PAPER

EVALUATION OF ENZYMATIC HYDROLYSIS APPLIED TO FISH BY-PRODUCT OIL THROUGH CHEMICAL PARAMETERS

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ABSTRACT

Large amounts of fish waste are produced by the fish processing plants. This waste could be used to obtain high value-added products, such as long chain polyunsaturated fatty acids. Thus, the aim of this work was to evaluate the enzymatic hydrolysis of low commercial value crude fish oil through chemical parameters. Crude fish oil was obtained from mixed fishmeal production and was chemically refined. *Candida rugosa* lipase AY "Amano" 30 was utilized to catalyze the enzymatic hydrolysis. The acidity index, iodine value, saponification index and peroxide value were used to characterize the samples studied. The chemical refining process yielded 62.75% and improved the quality of crude fish oil by reducing the acidity index around 91%. The best results of hydrolysis degree (23.45%) and iodine value (120 g I_2 g^{-1}) were obtained at 45ºC after 6 h of lipase action. The iodine value of crude oil was not affected by processing, indicating that the nutritional quality was preserved in the refined oil. Despite the hydrolysis process showed good results, it was not sufficient to concentrate the unsaturated fatty acids of refined oil, as indicated by the iodine value.

Keywords: acidity index, *Candida rugosa*, iodine value, lipase, refined fish oil

1. INTRODUCTION

The fish processing plants produce around 50% of waste from the total processed fish (ARRUDA *et al*., 2006). This waste is rich in organic and inorganic compounds, and its improper disposal causes negative environmental impacts, particularly on the margins of water bodies or in unlicensed landfills (FELTES *et al*., 2010). Fish waste could be utilized to obtain high value-added products, since fish oil can be produced from whole fish, roe and by-products from the processing of fish. Fish oil is widely used in aquafeeds as supply of long chain omega-3s fatty acids for aquaculture, especially for carnivorous fish *e.g.* salmonids. The direct use of fish oil in human foods and capsules is an increasingly significant outlet - the so-called, "nutraceuticals", which use has been increasing even more rapidly than that in aquaculture, at around 15% per annum (PIKE and JACKSON, 2010).

Fish oil is an important source of long chain polyunsaturated fatty acids (LC-PUFA), mainly the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (MONROIG *et al*., 2018). Recent studies revealed that LC-PUFAs prevented short and long term memory impairment induced by chronic sleep deprivation (AZOULBI *et al.,* 2019). There is also abundant evidence that increasing the intake of omega-3s fatty acids can soften the symptoms of neurodegenerative and neurological diseases, improving memory and cognitive function (CUTULI *et al*., 2014; ZHOU *et al.,* 2018). However, crude fish oil presents impurities *e.g.* free fatty acids, mono- and diglycerides, phosphatides, steroids, vitamins, hydrocarbons, pigments, carbohydrates, proteins and their degradation products and colloidal materials (MENEGAZZO *et al*., 2014). The production of high quality oils requires the largest possible removal of non-triglyceride components (PRIOR *et al*., 1991). The refining process aims precisely to remove these non-triglyceride components, conferring the oil best features.

One of the most promising techniques to fish oil processing is the use of lipase-catalyze enzymatic hydrolysis. This process favors the release of lipids from a protein matrix while preserving the nutritional value of fats for their application in food industry. The high specificity of lipases in relation to triacylglycerol substrate has suggested a large number of applications in the pharmaceutical and food areas, being used mainly for the production of particular fatty acids with low energetic consumption (PADILHA and AUGUSTO-RUIZ, 2007; FERREIRA-DIAS *et al*., 2013).

Concentrates of EPA and DHA may be prepared by selective hydrolysis of fish oils using lipases or by selective esterification of DHA and other free fatty acids (RANJAN-MOHARANA *et al*., 2016). The modification of lipids from oils usually involves a process catalyzed by lipase for fat hydrolysis, modification of triacylglycerol, and synthesis of esters. The process is attractive, since the lipase shows a high efficiency, using little amounts, especially in immobilization process (FERREIRA-DIAS *et al*., 2013; RAJENDRAN *et al*., 2009). Thus, this work aimed at evaluating the effect of enzymatic hydrolysis of low commercial value crude fish oil submitted to chemical refining. To this aim, samples submitted to chemical refining for increasing time and at different temperatures were characterized for chemical parameters.

2. MATERIALS AND METHODS

2.1. Crude fish oil and enzyme

Crude fish oil was obtained as a by-product of the mixed fishmeal production from the Torquato Pontes Fisheries Industry, located in Rio Grande, RS, Brazil. The crude oil was kept under refrigeration. The enzyme utilized was *Candida rugosa* lipase AY "Amano" 30, supplied by Amano Enzymes (USA).

