Research Article

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Comparative oil extraction from mutt (*Myliobatis goodei*) liver by enzymatic hydrolysis: free *versus* immobilized biocatalyst

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Abstract

BACKGROUND: The development and fine-tuning of biotechnological processes for fish oil extraction constitute a very important focus to contribute to the development of a food industry based on fish consumption. This work lies in a comparative analysis of the oil extraction yield of *Myliobatis goodei* livers using free and immobilized enzymes.

RESULTS: An immobilized biocatalyst was designed from the cell-free extract of a *Bacillus* sp. Mcn4. A complete factorial design was used to study the components of the bacterial culture medium and select the condition with the highest titers of extracellular enzymatic activities. Wheat bran had a significant effect on the culture medium composition for enzymatic production. The immobilized biocatalyst was designed by covalent binding of the proteins present in the cocktail retaining a percentage of different types of enzymatic activities (*Mult.Enz*@$MgFe_2O_4$). Among the biocatalyst used, Alcalase® 2.4 L and Purazyme® AS 60 L (free commercial proteases) showed extraction yields of 87.39% and 84.25%, respectively, while *Mult.Enz*@$MgFe_2O_4$ achieved a better one of 89.97%. The oils obtained did not show significant differences in their physical–chemical properties while regarding the fatty acid content, the oil extracted with Purazyme® AS 60 L showed a comparatively lower proportion of polyunsaturated fatty acids.

CONCLUSIONS: Our results suggest that the use of by-products of *M. goodei* is a valid alternative and encourages the use of immobilized multienzyme biocatalysts for the treatment of complex substrates in the fishing industry.

Keywords: fish liver oil; oil extraction; enzymatic treatment; multienzyme immobilization; *Bacillus*

INTRODUCTION

Nowadays there is a notable trend in driving the growth of global fish consumption that has been accompanied by many fundamental changes in the ways that consumers choose, buy, prepare, and consume fish products. The globalization of fish and fishery products, promoted by further trade liberalization and advances in food processing and transportation technologies, has expanded supply chains. So, it is necessary to contribute to this current by promoting the development of new products, valorizing traditional species, and reassessing by-products and discards.[1]

Cartilaginous fish species are traditionally caught around the world. However, only a few parts are eaten and most of the rest is considered waste (liver, viscera, and skin).[2] These by-products contain proteins of high biological value, vitamins, minerals, and lipids rich in essential fatty acids.[3] Cartilaginous fish livers contain large amounts of oil rich in n-3 polyunsaturated fatty acids (PUFAs) that are important in the human diet and have great commercial and scientific interest.[4,5] Fish oils are the most important source of n-3 PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which have several benefits for human health, being essential for the development and functionality of vital organs and metabolic processes.[6]

To extract oils from fish by-products different methodologies have been proposed. The method commonly used is extraction by wet pressing, which consists of cooking, pressing, and centrifugation to generate large volumes of crude oil.[7] However, the

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drastic conditions of temperature and pressure used can partially modify the PUFAs.[8,9] The use of solvent extraction is generally for analytical purposes, but not for industrial production, due to the usage of substances restricted in the food industry.[10] Fluid supercritical extraction is an emerging technology, which uses moderate temperatures, an oxygen-free environment, and allows the extraction of low-polarity lipids avoiding the extraction of impurities.[11] Finally, enzymatic hydrolysis usually employs a protease that enables the oil to be released from soluble components of aqueous phases and sediments at mild temperature and pH conditions.[3,12] This process is a good alternative to traditional methods since they prevent undesirable reactions such as oxidation and allow functional ingredients to be recovered.[12]

In biocatalytic processes, the immobilization of enzymes consists of a centuries-old technique that allows for achieving high operational and storage stability of the biocatalyst with the possibility of continuous reuse of the enzyme.[13,14] A particular approach of growing interest in this field consists of the use of multienzyme systems. Indeed, in cells, intracellular catalytic systems usually consist of a large number of multienzyme complexes, immobilized in a certain way through a cell compartmentalization mechanism.[14,15] Academic works carried out in the last decade have shown certain benefits conferred by performing a co-immobilization multienzyme on a wide range of supports. These developed technologies have opened a door in synthetic biology for the production of pharmaceuticals or for the treatment of complex substrates to generate products such as biofuels, biopolymers, or nutraceuticals.[16-18]

The *Bacillus* genus is known due to its wide potential for industrial applications, mainly for its remarkable ability to produce extracellular enzymes, among other compounds. Furthermore, many *Bacillus* strains have been characterized as GRAS (Generally Recognized as Safe) microorganisms, being suitable to be used in the food industry.[19] Among the enormous enzymatic machinery produced by these bacteria, the synthesis of extracellular proteases and lipases represents a highly promising option for their simultaneous application in the treatment of fishing industry by-products.[18] Enzymatic extraction of oil from various fishing waste, in particular, comprises a process in which three phases are typically obtained: an upper oily phase, an oil–water interface, and a lower aqueous phase. Proteases play an important role in enhancing the solubility of the water-soluble fraction and facilitating its separation from the oil phase by gradually hydrolyzing proteins to smaller peptides.[20] Lipases tend to congregate near the oil–water interface, where they may exhibit their catalytic activity by breaking down ester-bonds in different lipids (triglycerides, phospholipids, cholesteryl esters) releasing mostly fatty acids and glycerol.[21]

