for omega 3 purification or oil extraction such as, distillation, urea crystallization, soxhlet extraction are not safe for human health due to solvent toxicity. Also using high temperatures by some of these methods resulted in omega 3 decomposition (13). Organic solvents are common for oil extraction from seeds. Concern about the effect of these undesirable solvents on public health and environment necessitates food technologists to propose safe methods for oil extraction (14).

Recently, oil extraction methods with enzymes, such as alcalase, protex, protamex, lecitase ultra, and neutrase, are safe alternative methods to organic solvents. Moreover, high-quality protein is produced during oil extraction with enzymatic method (12, 15). Enzymatic technique using protease for oil extraction has become more interesting for achieving a safe product, which is chemical solvent free. Due to mild condition (low temperature) and short time of extraction, using protease is a suitable method for oil extraction (16).

This study aims to extract fish oil with optimum content of omega-3 from rainbow trout *(Oncorhynchus mykiss)* waste (head) by enzymatic hydrolysis using different bacterial protease concentrations and different amounts of water, as

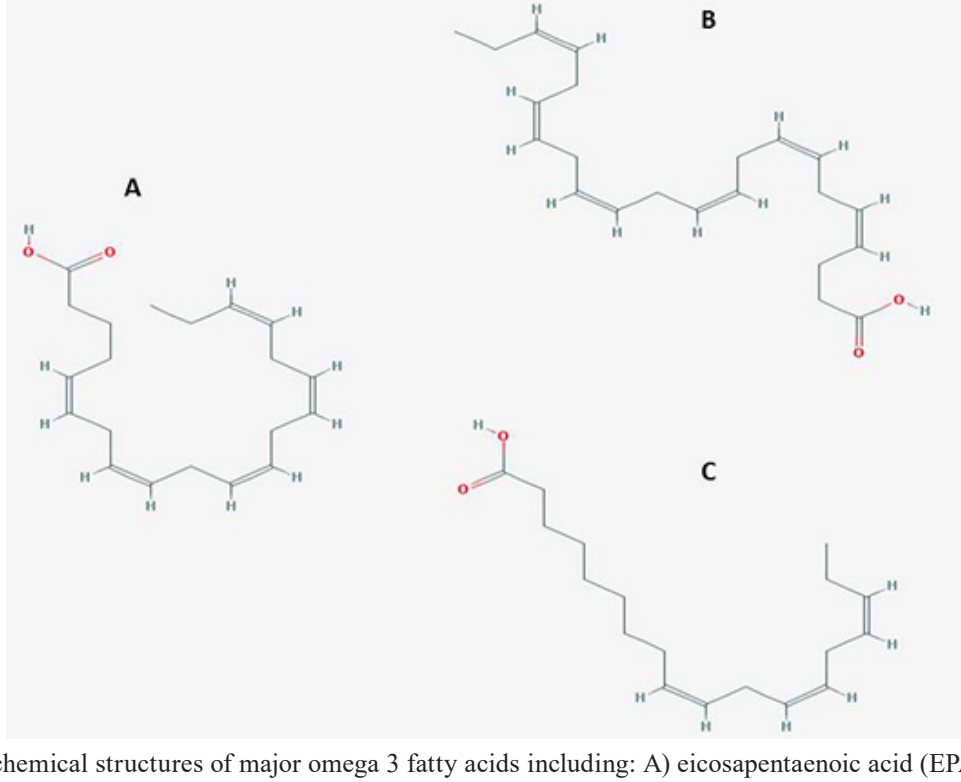

Figure 1: 2D chemical structures of major omega 3 fatty acids including: A) eicosapentaenoic acid (EPA, C20:5, n-3), B) docosahexaenoic acid (DHA, C22:6, n-3), C) α-Linolenic acid (ALA, C18:3, n-3) (Description: figure was adapted from PubChem, PubChem CID: 56842239).

extra volume for the hydrolysis reaction, and to compare their fatty acid contents to soxhlet as a common method.

Materials and Methods

In this study, 7 kg of rainbow trout *(Oncorhynchus mykiss)* heads were obtained from Liossa factory in Shiraz, Iran. The fish wastes were collected in sealed plastic bags, transported to laboratory, and stored in a freezer at -18°C. All the used reagents for Gas Chromatography (GC) were bought from Merck (Darmstadt, Germany) or Sigma Aldrich (Buchs, Switzerland). Besides, bacterial protease from Bacillus subtilis was obtained from Artin Chemistry Company, Iran. The oil extraction procedure was based on previous method with some modifications. In their study, oil extraction was done using alcalase with three concentrations (0.5, 1, and 2%) and four times (1, 2, 3, and 4 h)(17).