2.2. Chemical refining of crude fish oil

The chemical refining of crude fish oil was realized according to the methodology described elsewhere (MORAIS *et al*., 2001; MENEGAZZO *et al*., 2014). Fig. 1A shows all the process steps.

2.3. Enzyme application in the crude fish oil

The enzyme application in crude fish oil was adapted from SUN *et al*. (2002) and is shown in Fig. 1B. The chemically refined fish oil was hydrolyzed with *Candida rugosa* lipase AY "Amano" 30 by adding phosphate buffer solution (pH 7.0) with the enzyme, and 100 μmol of $CaCl_2$ solution.

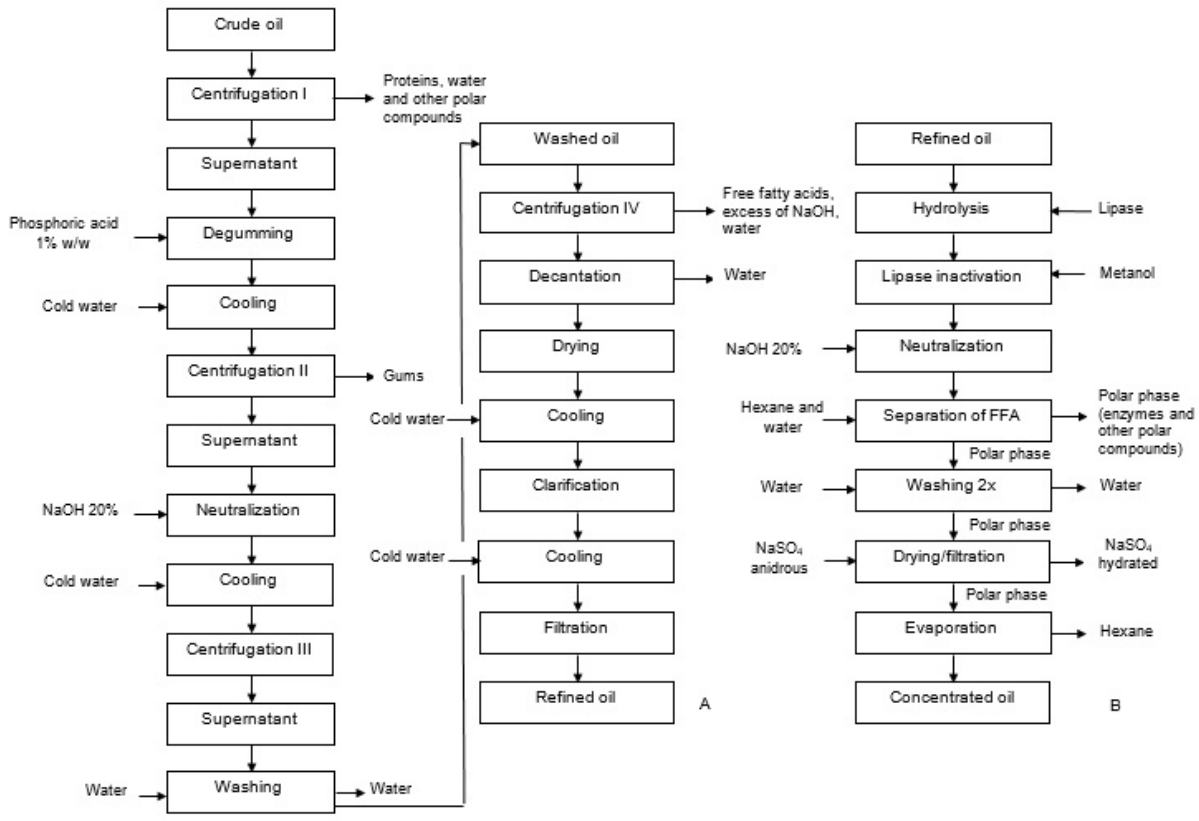

Figure 1. Chemical refining (A) and concentration (B) of fish oil.

The samples were placed in a reactor with stirrer and temperature-controlled bath. Aliquots were collected at defined intervals to determine the acidity index and the degree of hydrolysis (Enzymatic hydrolysis). After the reaction, lipases were inactivated with methanol (Lipase inactivation) and the free acids were neutralized with a NaOH solution 20% (Neutralization). The separation of glycerides from the fatty acids was performed by using a separator funnel. The oily mixture was added of 100 mL of hexane and 50 mL of distilled water (Separation of glycerides). The lower aqueous layer was separated and discarded, while the upper layer containing glycerides was washed twice with 50 mL of distilled water (Washing) and the remaining water was removed by a layer of anhydrous sodium sulfate (Drying/filtration). The glycerides were recovered after removal of solvent on a rotary evaporator (Evaporation). Then, unsaturated fatty acid was obtained as final product.

