Postural data from Stargardt's syndrome patients

Food and Bioproducts Processing

From tuna viscera to added-value products: A circular approach for fish-waste recovery by green enzymatic hydrolysis

--Manuscript Draft--

Manuscript Number:	FBP-D-22-00976R2
Article Type:	Full Length Article
Keywords:	Fish waste valorization; EPA and DHA; enzymatic extraction; design of experiments; life cycle analysis; principal component analysis
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Abstract:	Tuna represents one of the most consumed foods in many parts of the world. It is estimated that about 70% of the tuna's weight is discarded in the production of canned fillets. This work is focused on the optimization of production of an oil rich in omega-3 from the tuna viscera, provided by a canned tuna production company, using the Alcalase enzyme. Combined use of Design of experiments (DoE), life cycle analysis (LCA), and principal component analysis (PCA) on the collected analytical data made it possible to define the best combination of the values of pH, enzyme/substrate (E/S) ratio, and reaction time, respectively (pH=8.5), E/S (1%), and time (120 minutes), and to better understand the environmental bottleneck of the process. The outcomes of the study demonstrated that the duration of the hydrolysis and the associated electricity consumption is the factor that affects the most the environmental sustainability of the process.
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Cover Letter

POLITECNICO DI TORINO

4th August 2022

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Dear Editor,

We kindly ask you to consider the submission of our manuscript entitled "*From tuna viscera to added-value products: A circular approach for fish-waste recovery by green enzymatic hydrolysis"* for publication in the journal FOOD AND BIOPRODUCTS PROCESSING.

The present study proposes a circular economy strategy to valorize tuna viscera usually considered a food waste material by the production of oil with nutraceutical properties (rich in Omega 3, with EPA and DHA) through an enzymatic process. The extraction was optimized using a Design of Experiments and the data were analyzed throughout a life cycle assessment (LCA) and principal component analysis (PCA) to highlight the process bottleneck.

To the best of the author's knowledge, the present study proposes for the first time a combined approach of environmental sustainability analysis and PCA of the enzymatic extraction of tuna viscera, offering a novel strategy for the valorization of tuna waste material.

We believe that these results can be of great significance to any readership of your journal and make a profound resonance on hot topics such as "Biorefinery value chain optimisation", "Integration of biorefinery concepts with food processing" or "New functional food ingredients and non-food materials made feasible through the biorefinery context".

I confirm that this work has not been previously published, is not currently submitted for publication elsewhere, wholly or in part, nor will be submitted elsewhere while in the review process and the manuscript has not been submitted earlier to Food and Bioproducts Processing. Moreover, it has not previously appeared in printed or electronic online systems (such as in reviews, proceedings, or preprints).

All authors agree to the submission and the work is original.

On behalf of the authors,
Sincerely yours,

Tonia Tommasi

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Replies to Reviewer's Comments and Changes Done in the Revised Manuscript

(text modification in green colour throughout the manuscript)

Reviewers' comments:

Reviewer #1:

1) The last para of the introduction section should clearly mention the gaps in the literature and how the this paper is addressing the same. The para before the Material and Methods should give brief of the work done and why it is done. The paper should clearly mention the novelty and compare the results with the previous work.

Thank you for your precious suggestions. We have improved the paragraph by clearly indicating the novelties of this work compared to the studies already published in the literature.

We have added the following sentences in the introduction:

"Several authors, in recent years, have dealt with the enhancement of tuna viscera through enzymatic hydrolysis. Je et al. (2009) tested the enzymatic hydrolysis of tuna using commercially available proteases such as Flavourzyme, Alcalase, Protamex, and Neutrase. Guerard et al. (2001), Ovissipour et al. (2012), and Klomklao et al. (2016) instead tested only the alclase focusing on the optimization of the reaction. All these authors have focused on the protein fraction as the only product of hydrolysis. Only De Oliveira et al. (2016) has focused on the possibility of extracting the oily fraction from yellowfin tuna head by enzymatic hydrolysis, making the enzymatic extraction of oil from tuna viscera still unexplored. This work aims to fill this gap, focusing on the optimization of the enzymatic extraction of oil from tuna viscera, through a design of experiments (DoE). The present work also, for the first time, analyzes the results of the DoE through a comparative study of life cycle assessment (LCA), in order to identify the environmental bottleneck of the process. To the best of the author's knowledge, there are no LCA works that deal with the enzymatic extraction of oil from tuna viscera. To compensate for the lack of literature data, and to have at the same time a critical analysis of the results obtained both from the experimental campaign and from the sustainability analysis, a chemometric study was applied through principal component analysis (PCA)."

2) Whether the enzymatic activity of the purchased enzyme was checked as claimed by the manufacturer?

Thank you for the interesting question. We choose to measure the activity of the enzyme because not all the experiment was performed at the same time, and we were concerned about the possible degradation of the enzyme in the time elapsed between experiments (the enzyme was already in solution). The measurement of the activity allowed us to monitor this parameter before each day of experiments and avoid errors due to the degradation of the enzyme. We clarified this adding a sentence in "2.3 Protease characterization":

"The measurement of the activity was performed each day before of experiments and in order to avoid errors due to the degradation of the enzyme during time"

3) In LCA section: Please mention boundary, goal and scope.

Thank you for the suggestion. We stated goal and scope with the proper explanation of the reason for which we chose the functional unit (FU) and we described the boundary conditions and we draw them in Figure 1.

We added in 2.10 Life Cycle Assessment the following sentences:

"To be consistent with the goal of the study and with the function to which the process system was designed for, the chosen functional unit was 1g of fish oil. The adopted approach was from grave to cradle. The definition of the boundary conditions of the study includes the transported tuna waste feed as feedstock for the process, then the tuna pre-treatment, the enzymatic extraction, the thermal treatment, and last the centrifugation to recover the extracted oil. The scheme of the boundary condition is drawn in Fig 2."

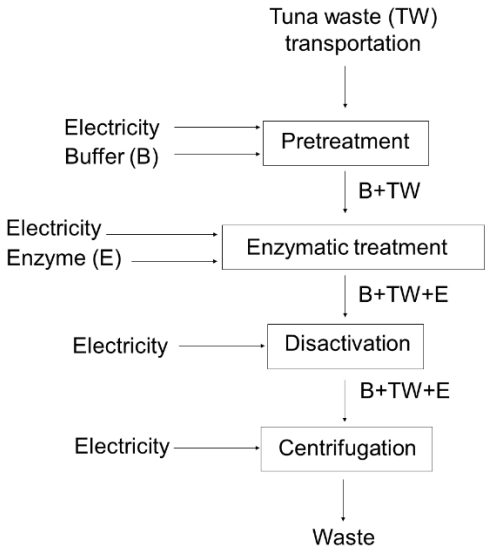


Figure 2. Scheme of the LCA boundary conditions. For each process, input and output of matter and energy are illustrated.

We also added the detailed life cycle inventory in the supplementary materials.

4) The assumptions made and what is excluded from the LCA studied should be clearly mentioned

Thank you for the suggestion. We highlighted the assumptions done. In detail we explained that tuna waste was considered a zero-burden according to (Buttol et al., 2007) and for tuna waste the only environmental credits were related to transport. To be more precautionary and avoided double discounting, all the environmental impacts and credit were allocated on the fish oil, which was the product target, and all the by-products and co-products generated in the studied system were considered as waste.

We added in 2.10 Life Cycle Assessment the following sentences:

"According to the zero-burden assumption, it was assumed that tuna waste did not contain any credits related to the impacts produced during the previous stages of its life cycle (Buttol et al., 2007). Furthermore, another assumption was the allocation criteria. To be more precautionary and avoided double discounting, all the environmental impacts and credit were allocated on the fish oil, which was the product target, and all the by-products and co-products generated in the studied system were considered as waste"

5) LCA results should be reported in absolute values and not in % as given in Table 6. Please refer any standard paper on LCA study.

Thank you for the advice. We modified Figure 4 (now figure 5) and we reported the results of each tested configuration for each impact category with the absolute value. Moreover, the contribute of each process step was referred as percentage of the absolute value.

We added in 3.5 Life Cycle Assessment the following sentences:

“Figure 5 reported the results of the 15 configurations for each impact category with the absolute value, where the contribution of each process step was referred as a percentage of the absolute value. Moreover, to underline the contribution of energy items in the total impact, it was highlighted in each process step in which it was required: pretreatment, enzymatic extraction phases, disactivation unit and oil recovery through centrifuge equipment.

Figure 5 proved that for all the tested configurations, the main contribution to the total environmental impact among the five investigated impact categories was the consumed energy and in particular the energy required for the enzymatic extraction and centrifugation phases.”

“Indeed, for the climate change category, the experiments with the longest enzyme extraction step (120 min) reached the highest energy contribution in that phase, which ranged between 30-52 % of the total impact in all the considered impact categories.

Furthermore, in all the considered impacts categories, the recovery of the oil is one of the highest energy consumer items, hence the present study underlined the importance of optimizing the downstream technology to recover the product target.

Another attentioned parameter in the present study of sustainability is the role of the enzyme. In the marine eutrophication the effect of the enzyme was highlighted, and it reached the highest contribution in the experiment in which the dose of the Alcalase was the highest one (1 mL) and the yield of fish oil was not the optimal one compared to the amount of tuna treated (0, 2 g/ g of tuna).

Among the investigated configurations, the one, reaching the highest impact of enzyme in marine eutrophication, was the experiment n°3 with a value equal to 72 % of the total enzymatic pretreatments (made up by the sum of the enzyme contribution and energy required to carry out the enzymatic extraction phase).