Previous reports carried out by our working group have shown the great biotechnological potential of *Bacillus* sp. Mcn4 strain in terms of its biocatalytic capacity.[22] Additionally, we have previously worked on lipase immobilization employing $MgFe_2O_4$ and $Ca_2Fe_2O_5$ nanoparticles as supports by both physical and covalent binding. The systems designed have been extensively characterized showing applications in food biotechnology.[23,24] In particularly, Morales *et al.*[23] optimized a culture medium for *Bacillus* sp. Mcn4 to produce lipases and the cell-free broth was immobilized by adsorption on $Ca_2Fe_2O_5$. The biocatalyst obtained in that work was able to enrich the content of PUFAs in refined ray liver oil.

Based on this background, the hypothesis of this work assumes that a *Bacillus* strain's cell-free extract with proteases and lipases allows a successful oil extraction when they are immobilized. In this sense, by hydrolyzing proteins, proteases prevent them from acting as emulsion-forming surfactants that cause oil loss, in addition to producing amino acids that are more water-soluble. Lipases, for their part, would help to reduce the volume of the emulsion by acting on triglycerides and phospholipids, hydrolyzing them into their constituents, and promoting phase separation. Thus, in this work, we assessed the yield of the enzymatic extraction process of oil from *Myliobatis goodei* livers using commercial enzymes and an immobilized biocatalyst from an enzymatic cocktail from a *Bacillus* strain. In addition to that, the physicochemical and nutritional characteristics of the oils obtained were evaluated and compared.

MATERIALS AND METHODS

Chemicals, substrates, and reagents

Carboxymethylcellulose (CMC), bovine serum albumin (BSA), Coomassie Blue G-250, *p*-nitrophenyl acetate (p-NPA), *p*-nitrophenyl palmitate (p-NPP), pentacyanonitrosil ferrate salts, azocasein, glutaraldehyde (GA), 3-aminopropyltriethoxysilane (APTES), xylans extracted from birchwood, dinitrosalicylic acid (DNS), and starch were purchased from Sigma-Aldrich (St Louis, MO, USA). Wheat bran was supplied by Obispo Colombres Agro-industrial Experimental Station (EEOC; San Miguel de Tucumán, Argentina).

Biological marine samples

The *M. goodei* specimens were captured during research campaigns carried out by the National Institute for Fisheries Research and Development (INIDEP) in 2018. The livers of each specimen were separated, packed in polyethylene bags, and frozen at −80 °C until use.

Microorganism and culture condition

An enzymatic cocktail was produced using a bacterial strain belonging to the Planta Piloto de Procesos Industriales Microbiológicos (PROIMI) collection, *Bacillus* sp. Mcn4. A full factorial design was used to identify the condition with the best enzyme production. As variables, three components of the culture medium were chosen at two levels: wheat bran (2 and 20 g/L), tryptein (0 and 5 g/L), and yeast extract (1 and 5 g/L). Ten experimental trials were obtained using the Minitab19® software, taking into account a central point for the variables tested (duplicated). Submerged cultures were carried out in 500 mL Erlenmeyer flasks with a culture volume of 100 mL. The incubation was carried out at 30 °C with constant agitation. Samples were taken at 24, 48, and 72 h. The cells were separated by centrifugation at 8000 × *g* at 4 °C and the cell-free supernatant was stored at 4 °C until the enzymatic and protein determinations were made.

Immobilized biocatalyst

Support synthesis

The mixed oxide $MgFe_2O_4$ used as a support for immobilization was synthesized following the methodology previously described by Romero *et al.*[25] Briefly, $MgFe_2O_4$ was obtained from the thermal decomposition in a muffle furnace of the heteronuclear complex $Mg[Fe(CN)_5NO]\cdot 4H_2O$, which was synthesized by an indirect double substitution method, reacting stoichiometric amounts of $MgCl_2$ with $Na[Fe(CN)_5NO]$.

Immobilization protocol

The surface of the mixed oxide used for enzyme immobilization was initially chemically modified with APTES and GA according

to the protocol described previously by Romero *et al.*[25] to generate chemical groups for the covalent binding of proteins. This functionalized $MgFe_2O_4$ was then mixed with a cell-free supernatant in a mass-volume ratio of 1:19. The immobilization conditions were 30 °C for 12 h with gentle agitation. The biocatalyst obtained (*Mult.Enz*@$MgFe_2O_4$) was washed twice with distilled water, and dried at 30 °C for 24 h, then stored at 4 °C until later use.