In the current study, the frozen rainbow trout heads were thawed overnight in the refrigerator at 4°C. For minimizing errors during laboratory analysis, similar samples were selected. At first, 100 g of rainbow trout heads were mixed with 200 mL of distilled water. The samples were heated in water bath at 80°C for 30 min then they were homogenized in a blender. Enzymatic hydrolysis reaction was started by adding 0.5% w/w (protease weight/head weight) bacterial protease with flour carrier when the temperature of the mixture reached 35-40°C (17).

The mixture was then stirred at 300 rpm by a magnet stirrer. The hydrolysis reaction was carried out at 35-40°C at the initial pH (6.8) for 90 min. After the hydrolysis period, the enzyme was inactivated by heating at 80°C in a water bath for 15 min. The samples were then centrifuged at 5000 rpm at 15°C for 20 min. After centrifugation, three phases were created: oil phase on the top, liquid protein phase in the middle, and sludge at the bottom. The oil fraction on the top was collected and oil extraction efficiency was measured (v/w %).

The oil was kept at -20°C for further experiments. Moreover, to compare the efficiency of protease for oil extraction, a control sample without enzyme was assessed using the same method. The experiments were carried out in triplicates. The following formula was used to determine the oil extraction efficiency. Oil extraction efficiency (%)=[content of oil in the waste (mL)/fish waste weight (g)]*100. The effect of water content on protease activity and oil extraction efficiency was studied. In doing so, 100 g of trout head and 0.5% w/w of bacterial protease with flour carrier were used and different water contents (0, 50, 100, and 200 mL) were considered for the hydrolysis reaction.

To compare oil extraction efficiency, two functional forms of protease (protease with flour carrier and concentrated protease) were used for the 90-min reaction at 35-40°C. In so doing, concentrated protease concentrations of 50, 150, and 250 ppm (using try and error experiment) and 0.5% w/w of protease with flour carrier (which was the optimum enzyme concentration suggested before were utilized (17).

In this experiment, hexane was used as a common chemical solvent in oil extraction studies by soxhlet method to compare the lipid contents of rainbow trout heads to those obtained by enzymatic extraction method, as a free-chemical solvent method. For sample preparation, 100 g of each sample was dried to constant weight by oven dryer at 70°C for 3 hours. During the process, the evaporated solvent was distilled and returned to the sample to continue oil extraction. Every 20 min, the solvent was circulated. Finally, the extracted oil and solvent were separated. This process lasted for 6 hours. After all, the volume of the oil-extracted content was measured.

For GC sample preparation, 0.2 mL of the sample was mixed with 10 mL of methanol-acetyl chloride solution, heated at 85°C for 60 minutes, and cooled. Then, 5 mL of deionized water and 1 mL hexane were added. After that, the samples were shaken and centrifuged, and the upper phase was collected for GC analysis. The samples were analyzed using a GC (Beifen 3420A, made in China) with film thickness of 0.25 µm, column (BPX70), and a Flame Ionization Detector (FID).

Nitrogen was used as the carrier gas. Besides, injector and detector temperatures were 250 and 300°C, respectively. At first, a sample volume of 5 µL was injected using split mode. Fatty acids were then identified by comparing their relative and absolute retention times to those of authentic standards (Sigma Chemical Co.). The fatty acids composition was reported as the percentage of total fatty acids. After all, Analysis of Variance (ANOVA) was performed on the major omega 3 fatty acids (18:3, 20:5, and 22:6) for fish oil samples.

All the experiments were carried out in triplicates. The obtained data were reported as mean±SD. One-way ANOVA was used to assess differences between the samples. All the analyses were performed using the SPSS statistical software, version 16.2.2.0 and $p<0.05$ was considered to be statistically significant. This study was approved by the Local Ethics Committee. All experiments were carried out in triplicates.

Results

In the present study, two kinds of samples (with

protease and protease-free treatment) were considered to determine the effect of a short-time heating process using bacterial protease (*Bacillus subtilis*) on trout head oil extraction efficiency. The results showed that oil extraction efficiency using aqueous enzyme (0.5% w/w) (10.94±0.7%) was significantly (p<0.05) higher compared to the control sample (protease-free) (1.88±0.02%). Enzyme treatment degrades cell wall components, thus facilitating oil release from the cell. However, oil extraction process in the absence of protease had considerably lower efficiency, because oil is not soluble in polar solvents and aqueous solvent alone without protease is not able to degrade cell wall components (Table 1).