Among the five impact categories, the pretreatment unit, consisting in pH regulation with the addition of a buffer, had environmental impacts detectable in terrestrial acidification and freshwater eutrophication due to the adopted chemicals. Specifically, the configurations n° 1, 2 and 6 exhibited pretreatment unit impact in terrestrial acidification and freshwater eutrophication ranging between 6.5-8 and 6-8,10 % of the total environmental impacts in that categories.”

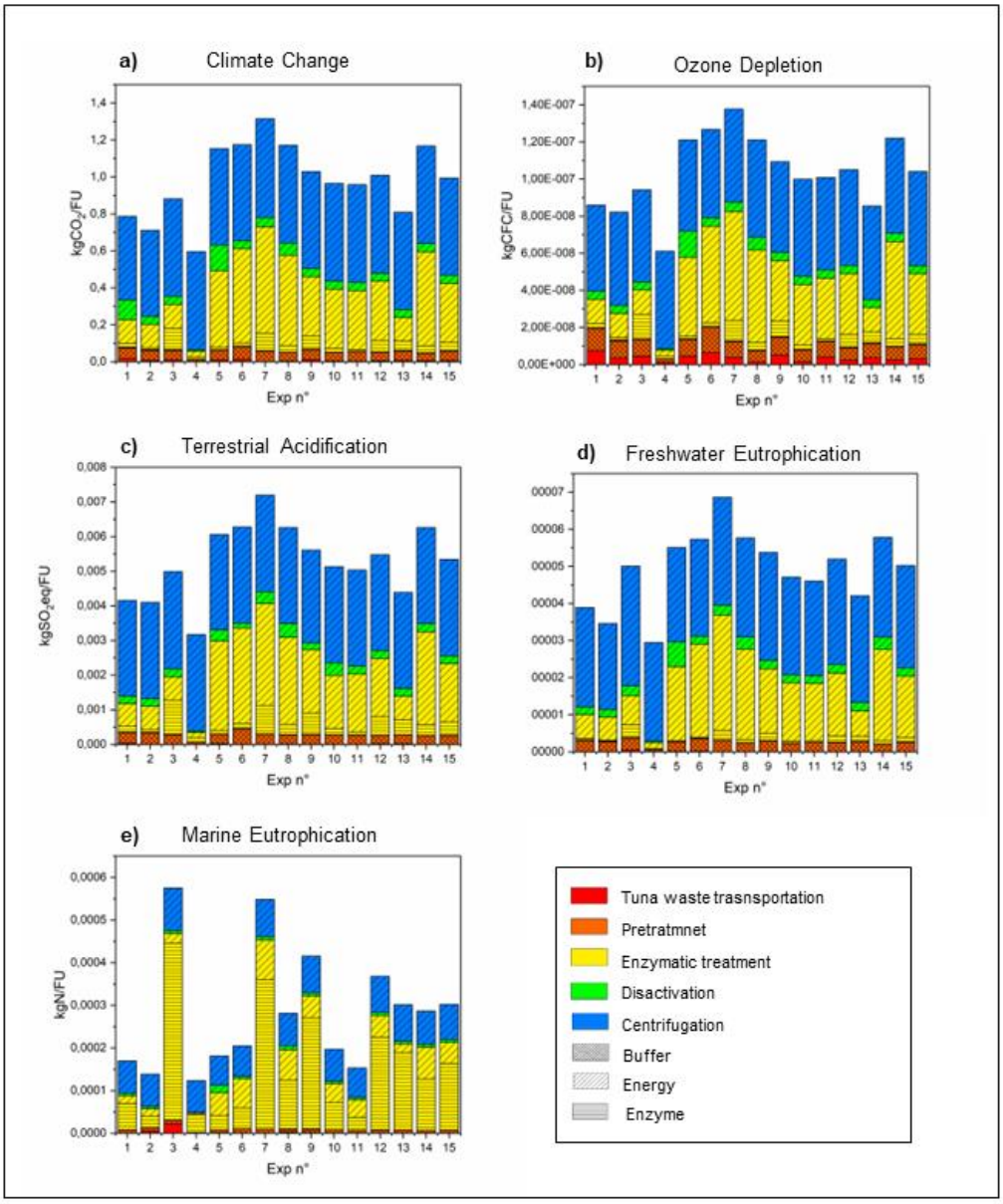


Figure 5 . Results of comparative LCA between the 15 experiments of the DoE in terms of five impact categories: (a) Climate change (kg CO2 eq), (b) Ozone depletion (kg CFC-11 eq), (c) Terrestrial acidification (kg SO2 eq), (d) Freshwater eutrophication (kg P eq) and (e) Marine eutrophication (kg N eq).

6) Compare your GHG KgCO2 eq and other 4 indictors with the reported literature

Thank you for the suggestion.
We improved the discussion of LCA results, but the comparison with literature was not realisable because in literature there are not available studies about LCA of extractive process on tuna viscera or /and Alcalase application.

Moreover, the LCA study and results are strongly dependent on the choice of functional unit, boundary conditions and time and location chosen. Considering these aspects, it is very difficult comparing our study with not similar process.
However, considering this lack we did a comparison among the investigated configurations, and we reported a study about LCA of biorefinery systems which faced sustainability problems in a general biorefinery systems.

We added in 3.5 Life Cycle Assessment the following sentences:

"To the best of the author's knowledge, there are still no LCA studies on the enzymatic extraction of oil from tuna waste, so our discussion will be limited to the results obtained in this work. in the literature, there are no available studies to compare the environmental impacts and credits of the process and substrate investigated in the present paper. For this reason, in the present work, the discussion of LCA results was done as a comparison between the 15 investigated configurations of the DoE."

"The transport of tuna exhibited a considerable impact in the ozone depletion category due to the necessity of transporting the feedstock with a refrigerated lorry for a distance of 1524 km (from the south of Italy to the north of Italy plant) in accordance with the study of (Khoo, et al 2019). The contribution of tuna transport in ozone depletion decreased by decreasing the amount of tuna, because according to the chosen FU (1g of fish oil), it means that the process is high performing.
According to the study of (Sillero et al,2021) about LCA of biorefinery systems in general and not specific for substrate or type of process, the enzyme application impacts on marine eutrophication due to its origin, whereas pretreatment impacts on terrestrial acidification and freshwater eutrophication due to the use of chemical and their post consume, whereas the product recovery exhibits great impacts on climate changes and ozone depletion due to the energy requirement."

7) You can have different scenarios to check the effect on values of different indicators.

Thank you for the suggestion.
We focused on the 15 possible configurations and on how the impacts of the 5 categories analysed in the considered processes can change. The changes and further details have already been described in the answers to the previous questions

8) The conclusion section should be rewritten.

Thank you for your suggestion. We have completely rewritten and expanded the conclusions.

"The enzymatic hydrolysis of tuna waste represents an innovative technology to exploit canned tuna processing wastes to obtain products with high added value, such as tuna oil and FPH. This study has shown that it is possible to obtain good quality tuna oil rich in omega-3 fatty acids through enzymatic hydrolysis of tuna viscera derived by a canned tuna production company with commercial enzyme Alcalase.

The DoE made it possible to optimize oil extraction and identify the best pH (8.5 °C), E/S (1%), and time (120 minutes). It was possible to obtain a model able to compute the yield as a function of the three-factor analyzed. The study of the progress of the DH% over time showed that after one hour, the reaction slows down. The experiments of the DoE were compared to each other through an LCA analysis, and the results were investigated with a PCA. This innovative approach allowed to demonstrate that

the duration of the hydrolysis and the associated electricity consumption is the variable that most affects the environmental sustainability of the process.

Further studies would be needed to reduce the time of hydrolysis, analyze in more depth the FPH obtained and its proprieties and design a possible scale-up in order to evaluate the technical, economic and environmental feasibility of this process at an industrial level."

9) The tables/Fig in LCA are not appropriate. Please check any published paper for reference.

Thank you for the advice.
We modified the Figure 4 (now 5), eliminated figure 6 and we separated the paragraph of LCA in two paragraphs: 3.5 for LCA results and 3. Evaluation of LCA results through PCA.
This separation could highlight both the results of LCA and the novelty of adopting PCA to analysed LCA results. Moreover, the application of PCA to LCA results could partially cover the impossibility of compare our results with literature data due to the absence of sustainability study of extractive process, fish oil production and enzyme application.

Reviewers' comments:

Reviewer #2:

Authors have used Alcalase enzyme for the digestion of Tuna waste. There are a lot of studies whose work is similar though this research team has done it on Tuna.

The data analyses has been done differently; however, many of the inferences are similar to the previous findings. LCA is comparatively newly employed.

Authors have not included a Table for ANOVA and have only considered R2 and R as criteria; however lack of fit of the model and the adjusted R2 should also be considered among other factors.

Thank you for suggesting the use of additional parameters to better describe the modelling of the percentage degree of hydrolysis (DH%) curve reported in Figure 3 (if we understood well!). We are not quite sure about including an ANOVA computed on the DH% data, as no groups or effects can be defined in relation to these experiments.

Regarding the use of an adjusted R2, it would probably be more suitable in the case of a modelling function with more than one explanatory variable x. In our case there is only time as explanatory variable, as expressed by equation 8, so we would state that the "basic" R and R2 values, together with the visual representation of Figure 3 should be enough to clearly provide a description of how well this function fits the experimental data.

Also, as there is no consideration of the quadratic terms, there is no requirement of the RSM graphs, they can be removed.

