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Research Article

Abstract Extraction and purification of enzymes from the Southern eagle ray (*Myliobatis goodei*) by-products and their compatibility with detergents: A practical approach towards circular economy

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Abstract

Nowadays, the need to simultaneously achieve the valorization and recovery of fishing waste is critical for addressing environmental challenges and promoting resource sustainability. Fishing waste hosts enzymes that hold the potential for extraction and utilization in various industries. In this sense, the aim of this work was to extract and purify enzymes from the gastrointestinal tract of *Myliobatis goodei* using low-cost processes. The proteolytic and lipolytic activities of the extracted enzymes were also investigated. In addition, the detergent compatibility of the purified enzyme extract was evaluated to determine its potential application as an additive in laundry soaps. The crude extract was obtained by homogenization of 100g gastrointestinal tract in buffer Tris-HCl (pH=8.0). Then, it was precipitated with ammonium sulfate and purified by filtration. Finally, it was subjected to centrifugation at 10,000 ×g for 30 min at 4°C in an ultrafilters membrane with a 100 kDa cut-off. This purification protocol showed good performance for proteases and lipases as their activity was recovered at each step. The proteolytic activity was assayed using azocasein as substrate at pH 8.0 and 40°C, while trypsin activity was analyzed against the specific substrate benzoyl-arginine-p-nitroanilide. The lipolytic activity was carried out by p-nitrophenol production through the reaction of p-nitrophenylacetate dissolved in 2-propanol at pH 7.0 and 37°C. The compatibility of the enzyme activity with some commercial detergents was evaluated. Additionally, proteolytic and lipolytic activities were tested on spiruline and soybean oil stains and showed a strong ability to remove them from cotton fabrics. Due to its promising properties, the purified enzymes isolated from the gastrointestinal tract of *M. goodei* may be considered as a potential effective active ingredient for its use in the detergents industry.

Keywords: Circular economy, Fish waste, Myliobatis goodei, Proteases, Lipases, Detergent compatibility

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Introduction

In recent decades, the fishing industry has experienced massive growth, driven by global population increases and rapid industrialization. As a result, the amount of onboard discards and land-based waste has also increased worldwide (Caruso *et al.*, 2020; Coppola *et al.*, 2021). The disposal and recycling of fisheries waste has become a key issue to be solved in order to avoid environmental problems and to keep the resource sustainable (Caruso *et al.*, 2020; Ideia *et al.*, 2020; Coppola *et al.*, 2021; Mutalipassi *et al.*, 2021). In this sense, the transition from the linear to the circular economy offers a valuable alternative to opt for the reuse or recovery of compounds present in these fishing residues.

Therefore, current research focuses on the recovery of different compounds, including marine enzymes (Wald *et al*., 2016; Lamas *et al*., 2017; Lamas and Massa, 2020; Kuepethkaew *et al*., 2022; Borges *et al.*, 2023). These enzymes possess unique catalytic properties and characteristics such as hyperthermostability, salt tolerance, barophilicity, cold adaptability, chemoselectivity, regioselectivity, and stereoselectivity (Lima and Porto, 2016; Zhang *et al.*, 2021). In addition, high enzyme activity has been reported under fluctuating oxygen availability, as well as in the presence of surfactants and oxidizing agents (Díaz-Lopez and García Carreño, 2000; Joo *et al.*, 2001; Ketnawa *et al.*, 2013), which are considered specific advantages of these enzymes.

Several studies have analyzed the potential use of marine enzymes in various biotechnology industries, including food, pharmaceutical and detergent industries (Jeloulli *et al.*, 2009; Wald *et al.*, 2016; Lamas *et al.*, 2017; Lamas and Massa, 2020; Kuepethkaew *et al.*, 2022; Borges *et al.*, 2023). In general, proteases represent about 60% of commercially used enzymes in industrial bioprocessing (Ferraro *et al.*, 2010; Salazar-Leyva *et al.*, 2014; Aissaoui *et al.*, 2017; Lamas *et al.*, 2017; Khangembam and Chakrabarti, 2018; Lamas and Massa, 2020). Trypsin (EC 3.4.21.4) is the predominant digestive proteolytic enzyme found in fish viscera. This enzyme is specific for the peptide bonds affecting the carbonyl group of arginine or lysine residues (Aissaoui *et al.*, 2017).

On the other hand, lipolytic enzymes also have a significant market in the industrial sector, particularly in the production of enzyme detergents (Rathi *et al*., 2001). An ideal detergent enzyme should be stable and active in the surfactants solution and thermally stable enough to function effectively over a wide range of temperatures (Kumar *et al*., 2008). Challenges in this industry focus on the use of enzymes to remove variable amounts of organic matter with low substrate specificity (Prazeres *et al*., 2006).

