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*Research Article*

# Valorization of the discards from the extraction oil processes of a ray fish (*Zearaja flavirostris*) liver as a culture media to produce *Saccharomyces cerevisiae* biomass

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**Abstract**

The great amount of unutilized rest raw materials in the cartilaginous fish industry led to investigate the possible utilization methods to generate a value-added product. In this study, the residual aqueous fractions of oil extraction processes from the liver of longnose ray (*Zearaja flavirostris*), were evaluated as a possible replacement for the commercial yeast extract peptone dextrose (YPD) culture medium for *Saccharomyces cerevisiae*. Oil extraction from the ray livers was carried out by different processes such as enzymatic hydrolysis, cold extraction, and extraction by applying high temperatures. The obtained culture media based on the aqueous fractions from the fish ray livers were compared with an industrial reference media. The pattern of cell growth and biomass yield was similar using both the standard culture medium for YPD as a positive control and the diluted and supplemented with the aqueous fractions obtained by the enzymatic extraction method. The results suggest that these by-product fractions provide an alternative source of carbon (peptides and free amino acids) as well as macro- and microelements for the growth of *S. cerevisiae*.

## Introduction

In recent decades, cartilaginous species, especially rays, have gained great importance and they are captured all over the world. However, only their fins are consumed, while the head and viscera, including the liver, are wasted (Lamas and Massa, 2017, 2019). Among the main ray species caught in the southwest Atlantic region (34 and 41º S), *Zearaja flavirostris* is found at depths of less than 50 m. In general, ray liver oil contains a high proportion of lipids (Ould El Kebir *et al.*, 2003; Navarro-García *et al.*, 2004; Le Nechet *et al.*, 2007; Özyılmaz and Öksüz, 2015; Sellami *et al.*, 2018; Lamas and Massa, 2019). Studies on *Z. flavirostris* from the Argentine continental shelf have shown that their livers contain high levels of polyunsaturated fatty acids (PUFAs), especially those of the omega-3 series (Lamas and Massa, 2019). These fatty acids, mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been extensively studied for their remarkable benefits for human health (Shahidi and Ambigaipalan, 2018). Oil extraction from ray livers generates discarded by-products, such as sludge and aqueous fractions, which are rich in proteins, and macro and micronutrients (Ghaly *et al.*, 2013). Among the potential applications of these by-products, their use as ingredients for fish feed or farmed animals or as a medium for growing commercially important microorganisms is highlighted (Martone *et al.*, 2005; Ghaly *et al*., 2013). Interestingly, fish by-products are an excellent source for microbial growth such as bacteria, yeast and fungi (Coello *et al.* 2000; Vazquez *et al.* 2006, Vazquez *et al.* 2008; Rebah and Miled, 2013). That is due the protein fractions contain free amino acids and peptides of different molecular weights. The proportion, composition, and potential applications of peptides vary according to the type of process used, and they are mostly composed of 2 to 20 amino acid residues. The small size of these peptides and the large amount of free amino acids allow them to be used as an enrichment of culture media for various microorganisms, or even as a complete medium for organisms with lower nutritional requirements (Petrova *et al.,* 2021). Several authors reported the use of fish proteins as a source of nutrients and amino acids sources in a microbial culture medium. Aspmo *et al*. (2005) studied the hydrolysates from the viscera of Atlantic cod (*Gadus morhua L.)* as components of microbial growth media. Vieira *et al*. (2005), investigated the potential use of fish industry by-products consisting of lobster carapace meat from *Panulirus argus* and *Panulirus laevicauda*, lobster tail parings, whole shrimp tails from cinnamon shrimp as sources of peptone for bacterial growth media. Also, Beaulieu *et al.* (2009) used mackerel (*Scomber scombrus*) and herring (*Clupea harengus*) for hydrolysis and investigate their suitability as bacterial culture media. In addition, Petrova *et al.* (2021), investigated the use of cod backbone and head protein hydrolysates as peptones for microbiological culture media. Different strains such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Lactococcus lactis* were used (Aspmo *et al.*, 2005; Beaulieu *et al.*, 2009; Petrova *et al.*, 2021).