Thank you for your precious suggestions.
The quadratic terms were introduced in Section 2.5 when describing in more general terms the multilinear (MLR) regression technique, and it was specified in Section 3.2 that they were removed as non-significant.
The phrasing of Section 3.2 was changed to make the changes to the model clearer:

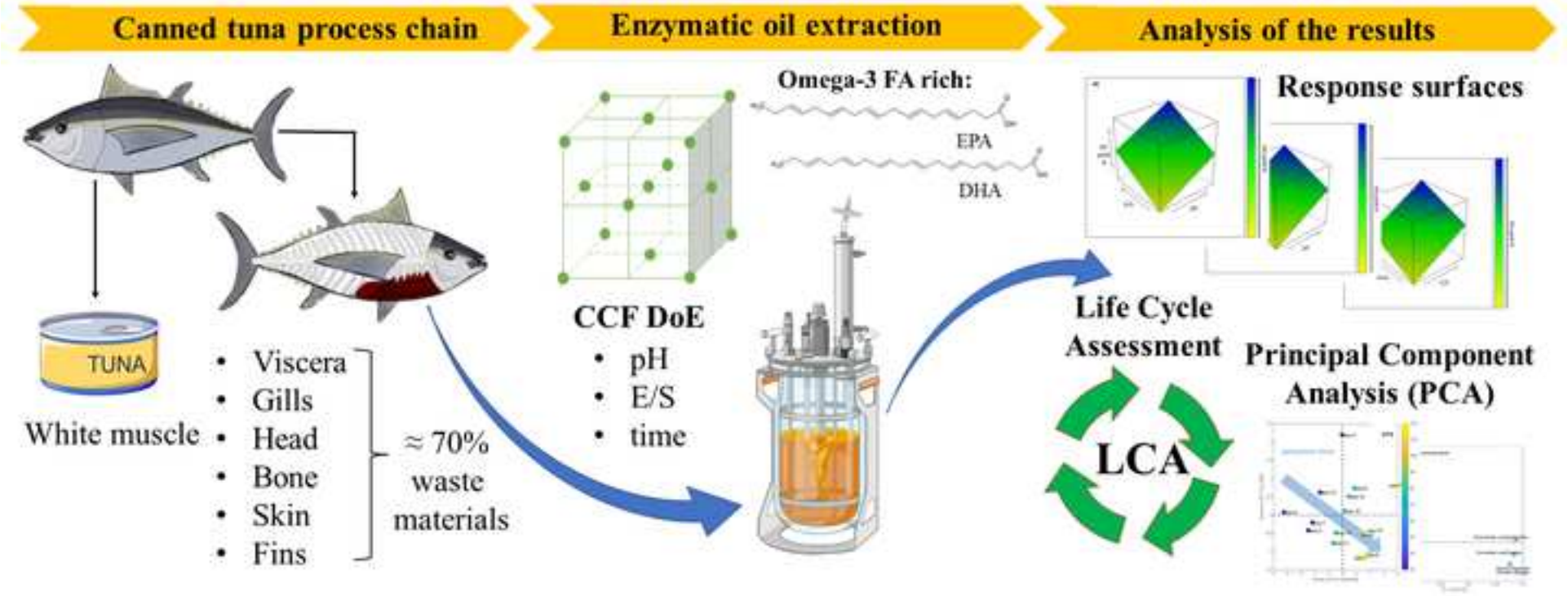
"To model the percentage oil yield a full MLR regression model was first computed and after a preliminary elaboration, the model was simplified excluding the non-significant terms, i.e., the interaction and quadratic terms. Only the statistically significant terms were included, as described by equation 7."

Regarding the inclusion of the RSM graphs of Figure 2, we believe that they can be very informative and useful to provide a complete and clear representation of the MLR model results, together with the regression parameters of Table 2, as the interpretation of the parameters alone can in general be misleading.

Highlights

- Application of enzyme hydrolysis in the recovery of oil from tuna waste material
- Process optimization through the Design of Experiments
- Oil extracted from tuna waste material is rich in omega-3 fatty acids
- A combined approach of Life Cycle Assessment and Principal Component Analysis
- Electricity consumption is the most environmental impacting factor of the process

Graphical Abstract



From tuna viscera to added-value products: A circular approach for fish-waste recovery by green enzymatic hydrolysis

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Abstract

Tuna represents one of the most consumed foods in many parts of the world. It is estimated that about 70% of the tuna's weight is discarded in the production of canned fillets. This work is focused on the optimization of production of an oil rich in omega-3 from the tuna viscera, provided by a canned tuna production company, using the Alcalase enzyme. Combined use of Design of experiments (DoE), life cycle analysis (LCA), and principal component analysis (PCA) on the collected analytical data made it possible to define the best combination of the values of pH, enzyme/substrate (E/S) ratio, and reaction time, respectively (pH=8.5), E/S (1%), and time (120 minutes), and to better understand the environmental bottleneck of the process. The outcomes of the study demonstrated that the duration of the hydrolysis and the associated electricity consumption is the factor that affects the most the environmental sustainability of the process.

Keywords: Fish waste valorization, EPA and DHA, enzymatic extraction, design of experiments, life cycle analysis

1. Introduction

Tuna and particularly canned tuna is one of the most consumed fish globally; this seafood is fished and processed in more than 70 countries and represents an affordable and common source of protein worldwide (FAO, 2020). The main tuna species processed are skipjack (Katsuwonus pelamis), albacore (Thunnus alalonga), yellowfin (Thunnus albacares), bigeye (Thunnus obesus), and bluefin (Thunnus thynuss), which are mainly fished in the Pacific Ocean (Allain et al., 2016; FAO, 2020). Around 5.2 million metric tons of tuna fish have been captured in 2018, and at least three-quarters of all landed tuna have been canned (McKinney et al., 2020). Thailand is the leading country for tuna processing, while the largest consumers among the European countries are Spain, Italy, France, UK, and Portugal (Ababouch and Catarci, 2008; Allain et al., 2016; FAO, 2020)

Tuna, after capture, is frozen and transported to production plants where it is subjected to quality controls and then deprived of heads, viscera, and fins through mechanical sawing systems (Ababouch and Catarci, 2008). After that, the tuna is cooked in various ways that depend on the choice of industry. After the cooking step, the fillets are manually cleaned with knives, then they get canned to be finally sterilized (Hospido et al., 2006; Ababouch and Catarci, 2008; Ramakrishnan et al., 2013).

The principal product of the canned tuna industry is the fillet, consisting mainly of white muscle, while viscera, gills, dark flesh/muscle, head, bone, and skin are considered as waste or low-value by-products (Herpandi et al., 2011). These wastes can

reach up to 70 % of the total weight of the fish and still contain a high portion of valuable protein, lipid and nutritional components, which can represent a profitable resource for the nutraceutical, pharmaceutical and cosmetic industries (Araujo et al., 2021; Herpandi et al., 2011). With the increasingly problematic effects of climate change and the scarcity of natural resources, many researchers are now focusing on improving technologies to extract bioactive compounds from fish by-products. Two of the main products obtained from tuna waste are tuna oil and fish protein hydrolysates (Herpandi et al., 2011). There has been growing interest towards tuna oil because it is rich in polyunsaturated fatty acids (PUFAs), in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), omega-3 fatty acids, both responsible for preventing some cardiovascular and various inflammatory diseases (Calder, 2012). Fish protein hydrolyzates (FPH) is obtained by subjecting fish proteins to chemical or enzymatic hydrolysis, producing peptides and free amino acids. Several studies demonstrated that enzymatic hydrolysis, in an optimum combination of process conditions, is able to produce FPH with excellent properties, like antioxidant activity with high amounts of antihypertensive, anticancer, anti-anemia peptides (Herpandi et al., 2011). Moreover, enzymatic hydrolysis can separate at the same time the protein fraction, the oil fraction and the insoluble solid, reducing the use of toxic solvents and the production of chemical waste compared to other extraction processes (Araujo et al., 2021). These characteristics make the enzymatic hydrolysis process environmentally sustainable and an excellent candidate for producing high added-value products from canned tuna waste materials.

Several authors, in recent years, have dealt with the enhancement of tuna viscera through enzymatic hydrolysis. Je et al. (2009) tested the enzymatic hydrolysis of tuna using commercially available proteases such as Flavourzyme, Alcalase, Protamex, and Neutrase. Guerard et al. (2001), Ovissipour et al. (2012), and Klomklao et al. (2016)

instead tested only the alcalase focusing on the optimization of the reaction. All these authors have focused on the protein fraction as the only product of hydrolysis. Only De Oliveira et al. (2016) has focused on the possibility of extracting the oily fraction from yellowfin tuna head by enzymatic hydrolysis, making the enzymatic extraction of oil from tuna viscera still unexplored. This work aims to fill this gap, focusing on the optimization of the enzymatic extraction of oil from tuna viscera, through a design of experiments (DoE). The present work also, for the first time, analyzes the results of the DoE through a comparative study of life cycle assessment (LCA), in order to identify the environmental bottleneck of the process. To the best of the author's knowledge, there are no LCA works that deal with the enzymatic extraction of oil from tuna viscera. To compensate for the lack of literature data, and to have at the same time a critical analysis of the results obtained both from the experimental campaign and from the sustainability analysis, a chemometric study was applied through principal component analysis (PCA).