Several investigations have been conducted to isolate, purify and characterize marine enzymes (Cao *et al*., 2008; Blanco *et al*., 2014; Lamas *et al*., 2015; Lamas *et al*., 2017; Kuepethkaew *et al*., 2022; Borges *et al*., 2023). These

purification methods are typically high-cost and time-consuming, which hinders the use of fishery waste as a source of proteases (Esposito *et al*., 2010). In general, purification reports detail precipitation by the addition of salt or organic compounds, filtration, chromatography, or column separation (Jeong *et al*., 2000; Siringan *et al*., 2006; Klomklao *et al*., 2007; Castro Ceseña *et al*., 2012; Aissaoui *et al*., 2017; Lamas *et al*., 2017).

The Southern eagle ray *Myliobatis goodei* inhabits a wide range from South Carolina in the United States (35°N) to Santa Cuz, Argentina (44°S). In Argentina, it is unintentionally caught and typically discarded. Nevertheless, comparable rays are commercially traded worldwide with high economic worth. Thus, the objective of this study was to isolate and purify enzymes from the gastrointestinal tract of Southern eagle ray using inexpensive techniques. Additionally, the proteolytic and lipolytic activities of the extracted enzymes were examined. Furthermore, the compatibility of the purified enzyme extract with detergents was evaluated to determine its potential as an additive in such products. This is consistent with the circular economy concept and will offer an alternative to valorize fish waste by obtaining an ingredient for use in other industries.

Materials and methods

Biological samples

To extract the enzymes, samples from the gastrointestinal tract of different *M. goodei* specimens were used. The samples (2 lots of n=15) were obtained from research campaigns carried out by the National Institute for Fisheries Research and Development in 2018. They were received at the "Fishery Products Technology Laboratory" and immediately eviscerated. The gastrointestinal tissue of each sample was separated, conditioned in polyethylene bags, and frozen at -80°C.

Preparation of crude enzyme extracts

Approximately 100 g of samples were thawed and homogenized in 10 mM Tris-HCl, pH 8.0 buffer (1:3 w/v) using an Omni Mixer Homogenizer. Homogenization was repeated three times. The homogenates were centrifuged at 10,000 g for 30 min at 4ºC. The lipid phases were discarded and the collected supernatants were referred to as “crude enzyme extract CEE”. The proteins of the crude enzyme extract were quantified by the method of Lowry (1951), using bovine albumin as a protein standard. Absorbance was measured at 500 nm in a Shimadzu UV Spectrophotometer.

Partial purification of the extracts

The CEE was first subjected to ammonium sulfate ($(NH4)_2SO_4$) precipitation according to Khangemban and Chakrabarti (2018), with some modifications. The sample was brought to 30% $(NH4)_2SO_4$ saturation by slow addition of the required amount of salt. The protein precipitate was obtained after centrifugation at 10,000 g for 30 min at 10°C. The supernatant was then brought to 50% $(NH4)_2SO_4$ saturation

by adding more salt and centrifuged again. The collected precipitates were dissolved in Tris-HCl buffer, pH 8.0, and the $(NH4)_2SO_4$ Extract was named "$(NH4)_2SO_4$ E". This sample was filtered through of membrane Microclar CL- 501 "FE". Finally, it was subjected to centrifugation at 10,000 ×g for 30 min at 4°C in a centrifugal ultrafilter membrane with a 100 kDa cut-off filter (Alltech Associates Ultrafilters) generating the ">100 kDaE" and "<100 kDaE" extracts, respectively.

Proteolytic activity assay

Total proteolytic activity was evaluated according to the method developed by Castro Ceseña *et al*. (2012) with some modifications: Aliquots (50 µL) of crude and purified enzyme extracts were incubated with 500 µL of 0.5% w/v azocasein (substrate) in 50 mM Tris-HCl, pH 8.0, for 20 min at 40°C. The reactions were stopped by the addition of 500 µL of 20% trichloroacetic acid (TCA). The samples were then stored at -4°C for 10 min and centrifuged at 10,000 ×g for 15 min. The supernatants were separated and the progress of the reactions was measured by monitoring the absorbance at 366 nm. A blank of activity was run by adding the enzyme solution after the addition of TCA.

Substrate hydrolysis units per mg of protein were calculated using the following equation:

$$\mathrm{U/\ mL\ extract} = (\mathrm{(\Delta Abs/\ min)} * \mathrm{(final\ reaction\ volume)/\ (mL\ extract)})$$

Evaluation of trypsin-specific activity

Trypsin activity was evaluated according to Castillo-Yañez *et al*. (2005), with slight modifications. Na-benzoyl-DL-arginine-p-nitroanilide (BApNA) was used as a substrate.