*Saccharomyces cerevisiae* is a model organism, a valuable tool for all aspects of basic research and, at the same time, a highly valuable species for a variety of industrial applications (Parapouli *et al.*, 2020). As a commonly studied organism, it is considered a powerful biological model of eukaryotic organisms (Gorter de Vries *et al.*, 2017). This yeast has been in use since ancient times, and is still in use today in the bread, wine, and beer industries. Biotechnology has maintained the traditional use that has been made of this yeast, improving and innovating baking and beverage production processes alcoholic. This yeast is one of the species considered as a GRAS microorganism, which has been approved for use as a food additive (Boyle *et al.*, 2006; FDA, 2018). It is inactivated by temperature and is used as a source of nutrients with antioxidant properties in animal and human nutrition, both in the form of integral yeast and from its derivatives (Czerucka *et al.*, 2007; Yalçm *et al.*, 2011; Datta *et al*., 2017).

This study aimed to evaluate the ability of the yeast strain *S. cerevisiae* to utilize a fish aqueous phase waste from the industrial process of *Z. flavirostris* liver oil extraction. The objective of the present investigations was to determine the yeast growth when the culture medium was enriched with the fish waste.

## Materials and methods

*Raw material*

Specimens of *Z. flavirostris* were captured in the southwestern Atlantic Ocean of Argentina. The rays and their livers were weighed shortly after collection. The livers were then placed in polyethylene bags, rapidly frozen and stored at -25ºC until used.

*Reagents*

The enzyme used was Purazyme AS 60 L (alkaline serine protease, from *Bacillus licherniformis*), a product considered generally recognized as safe (GRAS) for applications including protein modification and pet food production. The enzyme is active at pH 6, but the optimum pH for hydrolysis is 8.5, and exhibits activity over a wide temperature range (25-70ºC), with an optimum temperature of 60ºC. Commercial *S. cerevisiae* product (Levex) was used. All reagents used were of analytical grade.

*Oil extraction method*

For oil extraction, three lots of livers were cut into sections of approximately 2-3 $mm^3$ and crushed for 30 s using a domestic mill. Three methods of oil extraction were then used. The procedure performed for each method is detailed below.

*Procedure I: Enzyme extraction*

Enzyme extraction (E) was performed according to Gbogouri *et al.* (2006) with modifications. Hydrolysis was carried out in a thermostatic reactor with constant stirring. Equal parts of minced liver (approximately 50 g) and distilled water were mixed at 50°C for 15 min. The mixture was stabilized at pH 8.0±0.5 and temperature 60±2.5ºC. The hydrolysis process was initiated by adding the protease at a substrate mass concentration of 2 %, and the pH was controlled by adding 1 M NaOH. After 1 h of reaction, the temperature was increased to 85°C for 10

min to inactivate the enzyme. The hydrolysate was centrifuged at 20,000× g for 30 min at 4°C. The tubes were then placed vertically in a freezer (-20°C), and all fractions were separated by cutting the frozen contents of the tubes. The oil was collected from the top phase and weighed, and the aqueous phase was separated and stored until use.

*Procedure II: Cold extraction*

The cold extraction (C) method was carried out according to Głowacz-Rozynska *et al.* (2016) with modifications. Briefly, equal parts of minced liver (approximately 50 g) and distilled water were mixed at 50°C (1:1, w/v) with vigorous stirring for 5 min, in order to form a homogeneous pulp. During this procedure, the temperature did not exceed 15°C. The mixture obtained was then centrifuged at 10,000× g for 15 min at 15°C. The oil was separated from the aqueous protein phase together with solid residues. The soluble protein phase and residues were then centrifuged again under the same conditions. The separated oil was collected and combined with the previously obtained fraction. Finally, the oil obtained was weighed and the aqueous phase was separated and stored until use.

*Procedure III: High-temperature extraction*

High-temperature extraction (H) method was carried out according to Głowacz-Rozynska *et al.* (2016) with modifications. To form a homogenous pulp, water was added (1:1, w/v) to the minced livers, and the mixture was heated at 95°C for 30 min with constant magnetic stirring. It was then cooled to room temperature and centrifuged at 10,000× g for 15 min at 15°C. Centrifugation accelerated the natural decantation, and the oil was separated from the aqueous phase by density difference. The oil from the upper phase was collected, the solid residues were discarded, and the liquid phase was stored until use.