After determining the effect of protease on oil extraction efficiency, the effect of water content on fish oil extraction efficiency was assessed. In doing so, enzymatic extraction of oil was carried out with different amounts of water. The results showed that the amount of added water (0, 50, 100, and 200 mL) had no significant impact on oil extraction efficiency. Therefore, using less water content in the extraction process resulted in less oil hydrolysis. This also led to a decrease in the sample size and easier progression of the extraction process. Thus, waterless samples were utilized for further laboratory experiments (Table 1).

In this study, 0.5% w/w of protease with carrier was used. However, considerably higher amounts of the enzyme are required for higher fish volumes. For example, 1 kg of enzyme is needed for oil extraction from 200 kg of fish heads, which is not economic. Therefore, small amounts of concentrated protease (with high purity); i.e., 50, 150, and 250 ppm, were studied for oil extraction in this study as try and error experiments. According to the results, oil extraction efficiency was significantly higher in the samples with 150 ppm compared to those with 50 and 250 ppm of concentrated protease (p=0.04). Higher protein hydrolyses (with 250 ppm of protease) might have resulted in more emulsifying reactions between the hydrolyzed proteins and oil, which could eventually trap the oil. Hence, 150 ppm was determined as the optimum amount of concentrated protease (Table 1).

Using hexane as the oil extraction solvent is very common. Therefore, this study compared biological method and solvent extraction method (soxhlet method) regarding oil extraction efficiency and fatty acids composition. The results demonstrated that oil extraction efficiency was significantly higher in the soxhlet method compared to the optimum sample of the biological method (p=0.04); however, both results were close (Table 1). Therefore, the biological method could be a suitable alternative for oil extraction due to elimination of the chemical solvent and increase of consumers' health.

After oil extraction (by aqueous and non-aqueous enzyme-assisted extraction and hexane), the oil phases obtained by centrifugation were collected and analyzed by GC to determine the fatty acids content. Then, the contents of fish oil fatty acids were studied in three samples: 1- the oil extracted by the soxhlet method, 2- the oil extracted by the concentrated protease (150 ppm, without addition

Table 1: Comparison of the effect of different samples on enzymatic oil extraction efficiency.

Sample	Oil extraction efficiency (%)	ANOVA (p-value)
A:		
Trout fish head + 200 mL water (control sample)	1.88±0.02	0.03
B:		
Trout fish head + protease with flour carrier (0.5% w/w) + 200 mL water	10.94±0.72	NS
Trout fish head + protease with flour carrier (0.5% w/w) + 100 mL water	10.75±0.34	NS
Trout fish head + protease with flour carrier (0.5% w/w) + 50 mL water	9.18±1.28	NS
Trout fish head + protease with flour carrier (0.5% w/w)	11.03±0.09	NS
C:		
Trout fish head + 50 ppm concentrated protease	11.19±0.26	NS
Trout fish head + 150 ppm concentrated protease	13.65±0.77	0.046
Trout fish head + 250 ppm concentrated protease	11.29±0.68	NS
D:		
Oil extraction by soxhlet method	16.58±0.44	0.043

The results have been presented as mean values±SD. ppm: part per million; w/w: weight/weight; NS: not significant. All the experiments were carried out in triplicates. A, B: The effect of protease on fish oil extraction efficiency in comparison to protease-free (control) sample. B: The effect of different water contents on enzymatic oil extraction efficiency from trout fish head. C: The effect of different concentrations of concentrated protease on fish head oil extraction efficiency. D: Fish oil extraction efficiency by solvent extraction with soxhlet extractor. NS: Not significant.

of water), and 3- the oil extracted by protease with flour carrier (0.5% w/w, with adding 50 mL of water).