Aliquots of 100 µL of enzyme extract were mixed with 400 µL of 0.5 M Tris–HCl (pH 8.0), 0.1 mL of 0.2 M $CaCl_2$; and 40 µL of 0.02 M BApNA in dimethylsulfoxide DMSO. The final reaction volume was adjusted to 1 mL with distilled water. Assays were performed at 30°C. One unit of activity was defined as the production of 1 mol/min of free p-nitroanilide, which was measured by monitoring the change in absorbance at 410 nm for 10 min.

The units of substrate hydrolysis per mg of protein were calculated using the following equation:

$$\mathrm{U/\ protein\ mg} = \mathrm{((\Delta Abs/\ t)} * 1000 * \mathrm{(final\ volume))/\ ((8800)} * \mathrm{(protein\ mg))}$$

Where 8800 is the molar extinction coefficient of p-nitroanilide/M cm.

Lipolytic activity

The assay was carried out as described by Pedroza-Padilla *et al*. (2017), with slight modifications. Solution A was prepared with p-nitrophenyl acetate (pNPA) dissolved in 10 mL of 2-propanol to concentrations of 50 mM, and solution B for the pNPA assay

involved the 50 mM potassium phosphate buffer (pH 7.0). The reaction mixture consisting of 1 part solution A and 9 parts solution B was prepared fresh prior to the assay. To initiate the reaction, 0.04 mL of enzyme solution was added to 0.96 of the reaction mixture for 10 min at 37ºC. The reaction was stopped with the same volume of buffer. The activity was evaluated by estimating the change in absorbance at 410 nm in a UV Spectrophotometer. One unit of lipase activity was defined as the amount of enzyme that liberated 1 µmol of p-nitrophenol/min.

SDS-polyacrylamide gel electrophoresis (SDS-page) of protein extracts

SDS-page was carried out in gels with 30% of acrylamide/bisacrylamide (37, 5:1) solution according to the procedure of Laemmli (1970). Aliquots of the extracts were mixed with equal volumes of denaturation buffer (0.125M Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 10% β-mercaptoethanol) and boiled for 10 min. Then, samples (15 µL) were loaded onto the gel. A 4% stacking and 12.5% separating gels were subjected to electrophoresis at a constant current of 100 V per gel during 2 h. After electrophoresis, the gels were stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol and 7% acetic acid and destained with 7% acetic acid. A low molecular weight calibration kit was utilized to estimate the molecular weight of the enzyme extracts by using markers (10, 15, 25, 35, 40, 55, 72, 100, 130 and 180 kDa).

Compatibility with commercial detergents

The compatibility of CEE with various commercial detergents (Ala, Skip, Drive, and Zorro) was tested by incubating aliquots of the extract with these detergents. Prior to the addition of the extract, endogenous proteases present in the detergents were inactivated using heat. Aliquots of 100 µL of the sample were then incubated with the different detergents at a final concentration of 7 mg/mL for 1 hour at the respective temperature of each type of enzyme. Then, the proteolytic, trypsin, and lipolytic activity present in each detergent was assessed using the specified assay conditions and compared to the enzyme activity without detergent (control, 100% activity).

Enzyme performance in the washing process

The washing performance of the proteases as a detergent additive was studied according to Vishalakshi *et al*. (2009). White cotton cloth pieces (5×5 cm) were stained with 5 drops of spirulina solution containing 70% protein content and placed into separate trays. The following sets were prepared and analyzed:

(a) Tray with 50 mL of distilled water + spirulina stained cloth.
(b) Tray with 50 mL of distilled water + spirulina stained cloth+ commercial detergent (7 mg/mL).
(c) Tray with 50 mL of distilled water + spirulina stained cloth + partially purified enzyme.

(d) Tray with 50 mL of distilled water + spirulina stained cloth+ commercial detergent (7 mg/mL) + partially purified enzyme.

The trays were then incubated at 40°C for 45 min. After incubation, the cloth pieces from each set were extracted and centrifuged at 134 ×g for 15 min at 25°C. Finally, the cloth pieces were dried and visually examined. Untreated spiruline-stained cloth pieces were taken as control.

The washing performance of the lipases as a detergent additive was studied by the same protocol. Four white cotton cloth pieces, each measuring 5x5 cm and stained with 5 drops of degummed raw soybean oil, were left at room temperature for 24 hours. Then, the subsequent sets were arranged and examined.

(a) Tray with 50 mL of distilled water + oil stained cloth.

(b) Tray with 50 mL of distilled water + oil-stained cloth + commercial detergent (7 mg/mL).

(c) Tray with 50 mL of distilled water + oil-stained cloth + partially purified enzyme. (d) Tray with 50 mL of distilled water + oil-stained cloth + commercial detergent (7 mg/mL) + partially purified enzyme.

The above trays were incubated at 37°C for 35 min. The cloth pieces from each set were centrifuged at 134 ×g for 15 min at 25°C, dried and examined. Untreated oil-stained cloth pieces were taken as the control.