2. Material and methods

2.1 Raw materials and chemicals

Around 10 kg of frozen tuna (*Thunnus albacares*) waste materials were provided by the Company "Sebastiano DRAGO" (Siracusa, Italy), that produces canned tuna. The raw material was shipped and maintained at controlled temperatures of –20 °C until analysis and experiments. All reagents used in this work were high purity grade and provided by Sigma Aldrich-Merck (Germany). The enzyme applied for the hydrolysis was the Alcalase 2.4 L, an aqueous solution of Protease from *Bacillus licheniformis* with a declared specific activity ≥ 2.4 U/g.

2.2 Pretreatment of waste material

The frozen tuna waste material was thawed at room temperature and drained from the blood. After that, the viscera were manually separated from the bones and the skin using a sharp knife. To obtain a sample as homogeneous as possible, the viscera were blended and homogenized using a kitchen blender.

2.3 Protease characterization

The enzyme chosen for this study was Alcalase, a highly studied enzyme that has already been used to produce FPH from various fish species (Tacias-Pascacio et al., 2020). To choose the enzyme/substrate ratio and to monitor any degradation of the Alcalase during the time, an activity assay was conducted. First of all, it was necessary to measure the total amount of enzyme in the Alcalase solution, in terms of total proteins, through the Bradford assay using Bovine Serum Albumin (BSA) as standard protein (Kruger, 1994).

The Bradford assay is a protein determination method that involves binding Coomassie Brilliant Blue G-250 dye to proteins. When the dye binds to the protein, it is converted to a stable unprotonated blue form with the maximum absorbance at 595 nm.
Hence, 100 µL of diluted Alcalase was mixed with 1000 µL of the Bradford reagent into an Eppendorf tube. After 15 minutes the absorbance of the sample was measured at 565 nm through a spectrophotometer (Hach Lange DR5000). The protein amount was then determined by comparing the absorbance value with a calibration curve obtained using different concentrations of BSA (from 0.1 mg/mL to 0.01 mg/mL) mixed with the Bradford reagent. All the solutions were maintained at pH = 7 using a phosphate buffer. This assay allowed the correct choice of Alcalase dilution to apply in the activity assay.
A non-specific protease activity assay using casein as substrate (Cupp-enyard, 2008)

was chosen. The measurement of the activity was performed each day before of experiments and in order to avoid errors due to the degradation of the enzyme during time. In this assay, casein is digested by the Alcalase liberating tyrosine, which reacts with Folin & Ciocalteus reagent to produce a chromophore. This complex has a maximum absorbance in the blue region of the UV-Vis, and it is measurable with a spectrophotometer. Briefly, the diluted Alcalase was mixed with 0.65 % of casein solution and incubated at 37 °C for 10 minutes. After this time, Trichloroacetic Acid (TCA) was added to stop the enzymatic reaction, and more Alcalase was put in the solution and incubated for 30 minutes at 37 °C. Then the solution was filtered and was supplemented with sodium carbonate and Folin's reagent, mixed, and incubated again for 30 minutes at 37 °C. After the incubation time, the mixture was filtered, and the absorbance was measured at 660 nm. The µmol tyrosine equivalents released were obtained, by comparing the absorbance of the sample with a tyrosine calibration curve. Then the activity of the Acalase was calculated by the following equation (1):

$$\frac{Units}{mL\ enzyme} = \frac{\mu mol_{Teq}}{V_E \cdot t \cdot V_C} \quad (1)$$

where μmol_{Teq} are the micromoles of tyrosine equivalent obtained from the calibration curve, V_{tot} is the total volume of the assay (mL), t is the time (min), V_E is the volume of the enzyme (mL) and V_c is the volume of the cuvette (mL). It was possible to convert Units/mL in Units/mg of protein contained in the liquid enzyme by applying the following equation (2):

$$\frac{Units}{Protein\ (mg)} = \frac{\frac{Units}{Enzyme\ (mL)}}{\frac{protein\ (mg)}{Enzyme\ (mL)}} \quad (2)$$

2.4 Tuna viscera characterization

The total amount of lipids contained in the tuna viscera was determined using the Bligh and Dyer method (Bligh, E.G. and Dyer, 1959), originally developed to quantify the

total lipid in the fish muscle and later become the standard rapid method for fat determination in marine biological tissue (Bligh, E.G. and Dyer, 1959). In summary, 100 g of homogenized sample was mixed with 100 mL of chloroform and 200 mL of methanol. After that, 100 mL of chloroform and 100 mL of water were added to the mixture, agitated for 30 seconds, and then filtered. The liquid part was transferred into a graduated cylinder, where the separation between the polar and non-polar fraction of the solution occurred. The volume of chloroform was noted and the alcoholic fraction was removed using a glass pipette. The non-polar solution was evaporated through a Rotavapor (Labrota 4000, Heidolph) and the oily residue was weighed with an analytical balance. The measurement was performed in triplicate.

The total N content was first quantified through the Dumas or combustion method (Simonne, et al., 1997) utilizing an Elementar analyzer (Vario MACRO cube, Elementar). Then, the N total was multiplied for a nitrogen-to-protein conversion factor (equation 3). The factor chosen was 5.6, specific for fish protein, as suggested by Mariotti et al. 2008 (Mariotti et al., 2008) and not the standard 6.25 conversion factor, usually used for foodstuff.

$$Proteins\ \% = N \cdot 6.5 \quad (3)$$

The moisture and ash contents were determined following the "Rapporto ISTISAN 1996/34", and the total carbohydrate content was calculated by difference.

2.5 Experimental design and data modeling

To understand the effect of pH, enzyme/substrate ratio (E/S) and time on the amount of fish oil extracted and on the antioxidant activity of the fish protein hydrolysates, but also to optimize the extraction technique by minimizing and at the same time the number of experiments, a Design of Experiments (DoE) (Leardi, 2009) was put in

place. A three-factor and three-levels Central Composite Face Centered (CCF) design consisting of 15 experiments was employed, and 17 experimental runs, including three replicates at the center point, were performed. The design factors were pH (6.5, 7.5 and 8.5), E/S (0.1, 0.55 and 1) and time (30, 75 and 120 min), while the response variable was the yield of fish oil expressed in percentage (oil yield %). Based on literature information, the temperature was kept constant at 55 °C, which has been determined to be the optimal temperature for Alcalase (Li et al., 2018; Glowacz-Rozynska et al., 2016; Wang et al., 2019).

The design was created using MODDE 7 software (version 7.0.0.1), and the list of experimental runs, i.e., the experimental plan (with the original values of each factor) and matrix (coded version of the original values) are reported in Table 1. In the CCF design, the three levels are conventionally named as –1, 0, and +1, where –1 represents the lowest value of the factor range, +1 is the highest value and 0 is the central point (Taylor et al., 2021). In the presence of three factors, the experimental domain spanned by the CCF design can be represented in 3D space as a cube, like it is shown in Figure 1.

The data were analyzed using the R-based CAT (Chemometric Agile Tool, by Leardi R.) software, whose DoE modeling engine is based on the multiple linear regression (MLR) (Sergent, 1995) method. MLR is a regression method that allows modeling and inspecting each factor separately, but also in combination with one or more other factors. Its functioning provides enough flexibility to efficiently model the response under examination, while at the same time including or excluding the non-significant terms in the model equation, which can be represented as:

$$y = \beta_0 + \beta_A A + \beta_B B + \beta_C C + \beta_{AB} AB + \beta_{BC} BC + \beta_{AC} AC + \beta_{AA} AA + \beta_{BB} BB + \beta_{CC} CC \quad (4)$$

where y is the predicted response (oil yield %), ε represents the noise and I are the regression coefficients of each factor (A = pH, B = E/S and C = time), including the interaction between the factors (AB, AC and BC) and their quadratic terms (AA, BB and CC).

To better inspect the comparative LCA results (as described in Section 2.10) it was decided to study the obtained numerical values using the exploratory data analysis method principal component analysis (PCA, (Bro and Smilde, 2014). PCA is a decomposition method that allows obtaining clear representations of the samples' distributions, together with the correlation patterns among the variables by inspecting the so-called scores and loadings, respectively. In our case, the samples were the 15 experiments analyzed via LCA, and the variables were the five LCA impact categories.

2.6 Enzymatic extraction

The enzymatic extraction was conducted following the procedure of Ramakrishnan et al. (2013) with some modifications. Around 20 g of homogenized tuna viscera were put into a 50 mL falcon tube and heated at 90 °C for 10 minutes in a water bath, to deactivate any endogenous enzymes (Ramakrishnan et al., 2013). After cooling down at room temperature, a buffer was added to control the pH during the reaction in a ratio 1:1 (buffer:substrate). Three different buffers were used in different experiments of the DoE: phosphate buffer at different concentrations (pH = 6.5 and 7.5) and borate buffer (pH = 8.5). Then the mixture was thermostated at 55 °C for 30 minutes into an incubator with a shaking rate of 200 rpm (ES-20/60, Orbital Shaker- Incubator, Biosan). Different amounts of Alcalase were added to the mixture to obtain different E/S percentages (0.1, 0.55 and 1 %). The enzyme was then allowed to react for 30, 75 or 120 minutes. When the reaction time had elapsed, the falcon tube was put again into the water bath at 90 °C for ten minutes to stop the reaction. The mixture was cooled at room

temperature and centrifuged at 5000 rpm for 20 minutes with a SL 16R Centrifuge (Thermo Fisher Scientific).