Enzymatic parameters

Immobilized protein (IP) (mg protein/mg support) was determined according to the following equation:

$$IP = \frac{V_{enz}(C_0 - C_f)}{m}$$

where V_{enz} is the volume of the enzymatic solution in milliliters, C_0 is the initial protein concentration (in mg/mL), C_f is the residual concentration of protein in solution after the immobilization process (in mg/mL) and m is the mass of the support (in milligrams).

Apparent hydrolytic activity (HA) (IU/mg support) was calculated as the ratio between hydrolytic activity measured in the immobilized biocatalyst (IU) and the amount of support used.

The immobilized yield (IY) expressed as a percentage was calculated according to the following equation:

$$IY\,(\%) = \left(\frac{EA_0 - EA_f}{EA_0}\right) \times 100$$

where EA_0 and EA_f are the enzymatic activities of the solution before and after enzyme immobilization (IU/mL), respectively.

Finally, the recovered activity (RA) expressed as a percentage was calculated as follows:

$$RA\,(\%) = \frac{HA}{U_i\,IY} \times 100$$

where HA is the apparent hydrolytic activity of the biocatalyst (IU/mg support), U_i is the activity of the soluble enzyme before immobilization (IU/mg support) and IY is the immobilized yield.

Enzyme activities

Esterase and lipase activities

Esterase and lipase activities were measured using p-NPA and p-NPP as substrates, respectively.[26] The reaction mixture had a final volume of 1 mL and consisted of 100 µL of the cell-free extract (or 0.001 g of immobilized biocatalyst) contained in 100 mmol/L phosphate buffer pH 7 with 0.04% (*w/v*) Triton X100, 0.01% (*w/v*) gum arabic and 1 mmol/L p-NPA or p-NPP as appropriate. The reaction mixture was stirred at 37 °C for 10 min. The amount of *p*-nitrophenol (p-PN) released was measured spectrophotometrically at 405 nm. One international unit of enzymatic activity (IU) was defined as the amount of biocatalyst that released 1 µmol of p-NP per minute under the reaction conditions.

Protease activity

Protease activity was measured using 1% (*w/v*) azocasein as substrate in 0.20 mol/L Tris–HCl buffer.[27] One unit of protease activity (U) was defined as the amount of enzyme producing an increase of one unit in the optical density at 440 nm in 1 h.

Endoglucanase, xylanolytic, and amylase activities

Endoglucanase (CMCase), xylanase, and amylase activities were quantified using the DNS method.[28] The reaction mixtures consisted of 0.45 mL of 2% (p/*v*) CMC solution, 1% (p/*v*) birchwood xylan, or 1% (*w/v*) starch in 100 mmol/L sodium phosphate buffer pH 7 with 0.05 mL of culture supernatant or 0.001 g of the immobilized biocatalyst. The resulting solutions were incubated at 37 °C for 10 min and the reducing sugars released were quantified by adding DNS reagent and boiling for 10 min. The absorbance was then recorded at 540 nm and an international unit of enzyme activity (IU) was defined as the amount of enzyme that releases 1 µmol of reducing sugars (measured as glucose, xylose, or maltose as appropriate) per milliliter per minute under the reaction conditions.

Protein measurement

Protein measurements were performed according to the Bradford method[29] using BSA as standard.

Raw material characterization

Proteins were determined by the Kjeldahl method using a conversion factor of 6.25 to convert the nitrogen measurement to crude protein. Moisture was quantified by drying in an oven at 105 °C until constant weight. The ash content was quantified by calcination in a muffle at a temperature of 550 °C until white ash and constant weight were obtained.[30] However, the lipids were extracted and quantified by the method of Bligh and Dyer.[31] Lipid extracts from all samples were stored at −80 °C until further use.

Oil extraction procedure

Lipid extraction by enzymatic hydrolysis was performed using two commercial proteases (Alcalase® 2.4 L and Purazyme® AS 60 L) and *Mult.Enz*@$MgFe_2O_4$. The hydrolysis was carried out in a thermostatic reactor with constant stirring. Equal parts of ground livers and distilled water at 50 °C were mixed. For commercial enzymes, the incubation conditions were a pH of 8.0 ± 0.3 and a temperature of 55 ± 2.5 °C according to the supplier specifications. The hydrolysis process was started with the addition of the appropriate biocatalyst, maintaining an enzyme/raw material ratio of 2%. The reaction was run for 1 h and the temperature was then raised to 85 °C for 10 min to inactivate each enzyme. By using *Mult.Enz*@$MgFe_2O_4$, the pH of the reaction was adjusted to 7.0 ± 0.5 with phosphate buffer and the enzyme was also added at a substrate mass concentration of 2%. This enzymatic reaction was carried out at 40 °C ± 2.5 °C for 1 h with continuous stirring. The pH of reacting mixtures was adjusted with 2 mol/L sodium hydroxide (NaOH). Finally, the hydrolysates were centrifuged at 20 000 × *g* for 30 min at 4 °C, after which the tubes were placed in a vertical position and the separation of the different fractions continued.