Statistical analysis

All experiments were performed in duplicate and the results were expressed as mean value±standard deviation. Moreover, the one-way analysis of variance (ANOVA) test was used, and differences between means were tested using Duncan's multiple range test, being statistically different at the 5% significance level. Statistical analyses were performed using INFOSTAT software.

Results

Characterization of enzyme extracts

Gastrointestinal enzymes were recovered and purified in three steps: precipitation with ammonium sulfate followed by two filtration steps. The molecular weights of the proteins present in the CEE and ammonium sulfate precipitations were estimated. The electrophoretic profiles showed bands above 10 kDa (Fig. 1). A molecular weight band of approximately 27 kDa was identified in all samples analyzed.

Proteolytic activity

The results obtained of the proteolytic activity using azocasein as a substrate are presented in Table 1. After the precipitation with salt, greater specific activity was achieved with the recovery in the range of 64-68%.

Trypsin specific activity

All analyzed extracts efficiently hydrolyzed BApNA. The total trypsin activity of CEE was more than 50 U (Table 2). The ammonium sulfate

precipitation step increased twice. After the samples were subjected to ultrafiltration with a 100 kDa cut-off membrane, the enzyme was purified more than 3-fold with an activity recovery of about 60%.

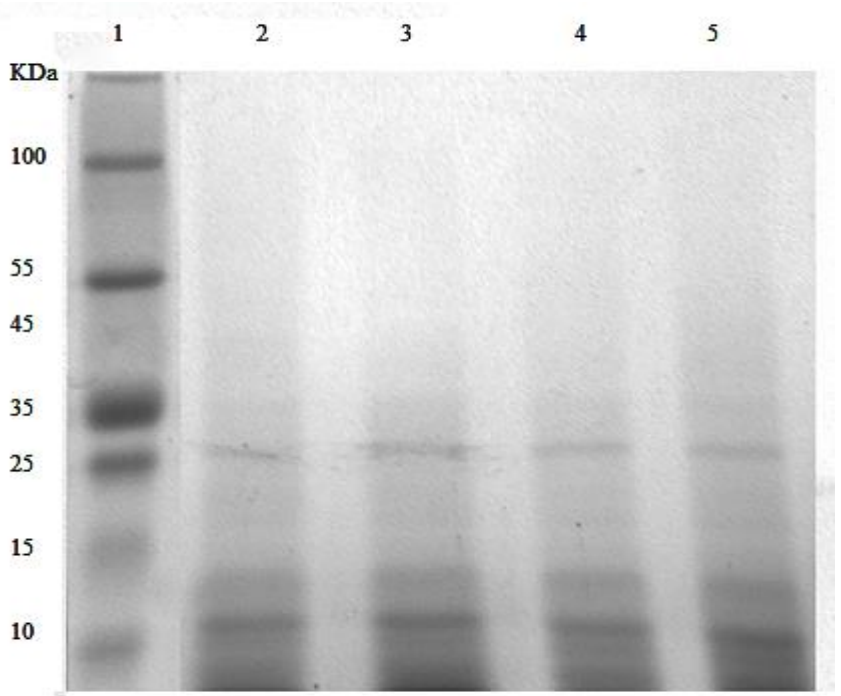

Figure 1: SDS-PAGE of enzyme crude extract from the gastrointestinal tract of *Myliobatis goodei.* Lane 1: standard proteins, lanes 2 and 3: crude extracts CEE from lots 1 and 2, respectively, lanes 3 and 4: ammonium sulfate precipitation extracts $(NH4)_2SO_4$ E from lots 1 and 2, respectively.

Table 1: Summary of purification procedure and protease activity from the gastrointestinal tract of *Myliobatis goodei.*

Purification stage[1]	Total activity U/mL extract	Protein mg/mL extract	Specific Activity U/mg	Purification fold	Yield %
Lot 1					
CEE	89.28	8.87	0.53	1	100
$(NH4)_2SO4$ E	60.95	3.45	0.93	1.75	68.26
FE	65.95	2.66	1.30	2.45	73.86
>100kDaE	74.52	2.06	1.90	3.58	83.46
<100kDaE	50.00	1.62	1.61	3.04	56
Lot 2					
CEE	98.10	9.27	0.55	1	100
$(NH4)_2SO4$ E	63.10	4.10	0.82	1.50	64.32
FE	68.33	3.13	1.15	2.10	69.46
>100kDaE	77.86	2.17	1.87	3.40	79.37
<100kDaE	65.47	1.82	1.90	3.45	66.75

The results are mean values (n = 2).

[1]CEE means crude extract, $(NH4)_2SO4$ E salt precipitate extract, FE Microclar CL- 501 filtrate extract, and > 100 kDa E and <100 kDaE the pools collected by 100 kDa cut off filter extracts

Table 2: Summary of purification procedure and trypsin activity from the gastrointestinal tract of *Myliobatis goodei*.