According to the results of GC, the predominant fatty acids in sample 1 were linoleic acid (18:2), oleic acid (18:1), and palmitic acid (16:0). In addition, omega-3 fatty acids content was calculated as 5.53% containing 3.52% α-linolenic acid (18:3), 0.11% EPA (20:5), and 1.9% DHA (22:6) (Figure 2a, Table 2). In sample 2, the predominant fatty acids were linoleic acid (18:2), oleic acid (18:1), and palmitic acid (16:0). Besides, omega-3 fatty acids content was calculated as 9% containing 3.33% α-linolenic acid (18:3), 1.76% EPA (20:5), and 3.91% DHA (22:6) (Figure 2b, Table 2)

In sample 3, the predominant fatty acids were linoleic acid (18:2), oleic acid (18:1), and palmitic acid (16:0), similar to samples 1 and 2. Additionally, omega-3 fatty acids content was calculated as 6.79% containing 3.84% α-linolenic acid (18:3), 0.7% EPA (20:5), and 2.25% DHA (22:6) (Figure 2c, Table 2). The results of fatty acids profile revealed higher omega-3 ratio in the biological method compared to the soxhlet method. In these two methods, fatty acids with high contents of trout head oil were linoleic acid (18:2) and oleic acid (18:1). The study results indicated a significant difference among the three mentioned methods regarding major omega 3 fatty acids content (p<0.05) (Table 2). Accordingly, the omega-3 content extracted from the optimum biological method (9%) was significantly (p<0.05) higher than that extracted from soxhlet method (5.53%).

Discussion

Although use of chemical solvents for oil extraction resulted in high extraction efficiency, it had disadvantages, such as safety concerns about food quality, high cost, environmental pollution, and being flammable (18). Water is a safe and environment-friendly solvent without having hexane fire and explosion hazards. However, it is not an efficient solvent for extraction of nonpolar foods, including oil. Therefore, enzyme-assisted treatment was carried out in several studies to increase oil extraction efficiency by removing proteins and facilitating oil release from cells (11, 2, 16-23).

Enzymatic extraction was considered by food supplement technologists and pharmacists because of low energy requirement for large-scale production (24). Moreover, applying protease in oil extraction