2.7 Oil yields

The upper oil layer was collected using a plastic pipette and transferred into a glass sample holder. To make sure there was only fat in the sample, the oil was washed with a small amount of dichloromethane and left to evaporate under a chemical hood. The samples were then weighed on an analytical balance and the amount of oil was related to the total oil content present in the tuna viscera (see paragraph 2.4) to obtain the yield % according to the following equation (4).

$$oil\ yield\ \% = \frac{oil\ extracted\ (g)}{total\ oil\ in\ the\ sample\ (g)} \cdot 100 \qquad (5)$$

2.8 Omega-3 quantification

The omega-3 content in terms of EPA and DHA was determined according to UNI EN ISO 12966-2:2017 standard methods. Fatty acids were analyzed in the form of methyl esters (FAME) using a gas chromatograph equipped with a mass analyzer. To prepare the FA methyl esters, around 100 mg of sample was dissolved in 3 mL of methanolic KOH (0.6 M) in a falcon tube and stirred for 10 s under an N_2 flow to avoid oxidation of compounds. The solution was heated under agitation for 10 minutes at 70 °C using a water bath. After this time, and the complete dissolution of the oil, 3 mL of 5 % H_2SO_4 in methanol was added and the solution was heated at 70 °C for another 5 min. Then 2 mL of a saturated solution of NaCl and 2 mL of hexane were added, and the falcon was centrifuged at 4000 rpm for 10 minutes. The supernatant was collected, diluted 1:2, and transferred in a vial for the GC-MS analysis. The omega-3 analysis was conducted using a gas chromatograph (Agilent 7890A GC System) equipped with a ZB-FAME column (30 m x 0.25 mm ID x 20 μm; Zebron Phenomenex) and a quadrupole mass

detector (Agilent 5975 C VL MSD). Helium was used as carrier gas with a flow of 0.4 mL/min, the injection volume was 1 μL using the split mode with a split ratio of 20:1 at 240 °C. The column was initially maintained at 100 °C for 2 min and then the temperature was then increased to 240 °C at 5 °C/min and kept constant for 8 min (Brotas et al., 2020). The mass analyzer worked in electron ionization mode with ionization energy of 70 eV and in full scan mode between 50 and 600 a.m.u.. The temperatures of the source and quadrupole were maintained at 230 °C and 150 °C, respectively. Omega-3 quantification was determined through calibration curves prepared using a DHA and EPA certified standard.

2.9 Progress of protein hydrolysis

To understand the behavior of an enzyme-substrate system, at least five indicators must be considered: the substrate concentration (S), the enzyme/substrate ratio (E/S), the pH, the temperature (T) and the time (t). These indices can be substituted by the degree of hydrolysis (DH) because, as affirmed by Alder-Niessen (1982) (Alder-Niessen, 1982) in some conditions, the development of an enzymatic protein hydrolysis reaction depends only on the pH and DH. The DH is defined as the ratio of the number of peptide bonds broken by the total number of peptide bonds in the original protein (North and Zealand, 2010) and allows to obtain information on the progress of the reaction in real time. To measure the DH, the pH-stat method was employed. This method is based on the fact that, during the hydrolysis of the peptide bond, there is a pH decrease and, to maintain it constant, it is necessary to add some base. It is possible to relate the amount of added base to the DH by the following equation (6) (North and Zealand, 2010):

$$DH\% = \frac{B \cdot N_B}{\alpha \cdot m \cdot h_{tot}} \quad (6)$$

Where B is the base consumption (mL), N_B is the normality of the base, m is the mass of the protein of the sample, α is the average degree of dissociation of α-NH_2 groups in the protein of the sample and h_{tot} is the total number of peptide bonds per unit mass of protein. The working conditions chosen for this experiment were the optimal conditions obtained from the DoE experiments. The base used was NaOH 0.5 N. The term 1/α is defined as a calibration factor and depends on the temperature and pH, in these working conditions (55 °C and pH 8.5) it values 1.03 (Navarrete del Toro and García- Carreño, 2003). The term h_{tot} value depends on the source of protein, for fish, Navarrete et al. (2003) suggest using 8.6 m_{eq}/g. This experiment was conducted into a 1 Lbioreactor capable of automatically regulating the pH by a proportional integral derivative (PID) controller connected to a pH probe and a peristaltic pump. The reactor was also equipped with a heating jacket to keep a constant temperature of 55 °C, and Rushton impellers to stir the medium. The amount of base added over time was recorded by a computer. The model then was fitted to the experimental data using SigmaPlot (Version 10.0, Systat Software Inc., USA).

2.10 Life Cycle Assessment

Life Cycle Assessment (LCA) was performed with SimaPro 9.0.4 software, database Ecoinvent 3.0. The goal of LCA was to compare all the experiments of the DoE, to choose the best extraction process in terms of environmental sustainability. To be consistent with the goal of the study and with the function to which the process system was designed for, the chosen functional unit was 1g of fish oil. The adopted approach was from grave to cradle. The definition of the boundary conditions of the study includes the transported tuna waste feed as feedstock for the process, then the tuna pre-treatment, the enzymatic extraction, the thermal treatment, and last the centrifugation to recover the extracted oil. The scheme of the boundary condition is drawn in Fig 1.

According to the zero-burden assumption, it was assumed that tuna waste did not contain any credits related to the impacts produced during the previous stages of its life cycle (Buttol et al., 2007). Furthermore, another assumption was the allocation criteria. To be more precautionary and avoided double discounting, all the environmental impacts and credit were allocated on the fish oil, which was the product target, and all the by-products and co-products generated in the studied system were considered as waste.

The life cycle inventory (LCI) defined all inputs and outputs involved in the processes. The primary data came from the present study, the produced emissions, the consumed material, and the required energy were referred to the chosen FU. The reagents which were not present in the Ecoinvent database were computed on a stoichiometric basis. The detailed inventory is provided in the supplementary materials. The secondary data, taken from Ecoinvent 3.0 were:

- Electricity, medium voltage {Row}| market for | Alloc Rec, S
- Tap Water from natural resource

Electricity RoW (rest of the world), was chosen to avoid geolocalizing the process, not being in the scope of the analysis.

Life cycle impact assessment (LCIA) was performed with the ReCIPE Midpoint (H) method. In the present study, the analyzed impact categories were: Climate change (kg CO_2 eq), Ozone depletion (kg CFC-11 eq), Freshwater eutrophication (kg P eq), Marine eutrophication (kg N eq) and Terrestrial acidification (kg SO_2 eq) because they were the most used impact categories in the fish processing industry (Avadí and Fréon, 2013; Ruiz-Salmón et al., 2021). The results of the LCA were also analyzed through a Principal Component Analysis (PCA) performed with Matlab (R2020a).

3. Results and discussion

3.1 Protease and Tuna viscera characterization

The commercial Alcalase solution shows an activity of 5.559 ± 0.004 (Units/mg protein).

The defrosted tuna was not deprived of blood and water, so the moisture content resulted quite high (77.5 ± 0.1 %). The carbohydrate content is not revealed (NR) and also the percentage of ashes resulted very low (2.22 ± 0.1 %). The most interesting values for this study are the percentage of proteins and lipids, respectively 17.70 ± 0.20 % and 2.58 ± 0.08 %.

These results are in line with other works investigating the composition of different body parts of several tuna species (Karunarathna and Attygalle, 2009; Vlieg and Murray, n.d.). Karunarathna and Attygalle (2009) affirm that the moisture percentage of tuna viscera is around 70 %, the protein content is between 16-19 %, the lipids percentage can vary between 0.9 and 1.34 % and ash content is around 1 % while the amount of carbohydrates is negligible in all tuna species. Slight variations are possible and depend on several factors, such as the species, diet, age and other parameters of the tuna (Karunarathna and Attygalle,2009).

3.2 Enzymatic extraction and interpretation of the Design of Experiments results

After extraction and centrifugation, the samples showed a four-layers separation with the oil floating on the top, followed by a light mixture of lipids and proteins, then by the FPH and a solid residue on the bottom of the falcon. The oil was collected using a plastic pipette, to be quantified and analyzed through a GC-MS instrument. The second and bottom layers were discarded, while the FPH layer was collected for future analysis.

Table 1 reports the results of the DoE in terms of oil yield %. The amount of extracted oil varies from 15 % up to 81.4 %. To model the percentage oil yield a full MLR regression model was first computed and after a preliminary elaboration, the model was simplified excluding the non-significant terms, i.e., the interaction and quadratic terms. Only the statistically significant terms were included, as described by equation 7.

$$y = \beta_0 + \beta_{pH} pH + \beta_{E/S} E/S + \beta_t t + \varepsilon \quad (7)$$

The significant linear terms are reported in the plot of the coefficients (Figure 3a) and in Table 2, where the presence of * indicates the significance of the coefficients: ** = $p < 0.01$, *** = $p < 0.001$.

Time was the factor with the lowest influence on the extraction yield. The positive values of all coefficients indicate that they positively influence the response, i.e., the oil yield % increases with the increase of pH, E/S and time. These results are confirmed by the response surfaces reported in Figures 3b,c,d. These surfaces represent a three-dimensional view of the relationship between pairs of factors (reported on the x and y axes) and the response (plotted on the z axis), providing a clear picture of how the response is related to the factors.

In this model the response surface takes the shape of a tilted plane, where the yield increases when the factors increase. The flat look of the surface is due to the fact that all quadratic and interaction terms were excluded from the MLR model, therefore no terms describing possible curvatures of the response were included. According to the model, it is possible to obtain the maximum oil yield % at maximum pH, E/S and time values (pH = 8.5; E/S % = 1; t = 120 min).