*Separation and protein characterization of aqueous phase (Aps) remaining from oil extraction processes*

The APs from each extraction were centrifuged for 20 min (4,000 × *g*; 20ºC). The supernatants were collected, pH adjusted to 4.5, and individually stored at -20°C until use. For the extraction and denaturation of AP proteins, 1 mL of each sample was centrifuged for 20 min (5,000 × g; 4ºC). SDS-page was carried out according to the procedure of Laemmli (1970). The supernatants obtained were treated with Loading Buffer (2.5 mL SDS 10 %; 0.2 mL β-Mercaptoethanol; 0.5 mL Bromophenol Blue 0.05 %; 0.5 mL Tris 0.5 M pH 6.8), heated at 100ºC for 5 min, and immediately cooled on ice. Electrophoresis was performed under denaturing conditions, at 10 % for the separating gel and 4 % for the concentrating gel (Acrylamide 30 %; Bisacrylamide 0.8 %; SDS 10 %; Ammonium Persulfate 100 mg/mL); gel separating buffer: Tris 1M pH 8.8; gel concentration buffer: Tris 0.5 M; pH 6.8. The electrophoretic run was carried out using Tris-Glycine Buffer (Glycine 14.4 g/L; Trizma Base 3 g/L; SDS 10%) for 2 h at 120 V. The gel was stained for 1 h (0.25 g Coomassie Blue; 45% Methanol; 10% Acetic Acid in distilled water), and developed overnight with several washes in

destaining solution (10% Methanol; 40% Acetic Acid in distilled water).

*Effect of AP from oil extraction processes on the growth of S. cerevisiae*

To obtain a metabolically stable culture of *S. cerevisiae*, 1 g of lyophilisate was inoculated into 50 mL of complete YPD medium (1% yeast; 2% peptone; 2% glucose) in a 250 mL flask for 24 h at 30±1ºC with gentle and continuous shaking To determine the viability of the culture, colonies were inoculated onto YPD agar at 24 and 48 h after culture initiation. The volume to inoculate $1\times10^4$ CFU (colony forming units) was determined by measuring the absorbance at 620 nm ($OD_{620}$). The culture method was monophasic, i.e., no new culture medium was added during the process. To evaluate the capacity of AP as a nutrient supplement for yeast growth, tests were carried out with the following culture media: YPDc (YPD media standard composition, positive control), YPDd (YPD media diluted in ¼, negative control), and modified medium (YPD diluted in ¼), supplemented with AP to a final concentration of 10 mg/mL of protein.

To obtain growth curves, culture media were inoculated with 1 x $10^4$ CFU in a final volume of 10 mL, incubated in an oven at 30±1ºC with shaking, and absorbance was measured at 620 nm by taking aliquots at different times for 48 h. Biomass yield was determined after the cells were harvested at 24 h by centrifugation at 4,000 × g and 4°C, which were then dried in an oven at 60±1°C until constant weight; and expressed as dry weight/Liter of culture medium.. Negative controls of the different culture media used were included, without inoculum. In parallel with each assay, cell viability was determined by plate count assays in YPD agar. Different dilutions of 24 h cultures were plated on the surface in duplicate.

*Statistical analysis*

All assays were performed in triplicate for each of the APs obtained from the different oil extraction methods. Data were expressed as mean±standard deviation. The results were statistically analyzed by analysis of variance (ANOVA) with a confidence interval of 95% ($p<0.05$) (GraphPad Prism). The Newman-Keuls multiple comparison test was used to compare the biomass yield of each method, which allows the means of the t-levels of a factor to be compared after the null hypothesis of equality of means has been rejected by the ANOVA technique.

**Results**

*Aqueous phase (AP) protein profile*

The electrophoretic separation of proteins from different APs is shown in Figure 1. The three enzyme-treated liver lots (E1/E2/E3) showed almost complete protein hydrolysis, resulting in peptides less than 10 kDa. Cold (C1/C2/C3) and high temperature (H1/H2/H3) oil extraction treatments showed no protein hydrolysis, as no peptides smaller than 10 kDa were produced.

According to the results obtained, only the AP obtained by enzymatic hydrolysates would have properties as a culture medium, since they are mainly composed of low molecular weight peptides that could be metabolized by yeast. However, all the APs obtained from the three different oil

extraction processes were studied in order not to exclude the possibility of making full use of the residual waste from cold and hot oil processing, which are cheaper processes than enzymatic extraction.