Lipid fraction analysis

Oil extraction yield

The yield of the different treatments was determined by measuring the amount of oil extracted after enzymatic hydrolysis. It was then expressed as the percentage of enzymatically extracted crude oil (WO_{Enz}) about the oil content in the residues obtained from the proximal analysis by the Bligh and Dyer method (WO_{Prox}) previously described.

Physicochemical analysis

The following physical quality indices were determined according to American Oil Chemists' Society (2009)[30]: moisture and volatile material content using the vacuum oven method; relative density using a pictometer calibrated at 20 °C; color measured from the Gardner scale (Gardner-Delta Color Comparator). To evaluate the oxidative stability, the following parameters were determined: the acidity index, the peroxide value (PV) as an indicator of primary oxidation; the anisidine index (AI) to detect secondary oxidation, and the total oxidation was determined by the TOTOX index (2PV + AI).

The fatty acid profile of the extracted oils was determined. First, the aliquots of each sample were methylated according to ISO 12966-2 (International Organization for Standardization, 2017). Briefly, the oil was dissolved in isooctane (1 mg/mL) and 0.5 mL of potassium hydroxide/methanol (KOH/MeOH, 2 mol/L) was added. The mixture was stirred for 1 min and then an equal volume of isooctane sodium chloride (NaCl) solution (40% saturated) was added. The resulting mixture was stirred for 10 s and the upper phase was transferred to a clean tube. Fatty acid methyl ethers were separated and identified in a Shimadzu GC-2010 gas chromatograph (Kyoto, Japan), equipped with a split injector using an Omegawax Supelco 320 (Darmstadt, Germany) fused silica capillary column (30 cm length, 0.32 mm internal diameter, 0.25 um film thickness of the stationary phase) and a flame ionization detector (FID). The temperature program used in the column started at 50 °C, followed by an increase of 5 °C/min up to 200 °C, holding this last temperature for 14 min, using helium as the carrier gas. The chromatographic run lasted 40 min and commercial standards of fatty acids present in marine organisms (Supelco® FAME Mix C4-C24 + PUFA N°1 Marine Source) were used for their identification. The resulting chromatograms were analyzed using GCSolution software (Shimadzu).

Statistical analysis

All the samples and assays carried out in this work were performed and analyzed in triplicate and the results are expressed as the mean and standard deviation (SD). The comparative analyses were carried out using the Infostat software, Córdoba, Argentina through a Duncan (1955) test, considered significant with an $\alpha = 0.05$.

RESULTS AND DISCUSSION

Enzymatic profile of *Bacillus* sp. Mcn4

Cell-free extract

The enzyme production by *Bacillus* sp. Mcn4 was studied through a complete factorial design, considering wheat bran, tryptein, and yeast extract as the study variables. The best activity titles were obtained after 72 h of culture and the results of the different enzymatic activities measured for the different design runs are detailed in Table 1. Except for runs 2, 4, and 9 (which had the lowest activity titers), there were no variations in microbe growth in terms of optical density in the other runs (data not shown). Thus, in most cases, the observed changes in activity titers were caused by alterations in the culture medium's components. CMCase was the only enzymatic activity not registered and the condition in which the highest activity values were obtained was (in g/L): 20, 5, and 5 for wheat bran, tryptein, and yeast extract, respectively (run 5). The statistical parameters associated with the study of each response are shown in Table 2; the lack of fit for each of them was not significant ($P > 0.05$). As general remarks, the incorporation of wheat bran as a component of the culture medium was significant in all the responses evaluated ($P < 0.05$), also showing a positive statistical effect, indicating that the enzymatic activities increased at the highest level evaluated (20 g/L). When lipolytic and proteolytic activity were evaluated, we found that only wheat bran had a significant influence ($P < 0.05$). Regarding the rest hydrolytic activities examined (amylase, xylanase, and esterase), the three components of the culture medium evaluated showed a significant effect. Wheat bran is an undervalued raw material derived from the agroindustry that contains carbohydrates (as fiber), lipids and proteins, among other nutrients. Thus, it constitutes an appropriate substrate to support microbial growth, serving as a carbon and nitrogen source.[32] In addition, due to its complex composition and structure, this agricultural by-product is usually used to promote the production of a wide repertoire of enzymatic activities.[33] In this view, the presence of proteins and lipids in the nutritional content of wheat bran could lead to microorganism adaptability via the synthesis of particular enzymes in response to substrate availability. This would result in the high protease, esterase, and lipase activity titers shown in our study. Thus, our findings highlight the significance of wheat bran in promoting enzyme production.