Purification stage[1]	Total activity U/mL extract	Protein mg/mL extract	Specific Activity U/mg	Purification fold	Yield %
Lot 1					
CEE	53.83	8.87	0.0041	1	100
$(NH4)_2SO4$ E	46.7	3.45	0.0092	2.21	86.69
FE	43.88	2.66	0.0110	2.69	81.52
>100kDaE	34.68	2.06	0.0114	2.74	64.42
<100kDaE	32.26	1.62	0.0136	3.25	59.94
Lot 2					
CEE	52.08	9.27	0.0038	1	100
$(NH4)_2SO4$ E	45.72	4.10	0.0076	1.98	87.78
FE	43.78	3.13	0.0095	2.48	84.07
>100kDaE	35.68	2.17	0.0112	2.92	68.51
<100kDaE	32.66	1.82	0.0122	3.18	62.72

The results are mean values (n = 2).
[1]CEE means crude extract, $(NH4)_2SO4$ E salt precipitate extract, FE Microclar CL- 501 filtrate extract, and > 100 kDa E and <100 kDaE the pools collected by 100 kDa cut off filter extracts

Lipolytic activity performance

The results of the lipolytic activity are shown in Table 3. The activity of the CEE, measured as specific activity showed similar values to those reported by Castro Ceseña *et al*. (2012). However, the salt-purified extract achieved almost to 4-fold improvements, which is in less than the value of the acetone-purified extract reported by that work from sardine by-products.

Stability of the alkaline enzymes with commercial solid detergents

To test compatibility with solid detergents, the CEE was pre-incubated in the presence of different commercial laundry detergents.

Table 3: Summary of purification procedure and lipolytic activity from the gastrointestinal tract of *Myliobatis goodei*.

Purification stage[1]	Total activity U/mL extract	Protein mg/mL extract	Specific Activity U/mg	Purification fold	Yield %
Lot 1					
CEE	39.11	8.87	0.015	1.00	100.00
$(NH4)_2SO4$ E	35.77	3.45	0.040	2.66	91.46
FE	31.16	2.66	0.075	5.00	79.67
>100kDaE	17.57	2.06	0.070	4.66	44.92
<100kDaE	13.40	1.62	0.048	3.20	34.26
Lot 2					
CEE	40.22	9.27	0.011	1.00	100.00
$(NH4)_2SO4$ E	33.17	4.10	0.032	2.91	82.47
FE	26.46	3.13	0.051	4.63	65.79
>100kDaE	13.25	2.17	0.047	4.27	32.94
<100kDaE	12.05	1.82	0.033	3.00	29.96

The results are mean values (n = 2).
[1]CEE means crude extract, $(NH4)_2SO4$ E salt precipitate extract, FE Microclar CL- 501 filtrate extract, and > 100 kDa E and <100 kDaE the pools collected by 100 kDa cut off filter extracts

Proteolytic activity during laundering is influenced by various factors such as pH, ionic strength, wash temperature, detergent composition, bleaching systems, and mechanical handling (Esposito *et al*., 2010). As indicated in Figure 2a, the enzymes showed stability in the presence of the detergents tested. After a 1 hour incubation at 40°C, their proteolytic activity remained at approximately 40% in the presence of Skip, 80% in the presence of Ala and Zorro, and 100% with Drive. The high activity and stability of *M. goodei* trypsin at pH 8.0 and its relative stability against to oxidizing agents indicate its potential as a detergent additive (Fig. 2b). Detergent formulations such as anionic or non-ionic surfactants and powdered microbial lipase are used to remove grease soils from fabrics. Interestingly, in this study, the lipolytic activity showed high activity with all of the commercial detergents tested, emphasizing its effectiveness in the presence of Ala (Fig. 2c).