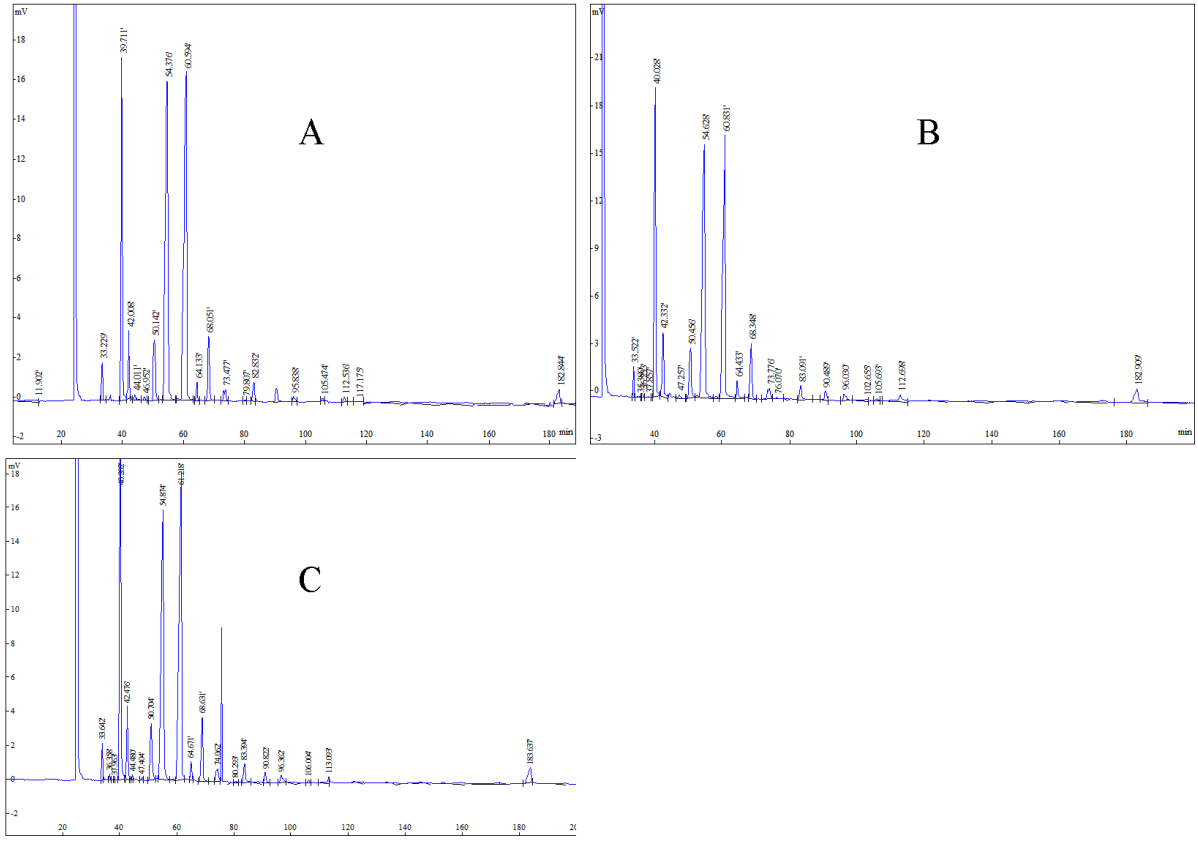

Figure 2: Chromatogram of the fatty acids analysis of the oil extracted in the three methods: A) with hexane by the soxhlet method (sample 1), B) the oil extracted with concentrated protease (150 ppm) method (sample 2), and C) the oil extracted with protease (0.5% w/w protease with flour carrier + 50 mL water) method (sample 3). Fatty acids were identified by comparing their relative and absolute retention times to those of authentic standards during 200 min.

Table 2: Fatty acids analysis of the oil recovered from trout fish heads by gas chromatography in the soxhlet method (sample 1), concentrated protease (150 ppm) method (sample 2), and protease (0.5% w/w protease with flour carrier + 50 mL water) method (sample 3).

Fatty acids (% of total fatty acids)	Fatty acids content of sample 1	Fatty acids content of sample 2	Fatty acids content of sample 3	ANOVA (*p*-value)
C14:0 (Myristic acid)	1.13±0.10	0.97±0.08	1.03±0.02	
C16:0 (Palmitic acid)	14.86±0.08	14.79±0.03	16.10±0.03	
C16:1 (Palmitoleic acid)	3.06±0.06	3.36±0.07	2.66±0.08	
C17:0 (Margaric acid)	0.28±0.08	0.28±0.05	0.40±0.06	
C18:0 (Stearic acid)	4.72±0.03	4.26±0.10	4.45±0.04	
C18:1 (Oleic acid)	31.59±0.02	28.63±0.08	28.46±0.10	
C18:2 (Linoleic acid)	32.46±0.07	31.59±0.06	34.39±0.05	
C18:3 (ω -3) (α-Linolenic acid)	3.52±0.07	3.33±0.06	3.84±0.03	0.006
C20:1 (Paullinic acid)	0.09±0.05	0.09±0.02	1.27±0.08	
C21:0 (Heneicosylic acid)	1.42±0.07	1.37±0.03	1.43±0.09	
C20:4 (Arachidonic acid)	0.55±0.02	0.87±0.06	0.84±0.07	
22:1 (Erucic acid)	0.41±0.08	0.23±0.05	0.24±0.08	
C20:5 (ω -3) (EPA)	0.11±0.06	1.76±0.08	0.71±0.04	0.0001
C22:6 (ω -3) (DHA)	1.90±0.04	3.91±0.02	2.25±0.03	0.0001

The results have been presented as mean values±SD. EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; ALA: Alpha-lipoic acid.

from marine waste improved oil extraction efficiency (11). Overall, enzymatic treatment with protease in a mild condition (low temperature) made it a suitable alternative method for oil extraction (Linder *et al*., 2005) (16). Alcalase, neutrase, protamex, and lecitase are common commercial enzymes used in enzyme treatment hydrolysis. Alcalase is a proteolytic enzyme with high performance, which is used for protein hydrolysis processes and lipid extraction from tissues (15, 19).

It was shown that increasing the enzyme concentration resulted in a slight increase in oil extraction. Therefore, the enzyme concentration of 0.5% was economically appropriate. Their results also showed that increasing the time led to a small increase in oil extraction efficiency. Thus, a 1-h reaction time was economically suitable (17). Batista *et al.* (2009) investigated extraction of oil from sardines (raw and cooked by-product) by food-grade commercial enzymes (alcalase, neutrase, and protamex) using 0.5% enzyme with water/fish ratio of 1:1. Their results demonstrated that alcalase and protamex were more efficient for oil extraction and the highest amount of oil was extracted from raw samples. Additionally, the oil sample was dark with high peroxide value (21).