Based on these results, the conditions of trial 5 of the design were adopted for the production of the cell-free extract, which

Table 1. Complete factorial design matrix for the three factors selected: wheat bran (A), tryptein (B) and, yeast extract (C) (g/L)

Run	A	B	C	Esterase	Lipase	Xylanase	Amylase	Protease
1	20	0	5	48.55	10.64	0.82	0.22	0.61
2	2	5	1	6.83	0.00	0.80	0.02	0.17
3	20	5	1	52.87	12.69	0.90	0.23	0.74
4	2	0	1	0.00	0.00	0.00	0.01	0.08
5	20	5	5	68.21	12.43	1.24	0.30	0.81
6	2	5	5	19.64	0.70	0.98	0.05	0.22
7	11	2.5	3	70.74	9.67	0.59	0.06	0.36
7′	11	2.5	3	67.47	10.72	0.50	0.07	0.25
8	20	0	1	46.76	5.72	0.47	0.16	0.41
9	2	0	5	9.22	1.56	0.45	0.03	0.44

Experimental results for different enzymatic activities (UI/mL) measured at 72 h are detailed for each trial.

Table 2. Estimated effects and statistical parameters for the three factors studied [wheat bran (A), tryptein (B) and, yeast extract (C)] as components in the culture media and their interactions for enzymatic production of *Bacillus* sp. Mcn4 by means a complete factorial design

	Esterase		Lipase		Xylanase		Amylase		Protease	
Factors	Effect	*P*-Value	Effect	*P*-Value	Effect	*P*-Value	Effect	*P*-Value	Effect	*P*-Value
A	45.17	0.002	9.809	0.007	0.2975	0.034	0.20002	0.000	0.4150	0.014
B	10.76	0.036	1.977	0.146	0.5503	0.010	0.04403	0.007	0.1000	0.185
C	9.79	0.043	1.733	0.178	0.3326	0.027	0.04547	0.006	0.1700	0.077
A*B	2.13	0.419	2.405	0.105	−0.1220	0.161	0.03202	0.013	0.1650	0.082
A*C	−1.23	0.620	0.600	0.553	0.0149	0.815	0.02348	0.023	−0.0350	0.558
B*C	4.29	0.179	−1.511	0.217	−0.0693	0.341	0.00747	0.178	−0.1100	0.160
Curvature	—	0.004	—	0.038	—	0.122	—	0.005	—	0.147
R^2	0.9974		0.9890		0.9886		0.9994		0.9813	
Adjusted R^2	0.9883		0.9504		0.9487		0.9975		0.9159	
Predicted R^2	0.8806		0.4185		0.4810		0.9949		0.4757	

Data analysis was performed using enzymatic activities (UI/mL) measured at 72 h.

was used to immobilize the enzymes present in $MgFe_2O_4$ nanoparticles.

Immobilized biocatalyst

The biocatalyst *Mult.Enz*@$MgFe_2O_4$ was successfully designed and the amount of protein adsorbed on the support was 2.77 ± 0.09 mg protein/mg support. As can be seen in Table 3, all the activities present in the cell-free extract were registered in the $MgFe_2O_4$ biocatalyst. Based on the drop-in activity that was seen in the supernatant before and after the immobilization, the IY represents the proportion of theoretical activity that would be kept on *Mult.Enz*@$MgFe_2O_4$. As can be seen in Table 3, the IY values ranged from approximately 14–31% for the different enzymatic activities. However, the RA is computed as the ratio of the activity measured in the adsorbed enzyme to the enzymatic activity that is hypothetically lost in the solution. Thus, RA less than 100% would indicate that the immobilization procedure harmed the enzyme's catalytic ability, whereas higher values would suggest that the enzyme was activated. When analyzing the RA measurements for each group of enzymes, we can observe that in most cases (xylanolytic, amylolytic, and proteolytic activities) there was a decrease in the expected activity. This may suggest that these enzymes were undergoing one or more inactivation processes (chemical modification, mass transfer problems, etc.).[34] RA greater than 100% was observed for the esterase and lipase activities, which would indicate a slight activation. This phenomenon may be due to the interfacial activation experienced by these enzymes when they are close to a hydrophobic environment. In a recent article, our research highlighted the existence of an interfacial activation of lipases mediated by protein-support hydrophobic interactions in this kind of oxides.[35]

Liver chemical composition

Table 4 shows and compares the proximal composition of *M. goodei* livers studied in this work with those reported for related species (Superorder: Batoidea; Orders: Myliobatiformes and Rajiformes). In general, the moisture and ash contents were comparatively lower than in the other species, except for *Rhinobatos cemiculus*. Likewise, the protein content was the lowest value reported for the livers of cartilaginous fishes belonging to these orders. The lipid content was 62.36% and represented the major component, being higher than that of other ray species captured on the Argentine continental shelf (*Zearaja flavirostris* and *Atlantoraja castelnaui*). Among the other species in Table 4, only the lipid content in *Rhinobatos cemiculus* (captured from the Mauritania coast) was higher, although older reports of this specimen from the coast of Tunisia showed a lower liver lipid content of around 40%.[37] The variation among related species and within the same species may be related to the physiological conditions of the fish (type of feeding, gonadal stage, season, among others).[38]