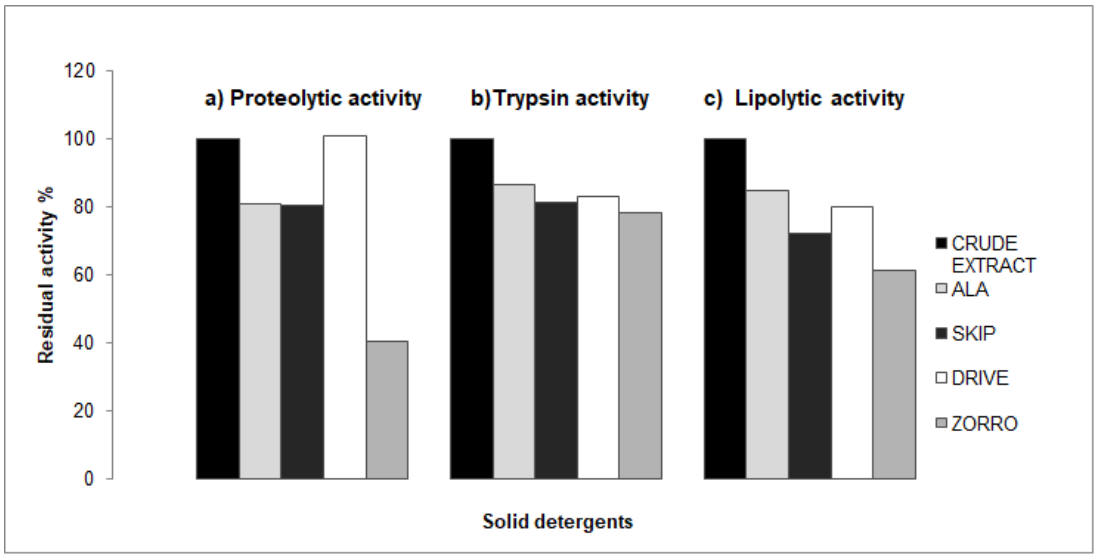

Figure 2: Enzymes stability from *Myliobatis goodei* against commercial solid detergents. The enzyme was incubated for 1 h at 40°C in the presence of solid detergents at a final concentration of 7 mg/mL, and then the remaining activities were determined. (a) Proteolytic activity. (b) Trypsin activity. (c) Lipolytic activity. In all cases, the activity of the crude extract sample without any detergent, incubated under the same conditions, was taken as 100%.

Washing performance test

The effectiveness of enzymes as a detergent additive in improving washing performance was evaluated against spirulina and soybean oil-stained cotton cloth pieces. Drive commercial detergent retained higher proteolytic activity, while Ala retained higher lipolytic performance; thus, these detergents were selected to evaluate the washing performance. As depicted in Figures 3a and b, spirulina stains were initially removed by water alone and then by components of the detergent matrix. Figure 3c shows the effect of using the enzyme alone. However, better elimination of stains was observed with a combination of detergent and enzyme (Fig. 3d).

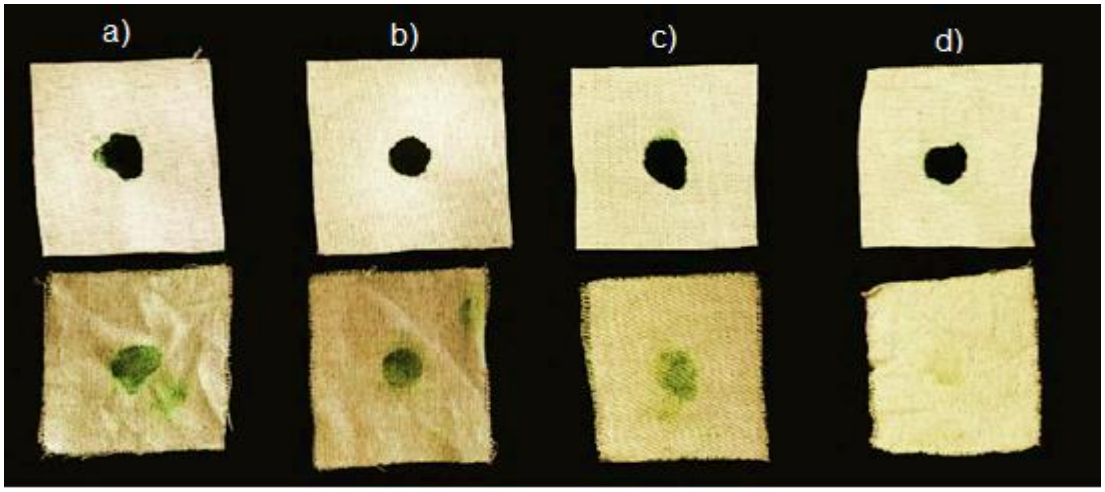

Figure 3*:* Cleansing potential of alkaline protease from *Myliobatis goodei*. The upper clothes were stained with spirulina. The lower were the cloths after treatments. a) Untreated stained cloth as control, b) stained cloth washed with Drive detergent, c) stained cloth piece washed with enzymes and water, and d) and stained cloth piece washed with Drive detergent and enzyme.

The lipase activity was also evaluated for washing performance using Ala. The control with water shos the oil-stain spilled and blurred throughout the cotton fabric (Fig. 4a). The detergent alone performed better that the enzyme alone as seen in Figure 4b and c respectively. The soybean oil was entirely removed after the treatment with enzyme extract and Ala, as demonstrated in Figure 4d. All washes released oil droplets to the surface during cleaning simulations. After centrifugation, a superior fine phase comprising of lipids was clearly observable.