The influence of pH is one of the most important parameters to be analysed in enzymatic hydrolysis. Indeed, enzymes contain a catalytic active site with charger amino acids. The pH can affect the dissociation state of these amino acids changing the

ionic bonds that maintain the three-dimensional shape of the protein. This may lead to alteration in protein function or inactivation of enzymes (Shu et al., 2016).

Alcalase was initially obtained from Bacillus subtilis and called “Subtilisin Carlsberg” (Tacias-Pascacio et al., 2020). Subtilisin, generally, work optimally in mildly alkaline. This kind of enzymes are serine proteases that contain catalytic triadin order of aspartic acid, histidine, and serine residues (Azrin et al., 2022) and in particular, Ser221, His64 and Asp32 (Wells and Estell 1988). The activity of subtilisin increases at alcaline pH because His64 is deprotonated. Therefore, by increasing the pH there is a greater activity of the enzyme which hydrolyzes more proteins, releasing larger quantities of oil and increasing the yield.

Results show a positive effect of the enzyme: substrate ratio on the enzymatich hydrolysis, the increase of the enzyme concentration resulted an higher oil yield.

At higher E/S, indeed, there are more enzyme active sites available to hydrolyze the substrate, resulting in a more effective cleavage of the peptide bonds, and consequently greater degradation of the proteins (Kurozawa et al, 2009).

To the best of the authors’ knowledge, this is the first study regarding the enzymatic extraction of tuna oil using Alcalase applying DoE. These results are in line with Ramakrishnan et al. (2013), who investigated the effects of E/S and time, separately, on the oil extraction from mackerel waste material using Alcalase at 55 °C and pH = 8.5 (Ramakrishnan et al., 2013). They demonstrated that oil yield increases with the increase in the enzyme concentration (0.5, 1 and 2 %) and time (1, 2, 3 and 4 h), reaching a plateau around an hour and a half of elapsed time. Qi-yuan et al. (2016) extracted oil from mackerel viscera applying a DoE to investigate how the extraction is affected by enzyme concentration, pH and temperature (Qi-Yuan et al., 2016). Their work confirms that as the E/S ratio increases, the oil yield also increases. In their study, De Oliveira et al. (2017) extracted oil from tuna by-product through enzymatic

extraction using Alcalase and they affirmed that this technique allows obtaining an oil of better quality than other obtained with non-green extraction techniques (De Oliveira et al., 2017). However, there are very few literature studies in which oil is extracted from tuna waste, while there are many on enzymatic extractions from other fishes. Wang et al. (2019) affirmed that Alcalase (at 55 °C and pH = 8) could be used as a good protease for extracting a good quality Antarctic krill oil (Wang et al., 2019). The present work results are also in line with Głowacz-Rozynska et al. (2016) who tried to extract the oil from salmon waste, showing that it is possible to achieve yields of around 82 % using Alcalase at 55 °C and pH = 8 (Glowacz-Rozynska et al., 2016). Other authors effectively extracted good quality oil from fish waste such as catla, rohu, perch, copepod, tilapia or trout, using Alcalase (Babajafari et al., 2017; Li et al., 2018; Taylor et al., 2013; Vlieg and Murray, n.d.). Araujo et al. (2021) treated fish scraps from a market with Alacase al pH = 8, 50 °C and 180 min and deduced that the E/S ratio was the main factor controlling the hydrolysis (Araujo et al., 2021).

To evaluate the prediction power of the MLR model, two replicated experiments of the central point of the design (not included to compute the model) were performed. Table 3 reports the experimental values of the two experiments, the upper and lower values with their predicted values, and their residuals. The predictive capacity of the model is hence confirmed.

3.3 Progress of protein hydrolysis

To monitor the progress of the hydrolysis, the DH% as a function of time was measured using the pH-stat method in a 1 L Bioreactor, applying the optimum condition found with the DoE (55 °C, pH = 8.5 E/S = 1 %). As shown in the graph in Figure 4 DH% increases very quickly in the first hour of reaction and then stabilizes around 22 %.

The experimental sets of (DH%, t) were fit to Eq. (9) using Sigma Plot and the results of the fitting procedure are listed in Figure 3 (Babajafari et al., 2017; Taylor et al., 2013). The squared points of Figure 3 represent the experimental values with their standard deviation and the continuous line the values obtained from the Equation 8. To evaluate the quality of the fitting, the correlation coefficient R, and the coefficient of determination R^2 were evaluated. These values, very close to 1, indicate that the regression predictions fit the data almost perfectly. The model passed also the Shapiro-Wilk test and the constant variance test with a significance level of 99 %.

$$DH\% = \frac{a + bt}{1 + ct} \tag{8}$$

These results are consistent with the work of Saidi et al. (2013), which hydrolyzed tuna by-products at the exact conditions of the present work and demonstrated that progress of hydrolysis increased roughly during the first hour and then no significant variation can be observed (Saidi et al., 2013). Ovissipour et al. (2010) obtained a trend in good agreement with our results, but the DH% stabilized around 16 %, probably because they did not work at controlled pH (Ovissipour et al., 2010). These results are also confirmed by Guerard et al. (2001) (Guérard et al., 2001). Klomklao and Benjakul (2016) obtained a curve with the same shape as the one in Figure 4, but their curve reached the plateau at an earlier time (20 min) and at higher DH% (35 %) (Klomklao and Benjakul, 2016). This is possibly caused by the fact that they used more severe T, pH and E/S conditions for the reaction compared to our experimental conditions. Valencia et al. (2014) identified the mechanisms responsible for the shape of the Alcalase hydrolysis curve for fish proteins (Valencia et al., 2014). They demonstrated that these curves are characterized by an initial "hydrolysis phase" followed by a slowdown and stabilization of the degree of hydrolysis. The cause for this decrease in reaction rate

could be associated with several factors, such as: a decrease in the concentration of peptide bonds of the substrate available for hydrolysis; a product inhibition caused by amino acids and soluble peptides released during the hydrolysis that act as an substrate competitor; and an enzyme deactivation (Klomklao and Benjakul, 2016; (Kurozawa et al, 2009).

3.4 Omega-3 quantification

The oil fraction was analyzed through GC-MS to quantify the omega-3 FA in terms of EPA and DHA; they represent 2,49 ± 0,32 % and 10,83 ± 1,13 % of the extracted oil. The percentages obtained in this work are lower than percentages achieved by De Oilivera et al. (2017). They extracted oil from tuna heads with Alcalase but applied different experimental conditions and reached 6 % of EPA and 27 % of DHA (De Oliveira et al., 2017). In general, the content of EPA and DHA in fish oil varies respectively from 5 to 26 %, and from 6 to 26 % of the total fatty acid, depending on fish species (Alkio et al., 2000). From the data found in the literature, tuna oil contains about 5 % of EPA and 25 % of DHA (Halldorsson et al., 2003; Zhang et al., 2017). In their work, Alkio et al. (2000) used tuna oil containing 4.6 % of EPA and 18.3 % of DHA, so these amounts are very variable (Alkio et al., 2000). Besides the study by De Oilivera et al. (2017), the tuna oil analyzed in these research works is never made from the tuna viscera, and the percentage composition of fatty acids can be very different. Moreover, even the extraction procedure is not consistent across the different studies, making the literature data hardly comparable to each other. In any case, the percentage of omega-3 found in the oil extracted in this study makes it a product with excellent qualities. As a matter of fact, EPA and DHA contribute to preventing some cardiovascular and various inflammatory diseases such as hyperlipidemia, atherosclerosis, inflammation, and cancer. Moreover, they present some beneficial

effects on the brain and nervous system (Alkio et al., 2000; Correa-Matos and Vaghefi, 2013; De Oliveira et al., 2017; Halldorsson et al., 2003; Zhang et al., 2017).

3.5 Life Cycle Assessment

The comparison of the environmental sustainability of the different extractions defined by the DoE was analyzed through Life Cycle Assessment (LCA).

Figure 4 shows the results of LCA among the 15 experiments of the DoE (the replicas of the central point were not considered) through five impact categories: Climate change (a, kg CO_2 eq), Ozone depletion (b, kg CFC-11 eq), Terrestrial acidification (c, kg SO_2 eq), Freshwater eutrophication (d, kg P eq) and Marine eutrophication (e, kg N eq). The analysis was limited to these five categories because, as indicated by Ruiz-Salmón et al. (2021), they are the most studied ones in LCA of fish and seafood processed products and, therefore, it would make the present study more comparable to what can be already found in the literature. By reviewing a considerable number of works on this topic, Ruiz-Salmón et al. (2021), highlighted that Ozone depletion was computed by 53 % of analyzed authors, followed by acidification (64 %) and eutrophication (66 %) (Ruiz-Salmón et al., 2021). However the study of Ruiz-Salmón et al. (2021) focused on the impact of landed fish, fish distribution and the process to make fish an edible product. The aim of the selected five impact categories is following defined. Climate change is the indicator of the global warming potential (GWP) caused by the emissions of greenhouse gasses into the atmosphere. Ozone depletion indicates the emissions to the atmosphere responsible for destroying the stratospheric ozone layer. Acidification is the indicator of the potential acidification of soils and water due to the release of gasses such as nitrogen oxides and sulfur oxides. The freshwater eutrophication indicator expresses the freshwater ecosystem's enrichment with nutritional elements attributable to the emission of compounds phosphor. Marine eutrophication is the indicator of

enhancing the marine ecosystem with nutritive elements due to nitrogen-containing compounds (Jessen et al., 2015). To the best of the author's knowledge, in the literature, there are no available studies to compare the environmental impacts and credits of the process and substrate investigated in the present paper. For this reason, in the present work, the discussion of LCA results was done as a comparison between the 15 investigated configurations of the DoE.