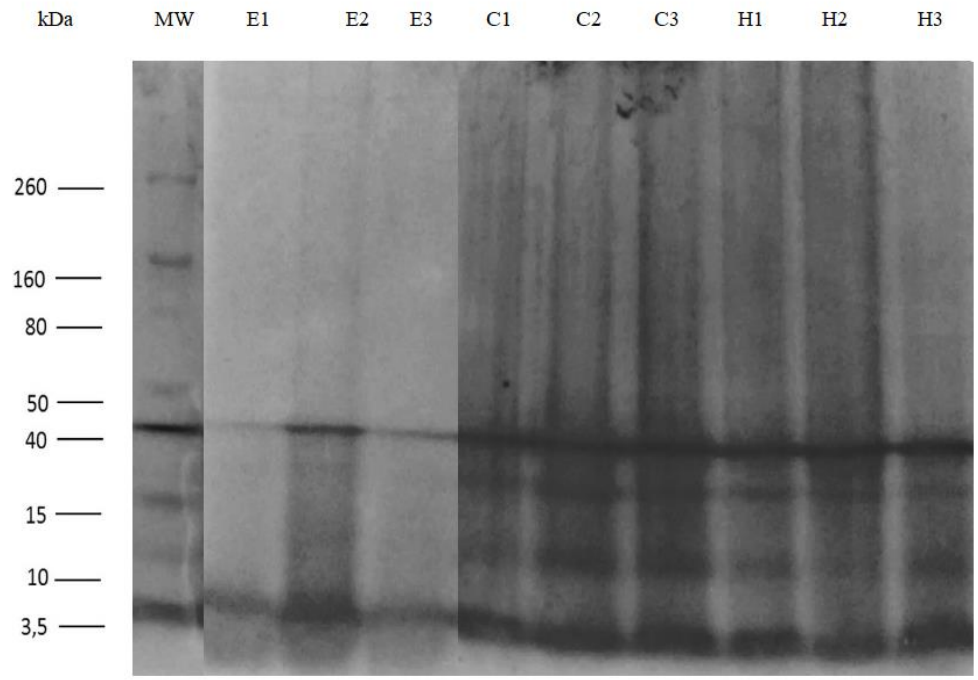


**Figure 1: SDS-PAGE of 10% aqueous phase (AP) remaining after oil extraction by different treatments. E1/E2/E3 indicates AP obtained by enzymatic extraction from liver 1, 2, and 3, respectively; C1/C2/C3 indicates AP obtained by cold extraction from liver 1, 2, and 3, respectively; H1/H2/H3 indicates AP obtained by hot extraction from liver 1, 2, and 3, respectively. MW indicates molecular weight marker, kDa.**

*Effect of AP from oil extraction processes on the growth of S. cerevisiae*

The growth curves of the yeasts, both in YPDc medium and in the modified medium supplemented with AP obtained by the enzymatic method (E1/E2/E3), showed the same growth kinetics up to 48 h (Fig. 2). As shown in Figure 2, in the YPDd media supplemented with the upper fractions of hot and cold oil extractions, (H1/H2/H3 and H1/H2/H3), no acceptable growth was observed, compared to those grown in the negative control (YPDd without supplementation. The experimental media curves obtained by enzymatic extractions APs were quite comparable to those obtained with the reference media. The $OD_{620}$ measurements using these APs showed similar performances in the all cases. The growth curves tested yeast in media with APs coming from cold and hot extraction methods appear remarkably similar, and did not differ greatly in biomass. These curves also correspond to those obtained with YPDd used as a negative control (Fig. 2).

The data obtained by calculating the biomass yield per litre of culture (Fig. 3), were consistent with those observed in the growth curves. The fractions from the hot and cold oil extraction treatments (H1/H2/H3 and C1/C2/C3), did not show

an acceptable growth when compared to the positive control (YPDc; $p>0.05$), being similar to the biomass obtained in the culture medium used as negative control (YPDd). The biomass produced with the modified media supplemented with AP obtained by the enzymatic oil extraction method (E1/E2/E3), did not show significant differences with the positive control (YPDc; $p>0.05$), suggesting that the AP of this oil extraction method is capable of supplementing the YPDd medium for use as a medium culture for this yeast. Yeast viability was determined after 24 h of culture for the modified media supplemented with AP (E1/E2/E3) and correlated with the data obtained by absorbance measurements of the growth curves. Table 1 shows the average cell counts corresponding to the biomass determination study (Fig. 2), which confirms that the biomass generated at least up to 24 h was viable in all cases.