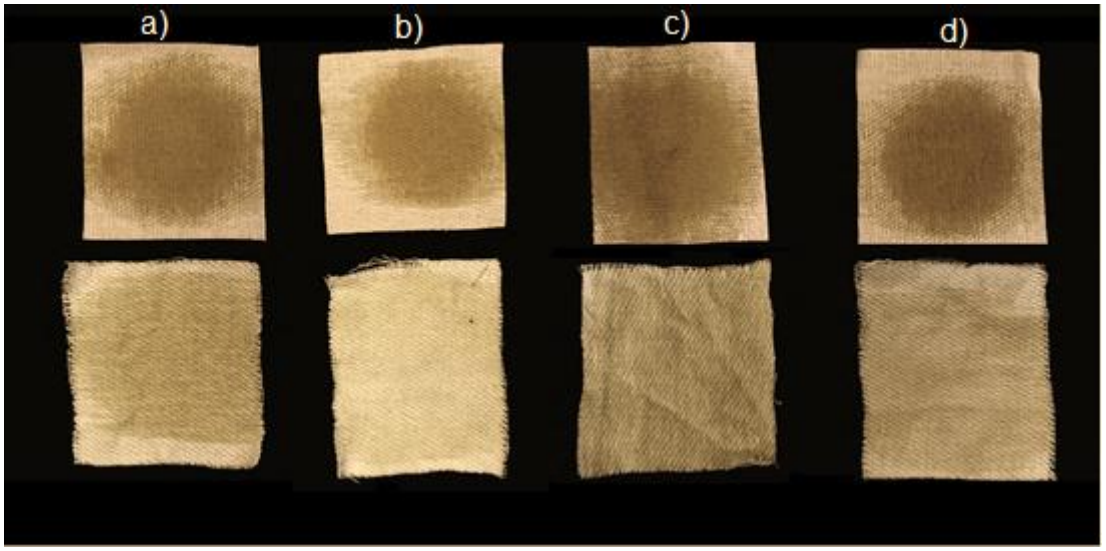

Figure 3*:* Cleansing potential of alkaline protease from *Myliobatis goodei*. The upper clothes were stained with spirulina. The lower were the cloths after treatments. a) Untreated stained cloth as control, b) stained cloth washed with Drive detergent, c) stained cloth piece washed with enzymes and water, and d) and stained cloth piece washed with Drive detergent and enzyme.

Discussion

In general, enzyme purification are high-cost process and requires long lead times, limiting the commercial and industrial use of fish waste as a source of enzymes (Esposito *et al.,* 2010). However, the processes used in this study are relatively low-cost and do not require long lead times. Therefore, considering that the raw material comes from the waste of the fishing industry, the proposed work could have a great economic impact. At the same time, this alternative use of marine enzymes contributes to the reduction of waste disposal and pollution problems, in line with the objectives proposed by the circular economy model.

The SDS page profile obtained is consistent with the molecular weight described for trypsin in other fish species (Simpson, 2000). Castillo-Yañez *et al.* (2005) identified a 25 kDa band associated with trypsin from Monterey sardine (*Sardinops sagax caerulea*), Bougatef *et al.* (2007) identified a band of similar molecular weight in *Sardina pilchardus* and Jeloulli *et al*. (2009) verified a 23.2 kDa band for trypsin from the intestine of *Balistes capriscus*. Previous studies in Argentine hake (*Merluccious hubbsi*) showed a band close to 25 kDa (Lamas *et al.*, 2015) and viscera from horse mackerel (*Trachurus lathami*) showed a band around 22 kDa. In addition, studies of salted anchovy (*Engraulis anchoita*) residues showed results consistent with this work (Lamas *et al.*, 2017).

In terms of the proteolytic activity, the results are similar to those reported by El-Beltagy *et al*. (2005) for the protease extracted from *Tilapia nilotica*, which indicated that the yield percentage was 62% after treating the crude enzyme extract with 40–60% $(NH4)_2SO4$ purification step. Al Ghais and Bhardwaj (2019) also reported a similar yield percentage after purifying the crude extract from *Tilapia mossambica* viscera. These high yield percentages confirmed that ammonium sulfate is an effective agent for the initial step of protease purification. The observed differences in activity between the CEE and $(NH4)_2SO_4$ extracts could be due to the presence of lipids in the unpurified extracts, which would interfere with the enzyme activity. The ammonium sulfate precipitate was then dissolved in 10 mM sodium Tris-HCl buffer, pH 8.0, and filtered in two steps. During the first filtration step, a slight increase in the purification factor and specific activity was observed. The recovery yield obtained after separation of the enzymatic extract with centrifugal ultrafilters of 100 kDa cut-off showed that both extracts contain enzymes with proteolytic activity, being greater in the >100kDa E.