Figure 5 reported the results of the 15 configurations for each impact category with the absolute value, where the contribution of each process step was referred as a percentage of the absolute value. Moreover, to underline the contribution of energy items in the total impact, it was highlighted in each process step in which it was required: pretreatment, enzymatic extraction phases, disactivation unit and oil recovery through centrifuge equipment.

Figure 5 proved that for all the tested configurations, the main contribution to the total environmental impact among the five investigated impact categories was the consumed energy and in particular the energy required for the enzymatic extraction and centrifugation phases.

As shown in Figure 5a-e, experiment n° 4 presented the lower contributions in all the impact categories analyzed in this work. This experiment was conducted at pH of 8.5 with an E/S of 1 % and for 30 mins. It is important to notice that, according to the DoE, this experiment does not correspond to the highest obtainable oil yield but with the same quantity of oil produced, it is the most sustainable combination of experimental conditions, from an environmental point of view. By analyzing Figure 5, it is possible to note that all the experiments with a reaction time of 30 minutes, which correspond to the lowest level of the time factor analyzed in the DoE, had minor impacts. It could be deduced that the duration of the reaction greatly influences the sustainability of this process. Indeed, for the climate change category, the experiments with the longest

enzyme extraction step (120 min) reached the highest energy contribution in that phase, which ranged between 30-52 % of the total impact in all the considered impact categories.

Furthermore, in all the considered impacts categories, the recovery of the oil is one of the highest energy consumer items, hence the present study underlined the importance of optimizing the downstream technology to recover the product target.

Another attentioned parameter in the present study of sustainability is the role of the enzyme. In the marine eutrophication the effect of the enzyme was highlighted, and it reached the highest contribution in the experiment in which the dose of the Alcalase was the highest one (1 mL) and the yield of fish oil was not the optimal one compared to the amount of tuna treated (0,2 g/ g of tuna).

Among the investigated configurations, the one, reaching the highest impact of enzyme in marine eutrophication, was the experiment n°3 with a value equal to 72 % of the total enzymatic pretreatments (made up by the sum of the enzyme contribution and energy required to carry out the enzymatic extraction phase).

Among the five impact categories, the pretreatment unit, consisting in pH regulation with the addition of a buffer, had environmental impacts detectable in terrestrial acidification and freshwater eutrophication due to the adopted chemicals. Specifically, the configurations n° 1, 2 and 6 exhibited pretreatment unit impact in terrestrial acidification and freshwater eutrophication ranging between 6,5-8 and 6-8,10 % of the total environmental impacts in those categories.

The transport of tuna exhibited a considerable impact in the ozone depletion category due to the necessity of transporting the feedstock with a refrigerated lorry for a distance of 1524 km (from the south of Italy to the north of Italy plant) in accordance with the study of (Khoo, et al 2019). The contribution of tuna transport in ozone depletion

decreased by decreasing the amount of tuna, because according to the chosen FU (1g of fish oil), it means that the process is high performing.

According to the study of (Sillero et al,2021) about LCA of biorefinery systems in general and not specific for substrate or type of process, the enzyme application impacts on marine eutrophication due to its origin, whereas pretreatment impacts on terrestrial acidification and freshwater eutrophication due to the use of chemical and their post consume, whereas the product recovery exhibits great impacts on climate changes and ozone depletion due to the energy requirement.

3.6 Evaluation of LCA results through PCA

To better compare the LCA results, it was decided to organize the values reported in Figure 4 into a data matrix of dimensions 15×5 (one column for each impact category) and to analyze it using PCA. Prior to analysis, the data table was scaled to unit variance and mean centered. The PCA results are reported in Figure 6. As already highlighted in Figure 5, Climate change (a), Ozone depletion (b), Terrestrial acidification (c) and Freshwater eutrophication (d) share the same overall trend, while Marine eutrophication (e) shows a rather different pattern. This aspect is confirmed by the PC1-PC2 loadings plot (Figure 6a), as all impact categories appear to be highly correlated, since they are located at positive PC1 values. PC2 accounts for the difference between Marine eutrophication and the other four impact categories. Regarding the DoE experiments' distribution in the scores plot of Figure 6b, it can be noticed a clear trend related to the extraction time (blue arrow and coloring). Moreover, from this plot it becomes clear that experiment n°4 has the lowest impact in all categories, while experiment n°7 has the largest impact in all categories.

The pattern of correlations among the impact categories described by PC2-PC3 (Figure 6a,b) seems to be related to the response, as the experiments' distribution colored

according to the oil yield % follow a rather clear trend (yellow arrow). The experiments with the highest response also have higher impacts in the Climate change and Terrestrial acidification categories, while the same experiments show much lower impacts in the Ozone depletion and Marine eutrophication categories.

Considering the results of the LCA expressed in figure 5 and of the PCA represented in figure 6, the following considerations can be made. Almost all impact is caused by electricity in all four categories. In this LCA study, non-geolocalized medium voltage electricity was used, referring to the whole world (Simapro). This electricity represents an average of all types of electricity used globally, from renewable and non-renewable sources (coal, oil, gas, biomass, nuclear, hydro, wind, geothermal, solar marine) (Schmidt et al., 2011). The production of this electricity causes emissions of greenhouse gasses, nitrogen oxides, and sulfur oxides, substances responsible for the depletion of the ozone layer and compounds rich in phosphorus and nitrogen, damaging the various ecosystems (Kalender and Alkan, 2019). For this reason, the DoE experiments with the shortest time are also those with the lowest environmental impact.

The only impact category where the enzyme also contributes in large quantities to the total is Marine eutrophication. This LCA study assumed that the Alcalase is produced from potato starch along with a bacterial strain, as suggested by Rosa et al. (2020) (Rosa et al., 2020). It is possible that the cultivation of potatoes and the use of fertilizers greatly influence this impact category, increasing the emission of substances rich in nitrogen into rivers or coastal areas or the nitrogen applications to the soil

These results are in line with the results obtained from the DoE. Indeed, the best experimental configuration is the one that uses a buffer at pH 8.5 and an E/S of 1 %. The duration of the process seems to be most responsible for the environmental impact, but time seems to be the least significant factor among the variables analyzed using the DoE. Moreover, the study of hydrolysis kinetics has shown that the DH% stabilizes

around a plateau after one hour. Therefore, it would be possible to reduce reaction times by reducing the environmental impacts without losing much in oil yield. Another way to reduce the contribution of electricity could be to geolocate the process and use renewable resources. For what it concerns the contribution due to the use of the enzyme, new scenarios could be envisaged, changing the type of enzyme or its way of production.

4. Conclusions

The enzymatic hydrolysis of tuna waste represents an innovative technology to exploit canned tuna processing wastes to obtain products with high added value, such as tuna oil and FPH. This study has shown that it is possible to obtain good quality tuna oil rich in omega-3 fatty acids through enzymatic hydrolysis of tuna viscera derived by a canned tuna production company with commercial enzyme Alcalase.

The DoE made it possible to optimize oil extraction and identify the best pH (8.5 °C), E/S (1%), and time (120 minutes). It was possible to obtain a model able to compute the yield as a function of the three-factor analyzed. The study of the progress of the DH% over time showed that after one hour, the reaction slows down. The experiments of the DoE were compared to each other through an LCA analysis, and the results were investigated with a PCA. This innovative approach allowed to demonstrate that the duration of the hydrolysis and the associated electricity consumption is the variable that most affects the environmental sustainability of the process.

Further studies would be needed to reduce the time of hydrolysis, analyze in more depth the FPH obtained and its proprieties and design a possible scale-up in order to evaluate the technical, economic and environmental feasibility of this process at an industrial level.

Acknowledgments

We thank "Ditta Drago Sebastiano di Giuseppe Drago" (Siracusa) for sending the tuna waste materials from their production chain.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Figure Captions:

Figure 1. A representation of the three-factors, three-levels, Central Composite Face Centered (CCF) experimental domain. To model and explain the results of the DoE, a multiple linear regression (MLR) was applied, as described in Section 2.5.

Figure 2. Scheme of the LCA boundary conditions. For each process, input and output of matter and energy are illustrated.

Figure 3. (a) The coefficients plot of the MLR model (**= $p < 0.01$, *** = $p < 0.001$) and the response surfaces for oil yield %: (b) interaction between E/S and pH, (c) interaction between time and pH, (d) interaction between time and E/S.

Figure 4. Enzymatic extraction progress curve and results of the fitting procedure Black squares represent the experimental values. Each experiment was conducted in triplicate, and the error bars correspond to standard error. The continuous line represents the modeled values (eq. 9). a, b and c are the coefficients of equation 9. R and R^2 represent the quality of the fitting.

Figure 5. Results of comparative LCA between the 15 experiments of the DoE in terms of five impact categories: (a) Climate change (kg CO_2 eq), (b) Ozone depletion (kg CFC-11 eq), (c) Terrestrial acidification (kg SO_2 eq), (d) Freshwater eutrophication (kg P eq) and (e) Marine eutrophication (kg N eq).

Figure 6. PCA scores and loadings plots of the comparative LCA results. Only the most informative combinations of principal components are reported: PC1-PC2 loadings (a)

and scores (b) plots; PC2-PC3 loadings (c) and scores (d) plots. The coloring in (b) corresponds to the extraction time, while in (d) it corresponds to the oil yield % (the response of the DoE). The coloring range values can be interpreted using the color bars on the right-hand side.