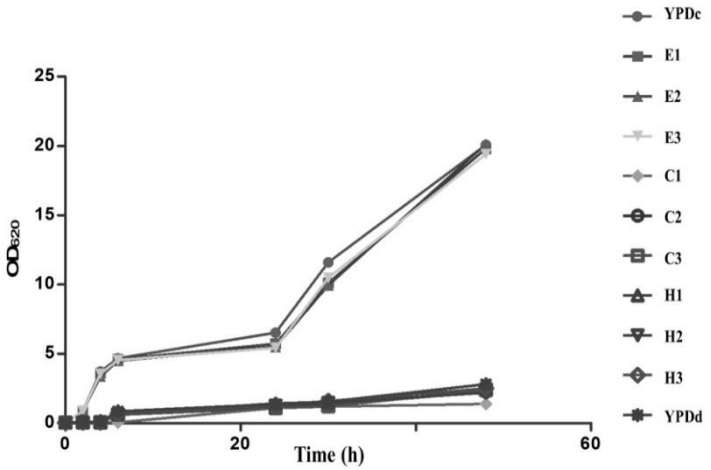


**Figure 2: Growth curves of S. cerevisiae obtained by absorbance measurement as a function of time. YPDc: Complete YPD medium used as positive growth control. E1/E2/E3: Modified media with aqueous phase obtained by enzymatic extraction method from liver 1, 2 and 3, respectively. C1/C2/C3: Modified media with aqueous phase obtained by cold extraction of liver 1, 2 and 3. H1/H2/H3: Modified media with aqueous phase, obtained by hot extraction of liver 1, 2 and 3, respectively. YPDd: YPD medium diluted ¼, used as negative growth control. Growth assays were performed in triplicate.**

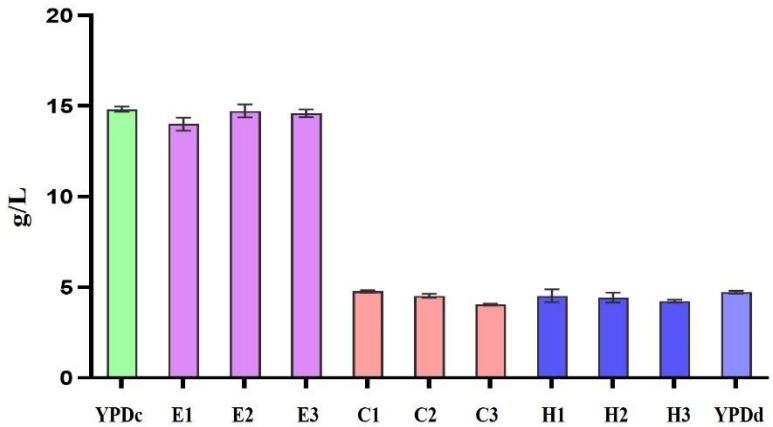


**Figure 3: Yield of yeast biomass per liter of culture medium. YPDc: YPD complete medium, positive control. E1/E2/E3: means modified media with aqueous phase AP obtained by enzymatic extractions from liver 1, 2 and 3, respectively. C1/C2/C3: are modified media with AP obtained by cold extractions from liver 1, 2 and 3, respectively. H1/H2/H3: are modified media with AP obtained by hot extractions from liver 1, 2 and 3, respectively. YPDd: YPD diluted medium in ¼, negative control. Each bar corresponds to an independently treated lot of liver and each assay was performed in triplicate.**

**Table 1: Viable yeast count after 24 h.**

| | Culture media | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **YPDc** | **E1** | **E2** | **E3** | **C1** | **C2** | **C3** | **H1** | **H2** | **H3** | **YPDd** |
| **CFU/mL** | $2.1\times10^9$ | $2.1\times10^9$ | $1.9\times10^9$ | $1.9\times10^9$ | $2.9\times10^6$ | $2.8\times10^6$ | $2.8\times10^6$ | $2.7\times10^6$ | $2.9\times10^6$ | $2.9\times10^6$ | $3\times10^6$ |

YPDd: negative control. CFU: Colony forming units; YPDc: positive control; E1/E2/E3: Modified media with APs from enzymatic extracts; C1/C2/C3: Modified media containing Aps from cold extractions; H1/H2/H3: Modified media containing APs from hot extractions.

## Discussion

*S. cerevisiae* is characterized by not having demanding nutritional requirements for its growth, where glycolysis is the main way to obtain energy, generally using simple sugars (glucose, fructose, sucrose), ethanol and/or glycerol as a carbon sources (Ferreyra *et al*., 2014; Paulo *et al*., 2015). This strain is able to metabolize low molecular weight peptides and free amino acids as an alternative carbon source to simple sugars, but not proteins larger than 10 kDa, as it does not release proteolytic enzymes into the medium, unlike pathogenic, genetically modified or selected mutant yeasts (Sturley and Young, 1988). When such carbon sources are absent or limited, regulations at the transcriptional level of genes involved in the metabolism of sources of non-fermentable carbon, adapt to the nutrients present in the culture medium (Turcotte *et al*., 2010; Paulo *et al.*, 2015). In this way, low molecular weight peptides and free amino acids that make up the AP of the protein hydrolyzate can be acquired and metabolized by the yeast, compensating for the low availability of carbohydrates in the YDPd medium.