2.4. Chemical characterization of samples

The characterization of the crude, refined fish oils and the unsaturated fatty acids was carried out according to AOCS (2004) methods: acidity index (AI) (Cd 3d-63), iodine value (IV) (Cd 1b-87), peroxide value (PV) (Cd 8-53), and saponification index (SI) (Cd 3-25). All analyses were performed in triplicate.

2.5. Determination of lipase activity

The lipase activity was performed using the method described by SUGIHARA *et al*. (1990) An olive oil emulsion was prepared and added of 50 mM acetate buffer (pH 5.6) and 100mM $CaCl_2$. The enzyme solution added varied from 5 to 50 μL. The reaction was maintained at 37°C under stirring of 500 rpm for 30 min. After that, the reaction was inactivated with ethanol, then titrated with KOH 50mM, to determine the amount of fatty acids released by enzymatic reaction.[19] The lipase activity was calculated using Equation 1:

$$\text{Activity} = \frac{N\left(\frac{\text{mol}}{L}\right)\times 10^6\left(\frac{\mu\text{mol}}{\text{mol}}\right)\times\left(\frac{L}{1000\text{mL}}\right)\times\Delta\text{volume}_{\text{NaOH}}(\text{mL})}{\text{time(min)}} \quad (1)$$

2.6. Hydrolysis degree

The hydrolysis degree of oils after the enzymic treatment was calculated according to Equation 2:

$$\text{Hydrolysis}(\%) = \frac{\text{AI(hydrolyzedoil}-\text{AI(non}-\text{hydrolyzedoil)}}{\text{SI(non}-\text{hydrolyzedoil)}-\text{AI(non}-\text{hydrolyzedoil)}}x100 \quad (2)$$

where: SI is the saponification index, and AI is the acidity index.

2.7. Statistical analysis

A factorial experimental design 2^2, with three central points, was applied to obtain statistical models for the parameters studied (hydrolysis time and temperature) in function of the considered responses (hydrolysis degree and iodine value). The studied factors with the respective real and coded levels are shown in Table 1.

Table 1. Variable levels and limits for the 2^2 experimental factorial design.

Independent variable	Level		
	-1	0	1
Hydrolysis time (h)	2	4	6
Hydrolysis temperature (ºC)	45	50	55

All statistical analysis was performed using the Statistica 6.0 software and the validity of quadratic model was performed by variance analysis (ANOVA) at 5% probability by Fisher test.

3. RESULTS AND DISCUSSION

3.1. Characterization of fish oils

Table 2 shows the yield of each step of chemical refining. The refined fish oil yielded 62.75%, which is acceptable, since refining stages causes many losses during process, as occur in neutralization step with NaOH. This alkali was more effective in bleaching, even if it caused saponification of a small part of neutral oil in the same time it promotes the neutralization of free fatty acids. The washing was essential to remove all NaOH residue. Notwithstanding, the importance of neutral oil drying lies in that the moisture, during the oil storage, can cause hydrolysis and increase the acidity, as well as oxidation of the heated oil. At the filtering stage, the yield dropped from 67.74% to 62.75%. This loss could be associated to the use of adsorbents in higher amounts than the minimum required, resulting in a greater loss of oil.

Table 2. Yield of chemical refining process.

Stage of the process	Yield (%)
Crude fish oil	100.00
Degumming	91.85
Neutralization	81.65
Washing	74.82
Decantation	70.12
Drying	67.74
Filtration	62.75
Refined fish oil	62.75

The chemical characterization of crude fish oil and refined fish oil is shown in Table 3.

Table 3. Chemical characterization of crude fish oil and refined fish oil.

Parameter	Crude fish oil	Refined fish oil
Acidity index (AI) (mg NaOH g-1)	5.05±0.02a	0.45±0.04b
Iodine value (IV) (g I2 g-1)	121±0.13a	121±0.11a
Peroxide value (PV) (mEq. Kg-1)	n.d*	n.d*
Saponification index (AI) (mg KOH g-1)	182±0.25a	182±0.19a

*Not detectable.
**Different letters in the same line indicated a significant difference ($p<0.05$) by the Tukey test.