Oil extraction yield

Results of oil extraction yields referred to the initial oil content established in the proximal composition of the raw material described in the previous section. Oil extraction yields of 87.39%

Table 3. Immobilization parameters for the different measured activities associated with the immobilization process by covalent binding of the proteins present in the cell-free extract of *Bacillus* sp. Mcn4 on $MgFe_2O_4$

	Activities				
Immobilization parameters	Esterase	Lipase	Xylanase	Amylase	Protease
Hydrolytic activity (IU/mg support)	1.126 ± 0.035	0.264 ± 0.024	0.014 + 0.010	0.005 + 0.001	0.021 ± 0.004[a]
Immobilization yield (%)	14.68 ± 2.15	16.32 ± 2.21	18.05 ± 5.12	24.10 ± 2.06	31.31 ± 3.14
Recovery activity (%)	113.74 ± 4.12	123.36 ± 7.15	58.33 ± 6.84	74.63 ± 2.14	79.48 ± 3.37

[a] Protease activity is expressed as U/mg support.

Table 4. Proximal composition of *Myliobatis goodei* livers compared with reports for related species (all liver samples)

Species	Moisture (%)	Ash (%)	Protein (%)	Lipid (%)	Reference
Myliobatis goodei	29.59 ± 0.36	0.43 ± 0.03	7.65 ± 0.33	62.36 ± 0.13	This work
Zearaja flavirostris	43.41 ± 4.48	0.59 ± 0.12	9.20 ± 1.20	44.59 ± 3.34	3
Atlantoraja castelnaui	61.20 ± 4.75	0.98 ± 0.13	17.33 ± 2.61	21.10 ± 6.50	3
Raja clavata	49.2 ± 1.1	0.5 ± 0.1	11.2 ± 0.4	39.7 ± 2.0	4
Dasyatis violacea	34.54 ± 2.70	0.51 ± 0.01	8.43 ± 0.45	57.33 ± 0.76	36
Rhinoptera marginata	76.23 ± 3.14	0.89 ± 0.04	12.02 ± 0.13	10.90 ± 0.25	36
Rhinobatos cemiculus	19.06 ± 0.232	0.234 ± 0.043	11.22 ± 0.87	69.57 ± 0.311	37

and 84.25% were obtained for Alcalase® 2.4 L and Purazyme® AS 60 L, respectively (Table 5). These data are in agreement with the values reported in previous works carried out by our working group using the same hydrolysis conditions and enzymes for oil recovery from ray livers.[3] In general, the differences that can be observed are related to the composition of the starting raw material. Rubio-Rodríguez *et al.*[11] reported yields close to 100% working with salmon residues and the enzyme Alcalase® 2.4 L, whereas Głowacz-Różyńska *et al.*[12] achieved only 70% oil extraction using these food-grade proteases working with salmon salar heads.

However, *Mult.Enz*@$MgFe_2O_4$ not only equaled the extraction yield achieved by commercial enzymes but also improved it, reaching a value of 89.97%. Although the microorganism produced proteases, the multiple enzymatic activities that were immobilized likely contribute in different ways to achieve this performance. Livers are complex matrices like wheat bran where the microorganism grew. In this way, the bacterial enzymatic machinery generated under culture conditions for the consumption of a complex carbon source would contribute to improving the separation of the aqueous protein phase from the oily lipid phase in the livers, due to the multiple hydrolytic reactions. In this sense, Hepziba Suganthi *et al.*[18] also carried out a multienzyme co-immobilization for the treatment of fish processing solid waste. These authors immobilized lipase and protease produced by *Streptomyces thermolineatus* in magnetic nanoparticles, achieving a lipid and protein hydrolysis percentage of up to 73.9% and 82.1%, respectively, by varying the nanoparticle functionalization protocol.

The raw material is rich in membranes made up of phospholipids which consist of a glycerol-3-phosphate esterified at its sn-1 and sn-2 positions with fatty acids. Its phosphoryl group can be esterified with head groups such as choline, serine, ethanolamine, or inositol. Phospholipids have an amphiphilic nature[39] that cause oil losses due to these types of molecules that could transport oil in the form of emulsions.[40] In the hydrolysates, these molecules remain at the interface between the extracted residual oil and the aqueous protein phase. In this way, these compounds do not allow the complete extraction of the oil since they retain oil by emulsion. Probably, the lipase activity of *Mult.Enz*@$MgFe_2O_4$ removed the fatty acids concerning glycerol and contributes to improved oil yield extraction.