Several investigations were reported using fish processing by-products to prepare microbial growth media. Ellouz *et al*. (2001) added heads and viscera powder of *Sardinella* to *Bacillus subtilis* culture medium. Also, *Pseudomonas aeruginosa* MN7 was cultured in media containing combined heads and viscera powder of *Sardinella* (Triki-Ellouz *et al*., 2003). Similarly, fish viscera of various marine species such as rainbow trout *Oncorhynchus mykiss*, swordfish *Xiphias gladius*, squid *Loligo vulgaris* and yellowfin tuna *Thunnus albacares* were added to enrichment media of *Vibrio anguillarum* and *Vibrio splendidus* (Vazquez *et al*., 2006). Beaulieu *et al*. (2009) reported the use of hydrolysates of *Scomber scombrus* and *Clupea harengus* as sources of peptones in the formulation of lactic and non-lactic bacterial culture media. Other experiments were reported on *Staphylococcus epidermidis* CMST Pi 2 growing in a medium containing raw and treated (defatted, alkali and acid hydrolysates) tuna by-products (Esakkiraj *et al*., 2010). Similarly, Petrova *et al.* (2021), studied the use of hydrolysates from cod fishes for the cultivation of *Staphylococcus aureus* and *Salmonella enteriditis.*

In the present work, the growth curves tested bacteria in media with APs coming from the enzymatic extraction method, achieved good performance for the *S. cerevisiae* biomass. Beaulieu *et al*. (2009), also found similar growth measured at $OD_{600}$ with respect to the control medium used (Man, Rogosa, and Sharpe Broth) for *Lactococcus lactis* in a medium supplemented with mackerel and herring

hydrolysates. This work also reported that *Pediococcus acidilactici* grew better in the supplemented media than in the control and that *Carnobacterium divergens* did not show acceptable growth in the supplemented media or in the control. Likewise, Petrova *et al*. (2021), reported that in general the MCMs (Microbiological Culture Media, a nutrient media for microorganism cultivation) based on cod backbones and heads hydrolysates showed similar or better efficacy compared to the commercial control MCM and it was better for *Salmonella entereditis* than for *Staphylococcus aureus* due to the lower selectivity of the former. Similarly, Vázquez *et al*. (2020) found that using whole and parts, of fish discarded and mechanically processed as hake, megrim, red scorpionfish, pouting, mackerel, gurnard, blue whiting, Atlantic horse mackerel, grenadier, and boarfish to supplement the culture media of lactic bacteria's led to similar growths. When hot extraction is used, drastic temperature conditions lead to protein coagulation and subsequent oil release (Lamas and Massa, 2019). Changes occur in the hydrodynamic properties of the protein, such as an increase in viscosity, a decrease in solubility as the hydrophobic residues from the interior appear on the surface, and others. In this way, no peptide residues are generated that could be used by microorganisms are generated. In a similar way, in cold extraction, oil is obtained by adding an aqueous phase before stirring and centrifuging, to which soluble components, such as proteins, carbohydrates and minerals migrate. Therefore, according to the data obtained in this study, none of these methods provide the availability of peptides for the growth of microbial biomass.

## Conclusions

Therefore, the disposal of wastes generated by fish processing industries represents an increasing environmental and health problem. However, several microbes are capable of using these substances as energy sources.

In this study, the remaining aqueous fractions of the ray liver oil extraction process from *Z. flavorostris* were evaluated as a possible replacement for the commercial YPD culture medium for *S. cerevisiae*. The pattern of cell growth and biomass yield was similar, using the standard culture medium for this yeast (YPDc) as well as the diluted and supplemented with the aqueous fractions obtained by the enzymatic extraction method (E1/E2/E3). The results suggest that these waste fractions provide an alternative source of carbon (peptides and free amino acids), and macro and microelements for the growth of *S. cerevisiae* in biotechnological processes. Finally, it is important to mention that this study is in line with several Sustainable Development Goals (SDGs) proposed by the United Nations.

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