A significant difference ($p<0.05$) was observed between the AI of crude fish oil and refined fish oil, showing that the chemical refining was effective in reducing that value. Free fatty acids contents are usually associated with undesirable flavour and textural changes (VISENTAINER *et al*., 2015). The IV, which is associated with the degree of unsaturated fatty acids of oil (CREXI *et al*., 2010), showed no significant difference ($p>0.05$) between the samples studied. The SI also presented no significant difference ($p>0.05$) between results (Table 3). However, they are in accordance with the values reported for oils from marine animals (160-196 mg KOH g^{-1}) (ARAÚJO, 2001). The peroxide value was not detected due to the addition of antioxidant, which inhibited the oxidation-reduction reactions used to determine this parameter. In general, the recommendation of peroxide value for human consumption is of 8 meq Kg^{-1} oil (BORAN *et al*., 2006).
OLIVEIRA *et al*. (2016) also observed similar results for AI of crude and refined tuna by-product oils. These authors observed a reduction of 85% in AI, whereas a higher reduction of 91% was obtained in the present study (Table 2). Moreover, the AI of refined fish oil is in accordance to other fish oils, *e.g.* Nile tilapia (0.08 mg NaOH g^{-1}) and hybrid sorubim (0.03 mg NaOH g^{-1}) (MENEGAZZO *et al*., 2014). Regarding IV results, they indicated that the unsaturated compounds were preserved during the process, which was also observed in other study with Nile tilapia and hybrid sorubim oils (MENEGAZZO *et al*., 2014). In general, IV of both samples are in accordance to the value reported by ACKMAN (1966) for oils from marine animals, varying from 110 to 193 g I_2 g^{-1}. MENEGAZZO *et al*. (2014)

3.2. Determination of the lipase activity

The lipase activity for the enzyme *Candida rugosa* Amano AY was 1.78 U mL^{-1} (35.7 U g^{-1}) according to SUGIHARA *et al*. (1990). Two experiments were carried out to verify the lipolytic activity. The relation among the enzyme concentration and the lipolytic activity was similar to the value supplied by the manufacturer, which is 30,000 U g^{-1} at the enzyme concentration of 2.5×10^{-4} g mL^{-1}.

3.3. Enzymatic hydrolysis

The results of enzymatic hydrolysis according to the experimental design are showed in Table 4. A decrease in the IV was observed with the temperature increased from 50 to 55ºC. This behavior is associated to the oxidation of unsaturated fatty acids present in the sample, reducing the IV.

Table 4. Factorial experimental design matrix and results for hydrolysis degree and iodine value for the hydrolyzed oil.

Experiment	Temperature (°C)	Time (h)	Hydrolysis degree (%)	Iodine value (g I_2 g^{-1})
1	-1 (45)	-1 (2)	22.79±0.12 [b]	121±0.19 [b]
2	-1 (45)	+1 (6)	23.45±0.15 [a]	120±0.20 [c]
3	+1 (55)	-1 (2)	14.65±0.10 [f]	179±0.13 [a]
4	+1 (55)	+1 (6)	20.4±0.09 [c]	107±0.17 [g]
5	0 (50)	0 (4)	22.63±0.18 [b]	117±0.12 [d]
6	0 (50)	0 (4)	18.90±0.11 [e]	113±0.22 [f]
7	0 (50)	0 (4)	21.00±0.12 [d]	114±0.15 [e]

*Different letters in the same line indicated a significant difference ($p<0.05$) by the Tukey test.

The experiment 2 showed the best results with hydrolysis degree (23.45%) and IV (120 g I_2 g^{-1}), whereas the other experiments showed minor values. According to those results, it was noted that the optimal temperature and hydrolysis time with *Candida rugosa* AmanoAY was at 45ºC and 6 h. Therefore, another experiment was realized to correlate hydrolysis degree and acidity index. Fig. 2 shows the hydrolysis results obtained with the optimal conditions (45°C and pH 7.0) for the enzyme *Candida rugosa* AY "Amano" 30 after 12 h, according to SUGIHARA *et al*. (1990).

According to Fig. 2, a sharp increase was observed in hydrolysis at the first 0.5 h (up to 14.1%). The maximal hydrolysis degree was obtained at 8 h (27.8%). In this meantime, the hydrolysis degree augmented at a relatively constant rate. After that, the values oscillated without great variability, showing that was not necessary to prolong the period of hydrolysis for a long time and that acidity index showed similar behavior, as expected.