Physicochemical characterization of extracted oils

Table 5 also details the physical–chemical characterization of the extracted oils. The acid value is related to the presence of free fatty acids produced during the hydrolysis of triglycerides and the contribution of other non-lipid compounds. Thus, a low acid value is desirable and depends on several factors such as the composition of the oil, the extraction process, and the freshness of the raw material.[11] The acidity indices of the different oils extracted in this work were below the limits established by CODEX (2017)[41] of 3 mg KOH/g oil for fish liver oils suitable for human consumption. The oil extracted with the *Mult.Enz*@$MgFe_2O_4$ was slightly more

Table 5. Oil extraction yield and physicochemical parameters associated to oils extracted from *Myliobatis goodei* livers by different enzymatic hydrolysis treatments

	Enzyme		
Parameter	Alcalase® 2.4 L	Purazyme® AS 60 L	*Multi.Enz*@$MgFe_2O_4$
Oil extraction yield	87.39[ab] ± 1.30	84.25[a] ± 1.75	89.97[b] ± 0.06
Acid value (mg KOH/g)	2.38[a] ± 0.13	2.36[a] ± 0.12	2.62[a] ± 0.02
Peroxide value ($meqO_2$/kg)	7.86[b] ± 1.60	9.40[b] ± 0.60	6.52[a] ± 0.39
Anisidine index	10.98[a] ± 0.88	10.16[a] ± 0.28	9.91[a] ± 1.88
TOTOX	26.70	28.98	22.95
Moisture (g/100 g)	0.89[a] ± 0.02	0.86[a] ± 0.07	0.89[a] ± 0.02
Color (Gardner scale)	6–7	7–8	7–8
Density (kg/m^3)	924.20[a] ± 2.20	921.8[a] ± 3.10	925.02[a] ± 1.98

Values in the same row with the same letter are not significantly different ($P = 0.05$).
[a] Lowest value.
[b] Highest value.

Table 6. Fatty acid content of *Myliobatis goodei* liver oil samples extracted by different enzymatic treatments

Fatty acid	Alcalase® 2.4 L	Purazyme® AS 60 L	*Multi.Enz*@$MgFe_2O_4$
C12:0	0.46 ± 0.04	ND	0.60 ± 0.11
C14:0	4.18 ± 0.09	4.36 ± 0.36	4.56 ± 0.29
C15:0	0.96 ± 0.06	0.91 ± 0.11	1.03 ± 0.02
C16:0	17.79 ± 0.57	20.88 ± 3.71	19.48 ± 0.86
C17:0	0.53 ± 0.03	0.56 ± 0.02	0.55 ± 0.04
C18:0	4.24 ± 0.29	5.13 ± 0.50	4.56 ± 0.33
C20:0	0.24 ± 0.00	2.03 ± 0.05	0.47 ± 0.14
C22:0	0.04 ± 0.01	0.12 ± 0.06	0.05 ± 0.02
∑SFA (%)	28.45	33.99	31.31
C14:1	0.62 ± 0.03	0.63 ± 0.04	0.70 ± 0.06
C15:1	0.87 ± 0.01	0.60 ± 0.02	0.85 ± 0.00
C16:1	13.71 ± 1.21	11.55 ± 0.13	10.40 ± 2.16
C17:1	0.71 ± 0.03	0.66 ± 0.12	0.70 ± 0.00
C18:1n9	18.13 ± 0.01	20.01 ± 1.90	18.47 ± 0.31
C18:1n7c	2.72 ± 0.43	3.53 ± 0.04	2.84 ± 0.27
C18:2n6c	0.21 ± 0.02	0.92 ± 0.2	0.24 ± 0.03
C20:1n9	1.79 ± 0.11	0.97 ± 0.14	1.91 ± 0.13
C22:1n9	0.14 ± 0.00	0.13 ± 0.02	0.75 ± 0.37
C22:1n11	0.13 ± 0.00	0.14 ± 0.01	0.15 ± 0.02
∑MUFA (%)	39.02	39.14	37.01
C18:3n6	0.59 ± 0.00	0.59 ± 0.10	0.75 ± 0.10
C18:3n3	0.26 ± 0.01	0.35 ± 0.08	0.30 ± 0.02
C18:4n3	1.51 ± 0.16	0.31 ± 0.02	1.73 ± 0.21
C20:2n6	0.99 ± 0.05	1.03 ± 0.11	1.02 ± 0.00
C20:3n6	0.40 ± 0.04	ND	0.28 ± 0.08
C20:4n6	1.85 ± 0.01	1.01 ± 0.05	1.81 ± 0.00
C20:5n3	7.08 ± 0.02	6.14 ± 1.29	6.40 ± 0.49
C22:2	0.02 ± 0.00	0.29 ± 0.08	0.03 ± 0.01
C22:5n3	2.10 ± 0.15	1.11 ± 0.03	2.26 ± 0.04
C22:6n3	17.73 ± 0.77	15.90 ± 2.65	17.10 ± 0.70
∑PUFA (%)	32.53	26.88	31.68

Values are expressed as percentage (%*w/w*) of total fatty acid content. The percentage composition of the different types of fatty acids (SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid) is also detailed. ND, not determined.

acidic than the rest, probably due to the activity of immobilized lipases that would increase the amount of free fatty acids.