A study conducted on codfish by-products assessing the effect of different parameters, such as initial heat inactivation of enzyme, water content, and different combinations of enzymes, on purity and fish oil extraction efficiency showed that, the best results were obtained by hydrolysis of unheated raw materials with alcalase and addition of water (15, 19). Liaset *et al.* (2002) also evaluated extraction of fish oil and nitrogen recovery with protamex protease from frames without heads of Atlantic salmon. Their study parameters included pH, temperature, frames/water ratio, and enzyme/substrate ratio. According to their results, the highest nitrogen recovery was achieved by the highest level of enzyme and the lowest frame/water ratio (25).

In the same line, Mbatia *et al.* (2010) extracted oil from Nile perch and salmon head using two kinds of protease, including bromelain and protex. They found that increase of water content during the hydrolysis led to a decrease in oil extraction efficiency. Moreover, chromatogram of oil fatty acids composition showed that saturated fatty acids were higher in Nile perch (36.8 mol%) compared to salmon head (19 mol%). Besides, palmitic acid, EPA, and DHA were 50, 13, and 48 mol% in crude oil, respectively (12). Gbogouri *et al.* (2006) used proteolytic enzyme treatment for salmon head oil extraction. The results showed that fish oil consisted of saturated fatty acids (24.7-27.3%), monounsaturated fatty acids (39.9-40.8%), and poly-unsaturated fatty acids (32.3-35.4%) (11).

Similarly, Nguyen *et al.* (2013) performed a research on oil recovery from tuna head using industrial protease. Their results indicated that oil extraction efficiency increased during the first 90 min of enzyme reaction. However, further hydrolysis (90-120 min) did not result in higher oil recovery. Decrease in oil extraction efficiency after 90 min could be due to the lipid-protein complex created through interaction of hydrolyzed protein with lipid. According to their

results, hydrolysis of the heads with 0.5% protamex at 45°C for 120 min resulted in 65.4% oil recovery. Furthermore, tuna head oil analysis revealed that 18.99% and 4.37% of fatty acids were related to omega-3 and omega-6, respectively (22).

In another study, Batista *et al.* (2009) showed that after 1-hour enzyme treatment of sardine by-product with protamex, oil extraction efficiency was 35% and only a small increase was observed in oil extraction efficiency in the next 3 hours (21). Furthermore, Linder *et al.* (2005) found that after a 2-h treatment with alcalase 2.4 l, the oil yield reached 17.4% that was close to oil extraction with chemical method. In addition, salmon heads treatment with 0.5% (w/w) bromelain at 55°C for 1 h (without adding water) resulted in oil extraction of 11.8±0.4% g lipids/100 g wet weight (16). Daukšas *et al.* (2005) also indicated that after hydrolysis of different by-products, lipid recovery increased from 36.4% to 82.8% (20). Overall, the previous studies reported that oil extraction efficiency depended on the sample and enzyme reaction conditions, including pH, temperature, reaction time, and enzyme content (12, 20, 21).

After determining the effect of protease on oil extraction efficiency, the effect of water content on fish oil extraction efficiency was assessed in our study. Similarly Slizyte *et al.* (2005) revealed that in the absence of water, the amount of oil recovery increased and emulsion phase decreased (15, 19). According to the report by Senphan and Benjakul (2015) palmitic acid and oleic acid were the major components of lipid in striped catfish muscle, which was processed with protease from pacific white shrimp (23). Additionally, Chaijan *et al.* (2010) disclosed that DHA and EPA obtained from striped catfish were 0.29-0.33% and 0.46-0.60%, respectively. They also reported that palmitic acid and oleic acid (18:1) were the predominant fatty acids in catfish. In general, fatty acid compounds of fish are affected by fish species, wild or cultured fish status, and season of the year (26).

Conclusion

Due to high consumption of trout fish *(Oncorhynchus mykiss)* and its usage in fish processing factories, fish waste including heads is available in large quantities. Therefore, this study used trout fish waste (head) for fish oil extraction using biological methods (with protease) without using chemical solvents and analyzed their fatty acids content. In addition, the results of oil extraction efficiency and fatty acids content in the biological method were compared to those obtained in soxhlet extractor, as a common method. Comparison of these two methods showed that oil extraction efficiency using the enzymatic method was close to that by the soxhlet method. Moreover, the results of fatty acids content revealed higher omega-3 content in the biological method compared to the soxhlet method ($p<0.05$). Fatty acids with high contents in trout head oil in both methods were linoleic acid (18:2) and oleic acid (18:1). These results suggested that biological methods, as a green and environment-friendly extraction technique with suitable oil extraction efficiency, could be used to promote consumers' health.

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Conflict of Interest

None declared.

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