Similar to the present work, previous investigations have demonstrated the efficiency of ammonium sulfate precipitation as a step in the purification of fish trypsins (Souza *et al.*, 2007; Bougatef *et al.*, 2007; Esposito *et al.*, 2010; Khangembam and Chakrabarti, 2018). Rawdkuen *et al.* (2012) reported that the application of t-butanol ammonium sulfate for the extraction of alkaline proteases resulted in a yield of

about 163%. On the other hand, the precipitation of trypsin from the pancreas of the small spotted catshark with ammonium sulfate yielded only 8% (Blanco *et al.*, 2014). This behavior is probably due to the denaturing effect caused by the high salt concentration (Klomklao *et al.*, 2004). From the results obtained, it can be concluded that the viscera of *M. goodei* is rich in trypsin and that the purification steps are advantageous due to the significant increase in specific trypsin activity.

Saxena *et al.* (2003) reported that lipases purified from *Aspergillus carneus* using ammonium sulfate achieved were similar to those purified in the present study. Also, a previous study from *T.lathami* guts and heads using a similar purification protocol showed similar behavior for lipase activity (Lamas and Massa, 2020). A significant reduction in total activity after filtrate steps was also reported in this study. This behavior suggests that the purification of the enzymes is influenced by the raw material. Notably, there were no differences in recovery yield after the filtrate steps here for the gastrointestinal tract of *M. goodei*. Thus, target proteins were lost in this step, but it was a necessary cleanup step as a preparative method if it was to be filtered later by membrane cut-off.

As in the present study, we previously found high stability of alkaline proteases from horse mackerel viscera in the presence of commercial detergents (Lamas and Massa, 2020). Other authors have investigated the stability of different enzymes in detergents. El Hadj Alí *et al.* (2007) worked with trypsins isolated from *L. mormyrus* that retained 87.5%; 84%; 77%; 73.4% and 69% of their initial activity after 1 h of incubation at 30°C in the presence of Axion, New Det, Dixan, Ariel and Nadhif, respectively. On the other hand, Jeloulli *et al.* (2009) reported that the trypsin from *B. capriscus* was extremely stable in the presence of Ariel and Axion, retaining 100% of its activity even after 1 h of incubation at 40°C, but retained about 84.5% and 76.9% of its initial activity with Dixan and New Det, respectively, and was found to be less stable in the presence of Nadhif. Regarding the lipase activity, Cherif *et al.* (2011) reported that the lipolytic preparation from *Staphylococcus sp.* was extremely stable to all solid detergents tested at 50°C, retaining 100% of its activity in the presence of Axion and Ariel and more than 80% with Dixan and Nadhif after 1 h of incubation. Lailaja and Chandrasekaran (2013) showed that the marine bacterium *Bacillus smithii* retained more than 90% of its lipase activity in the presence of some of the detergents tested.

These results clearly indicate that the enzyme is stable as a component in the presence of detergents and contributes positively to the removal of protein materials. There are very few reports on the use of marine enzymes from fishery waste and their use in the detergent industry. However, there are studies on the use of enzymes from other sources in the detergent industry. In this sense, Patil *et al.* (2016) demonstrated the removal of blood and egg albumin stains within 1

h and the improved removal of these stains using a commercially available detergent (Rin) supplemented with purified protease from *Bacillus circulans*. Similarly, Vishalakshi *et al*. (2009) demonstrated that alkaline protease from *Streptomyces gulbargensis* removed blood stains from cotton fabrics and on surgical instruments. In the same way, Abidi *et al*. (2011) reported a positive effect of adding *Botritis cinerea* protease to remove blood, egg yolk, and chocolate stains. Removal of proteins by non-enzymatic detergents can result in permanent stains due to oxidation and denaturation caused by bleaching and drying. Microbial proteases hydrolyze proteins and break them down into more soluble polypeptides or free amino acids. The combined action of surfactants and enzymes can remove such hard-to-remove stains from fibers. Normally grease stains are difficult to remove at low temperatures with conventional detergents, so lipases that are active at lower temperatures are required and can be used in detergent formulations (Hasan *et al.*, 2010). In the present work, the use of low-temperature active lipase in detergent formulation was significant. This condition maintains the texture and quality of fabrics.

The gastrointestinal tract of *M. goodei* could be considered a good source of enzymes, with an important proteolytic and lipolytic activity in the conditions of pH and temperature studied. The present study confirms the efficiency of ammonium sulfate precipitation as a step for the purification of fish enzymes. The filtration processes also showed a significant increase in the purification factor and the specific activity of the enzymes. Thus, the present investigation proposes a simple, low-cost, and timeless process for the recycling of enzymes. On the other hand, enzymes showed promising results in the removal of stains of protein and lipid origin. Therefore, the properties of enzymes indicate that it could potentially be used as an additive in the production of detergents. In this way, the proposed recovery and use of enzymes from the gastrointestinal tract of *M. goodei*, usually considered waste from the fishing industry, could have a great economic impact by obtaining value-added bioproducts, in line with the concept of circular economy.

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