PV measures the oxidation or degree of rancidity of the oil at the time of testing.[42] As can be seen in Table 5, high PVs were obtained for all oils, especially for the one extracted with Purazyme AS 60 L. This could suggest that these oils need to be treated with antioxidants to prevent immediate oxidation. It was also registered that the oil extracted with *Mult.Enz*@$MgFe_2O_4$ showed lower values in terms of its PV, indicating that the multi-catalytic process could have contributed to reducing the oxidation processes associated with this index. Although PV is a standard measure to quantify lipid oxidation, its use is limited to the initial stages of the reaction. The AI determines the secondary oxidation products. In all the oils evaluated, the AIs were close to 10, which reflected a low degree of secondary oxidation and an acceptable freshness according to Masson.[43] In general, several limits and quality standards have been established for fish oil intended for food, according to which fresh oil must have PV between 3.9 and 5 meq O_2/kg, an AI between 10 and 20, while an oxidized oil presents levels higher than 26 meq O_2/kg. In this sense, the TOTOX value is a good indicator of the deterioration of oils by relating PV and AI to give an estimate of the total degree of oxidation of the oil, both by primary and secondary oxidation.[44] All values recorded in our work are within the limit established by CODEX (2017).[41]

The average moisture content of all oils tested was slightly less than 1% (Table 5). These values are above the range established by Masson[43] for crude oils suitable for animal nutrition. It should be noted, however, that crude oils generally undergo a drying stage during the refining process before being marketed. As for the color of the oils, the one extracted with Alcalase® 2.4 L was subtly different from the other two, showing values from 6 to 7 on the Gardner scale, with a yellowish, translucent, and light color. The other two oils, meanwhile, were darker but also with a yellowish hue. All the extracted oils were within the quality standards for crude fish oils which require a Gardner scale of less than 14.[45] The density of the oils obtained did not show significant differences, giving values within the expected range for this type of oil (0.90–0.93 g/mL).

Fatty acid profile of extracted oils

The profiles of fatty acids found in the oils extracted by the different enzymatic treatments are shown in Table 6. It also shows the sum of the percentage content of the different groups of fatty acids for the three oils. As can be seen, the oils extracted with Alcalase® 2.4 L and *Mult.Enz*@$MgFe_2O_4$ had an abundance of each fatty acid fraction of monounsaturated fatty acid (MUFA) > PUFA > saturated fatty acid (SFA) while for Purazyme® AS 60 L of MUFA > SFA > PUFA. Indeed, the oil extracted with Purazyme® AS 60 L showed the lowest content of PUFAs ($P < 0.05$), while the others did not show significant differences in the percentage content of this fraction, with values around 32%. The slight differences in the fatty acid profiles of the extracted oils may be attributed mostly to the kind of enzyme utilized, its starting concentration, and the hydrolysis time. Furthermore, the oxidative reactions that occur in the different reaction settings might also lead to a drop in PUFA concentration.[46] Because each enzymatic system has unique catalytic features, more in-depth investigations are required to elucidate the differences in the fatty acid profile found. Also, variances in the composition of other lipids not investigated in this work may have a lesser role in causing variations in the fatty acid profiles.[47]

Within the SFA, the oils showed a predominance of palmitic acid (16:0), followed by stearic acid (14:0) and myristic acid (18:0) which had similar content (Table 6). This is of interest since palmitic and stearic fatty acids can be used as an energy source.[48] The oleic acid (18:1 n9) and palmitoleic acid (16:1) were the predominant MUFAs, which have a great nutritional value since diets rich in oleic acid are associated with a reduced risk of developing type 2 diabetes.[49] In addition, in terms of PUFAs, the main components were EPA (20:5 n3) and DHA (22:6 n3).

CONCLUSIONS

In the current work, a multienzyme complex was immobilized on $MgFe_2O_4$ to assess its effectiveness in the oil extraction yield from *M. goodei* liver and compare it to commercial enzymes. The enzyme cocktail containing lipase, esterase, protease, amylase, and xylanase activities was produced from a *Bacillus* sp. Mcn4 strain and by optimizing the culture medium components. Our findings demonstrated that wheat bran incorporation was

significant in achieving high-activity titers in cell-free supernatant. The multienzyme cocktail was effectively immobilized, demonstrating the preservation of all enzymatic activities. Its application in oil extraction produced equivalent or slightly better yields than the commercial enzymes Purazyme® 60 L and Alcalase® 2.4 L. In all cases, the extracted oils showed potential nutritional qualities for use in the human diet.

In this way, our results suggest that the use of *M. goodei* by-products may be a source of potential exploitation. Moreover, among the enzymes studied for oil extraction, this work also gives the kick for using multienzyme complexes to treat marine samples in the extraction of oils. Indeed, the existence of accessory activities in the biocatalyst designed in our work contributed to a better oil separation compared to the use of only proteases. Thereby immobilized multienzyme biocatalysts constitute a highlighted alternative for the treatment of complex substrates in the fishing industry.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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