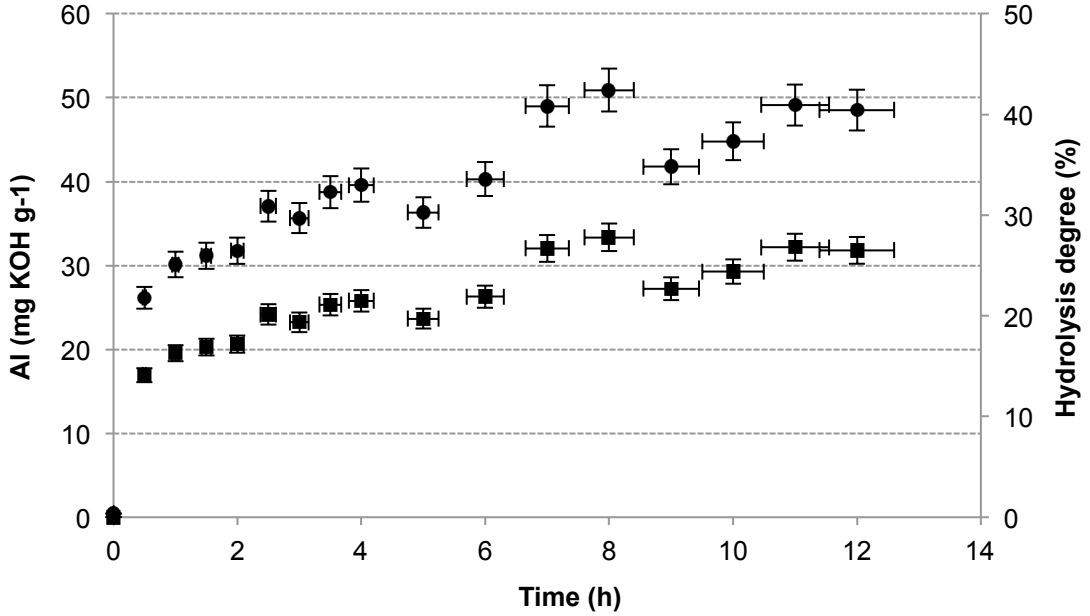

Figure 2. Kinetics of hydrolysis degree (■) (%) and acidity index (●) (mg KOH g^{-1} oil) of refined fish oil.

Other researchers also studied the enzymatic hydrolysis for extraction of unsaturated fatty acids from fish oil (IBERAHIM *et al*., 2018; BABAJAFARI *et al*., 2017). BABAJAFARI *et al*. (2017) compared enzymatic hydrolysis and chemical methods of oil extraction from rainbow trout. The chemical methods showed higher yield (16.58%) values than enzymatic hydrolysis (13.65% - 150ppm concentrated protease). However, the fatty acids composition showed that enzymatic hydrolysis concentrated the LC-PUFA (EPA and DHA), whereas the chemical methods concentrated MUFA and PUFA (linoleic acid and α-linolenic acid). Moreover, enzymatic hydrolysis is a safety method about food quality and environment-friendly extraction.

IBERAHIM *et al*. (2018) observed that catfish oil submitted to enzymatic hydrolysis showed no significant difference ($p<0.05$) in their fatty acids content, it means, MUFA content before hydrolysis was 48.41% and after 47.99%; PUFA content decreased from 20.32% to 19.32% after enzymatic hydrolysis by lipase. This behavior could be associated to oil auto-oxidation and photo-oxidation, since PUFA are more likely to undergo oxidation. PV and AI increased during the enzymatic hydrolysis, indicating the oil oxidation. Those results are similar to obtained in this study (Tables 3 and 4), since AI increased during the enzymatic hydrolysis process and IV indicated no change in fatty acids chains.

Despite results showed that hydrolysis of refine oil was affected by temperature and time (Table 4), no significant difference was observed between the IV of refined oil (Table 3) and hydrolyzed oil (Table 4). An increase in IV was expected with the hydrolysis process, since the enzyme has the ability to concentrate the unsaturated fatty acids. So, those results indicated that the hydrolysis process was not efficient. Thus, other process such as winterization (AMORIM *et al*., 2015) and ultrafiltration processes (KORIS *et al*., 2006) might be also evaluated to this kind of raw material to improve its nutritional quality, increasing the unsaturated fatty acids content.

4. CONCLUSIONS

The chemical refining yielded 62.75% and was efficient at removing undesirable compounds from crude fish oil, since the acidity index decreased by around 91% during the processing. The iodine value and saponification number of refined oil were not affected, ensuring its nutritional quality as a food ingredient. The best results of hydrolysis degree (23.45%) and iodine value (120 g I_2 g^{-1}) were obtained at 45ºC after 6 h of lipase action. However, this hydrolysis process was not efficient in concentrating the unsaturated fatty acids of the refined oil since no significant difference ($p>0.05$) was observed between the iodine value of the refined oil and the hydrolyzed oil. Thus, further research using this kind of residue is necessary in order to obtain isolated fatty acids (omega-3) or increasing the content of health beneficial fatty acids.

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