Tables:

Table 1 The experimental plan (i.e., the factors described with their original values) and matrix (i.e., the coded version of the factors' values) for tuna oil extraction optimization, whose yield values are reported in the last column on the right.

Experiment N°	pH (coded)	E/S (coded)	time (coded)	pH (original)	E/S (%) (original)	time (min) (original)	oil yield % (response)
1	–1	–1	–1	6.5	0.1	30	15.0
2	1	–1	–1	8.5	0.1	30	31.7
3	–1	1	–1	6.5	1	30	25.0
4	1	1	–1	8.5	1	30	49.3
5	–1	–1	1	6.5	0.1	120	24.1
6	1	–1	1	8.5	0.1	120	33.5
7	–1	1	1	6.5	1	120	56.0
8	1	1	1	8.5	1	120	81.4
9	–1	0	0	6.5	0.55	75	21.9
10	1	0	0	8.5	0.55	75	76.6
11	0	–1	0	7.5	0.1	75	27.7
12	0	1	0	7.5	1	75	47.0
13	0	0	–1	7.5	0.55	30	31.3
14	0	0	1	7.5	0.55	120	43.0
15	0	0	0	7.5	0.55	75	34.8
16	0	0	0	7.5	0.55	75	38.7
17	0	0	0	7.5	0.55	75	42.5

Table 2. Coefficients of the MLR model and their significance with the explained variance of the data. *= p < 0,05 **= p < 0.01, *** = p < 0.001

Coefficients				Explained Variance %
β_0	β_{pH}	$\beta_{E/S}$	β_t	
39.968	13.043	12.665	6.575	71.58
0.0000***	0.00009***	0.0011**	0.0148*	

Table 3. Prediction results of experiments 18 and 19.

Exp N°	Experimental value	Lower	Predicted value	Upper	Residuals
18	24.16	34.90	39.97	45.04	–2.20
19	39.25	34.90	39.97	45.04	0.71

Figures:

Figure 1.

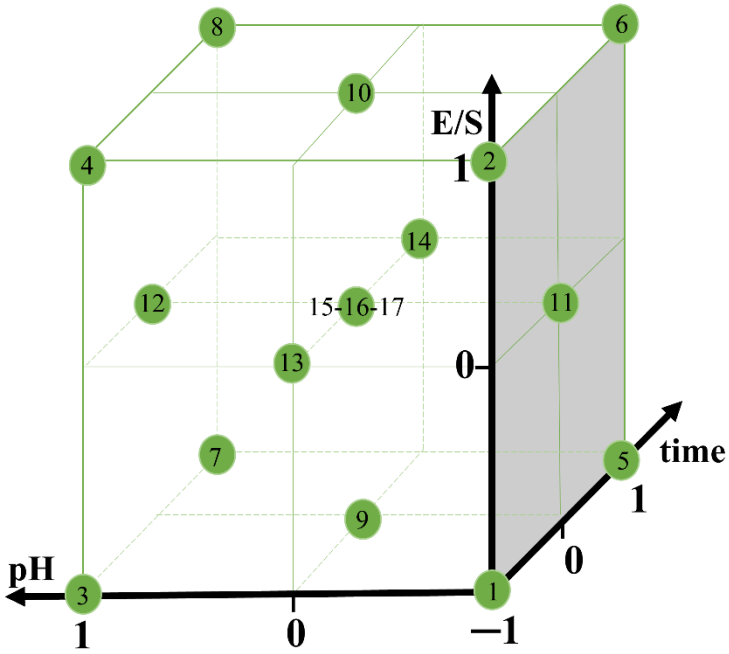


Figure 2.

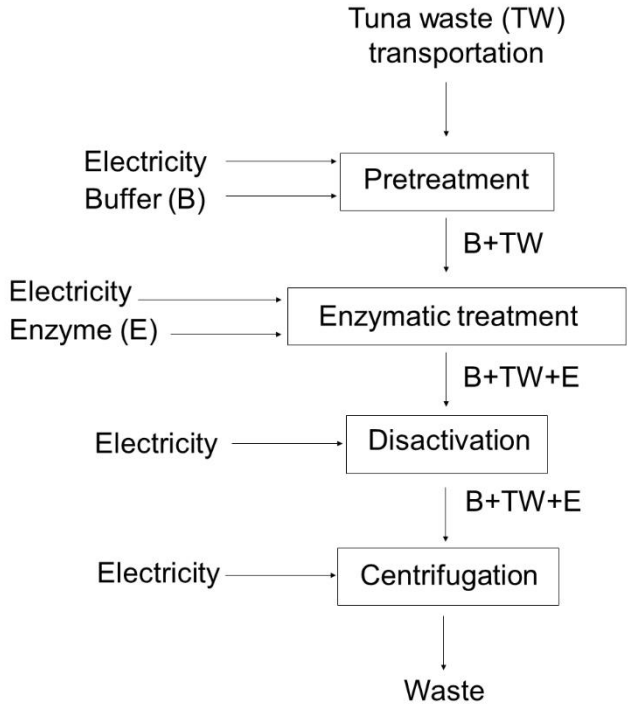

Tuna waste (TW) transportation
Electricity
Buffer (B)
Pretreatment
B+TW
Electricity
Enzyme (E)
Enzymatic treatment
B+TW+E
Electricity
Disactivation
B+TW+E
Electricity
Centrifugation
Waste

Figure 3.

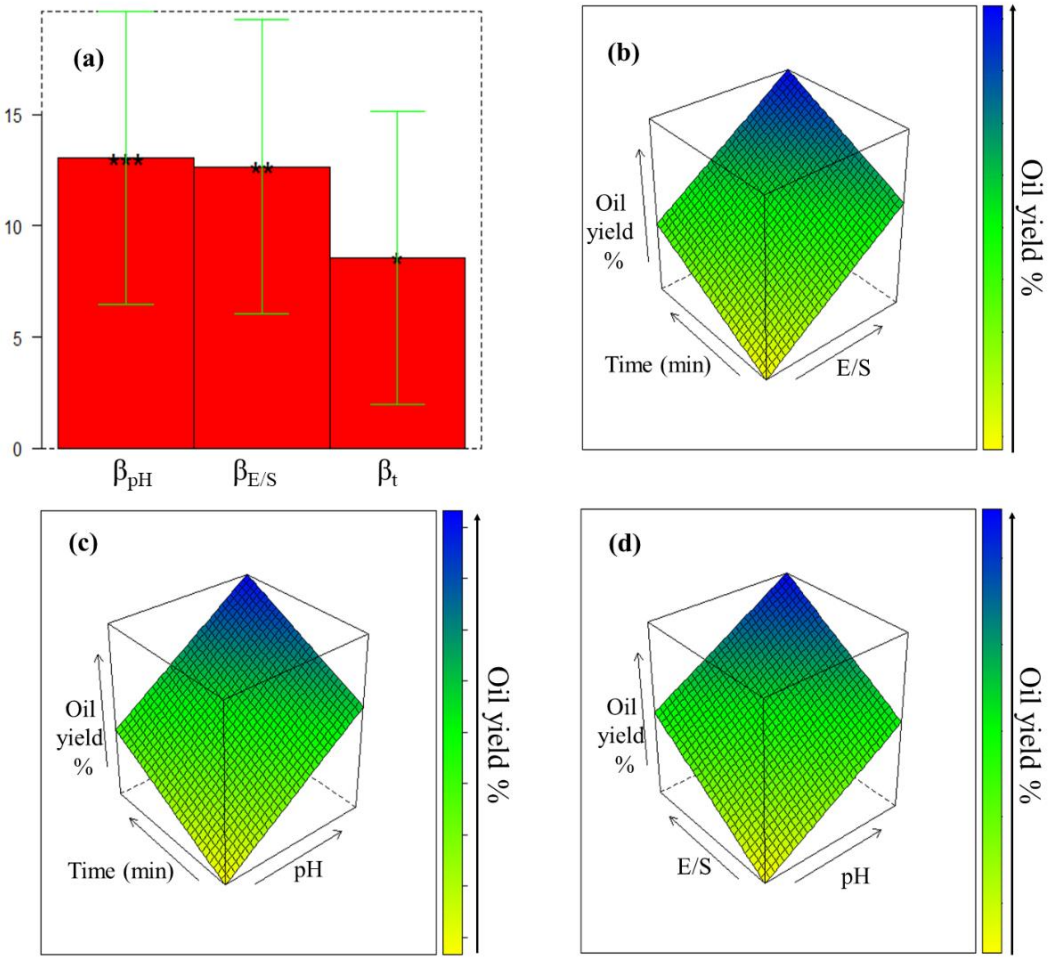


Figure 4.

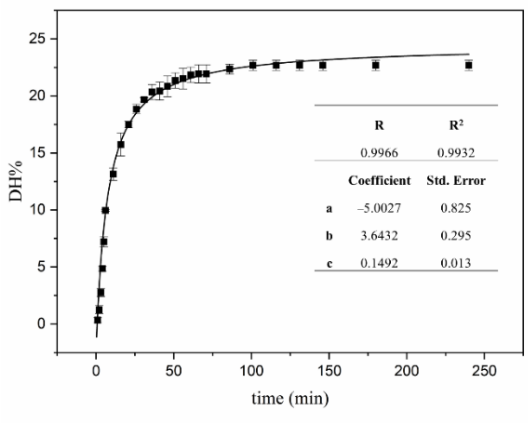


Figure 5.

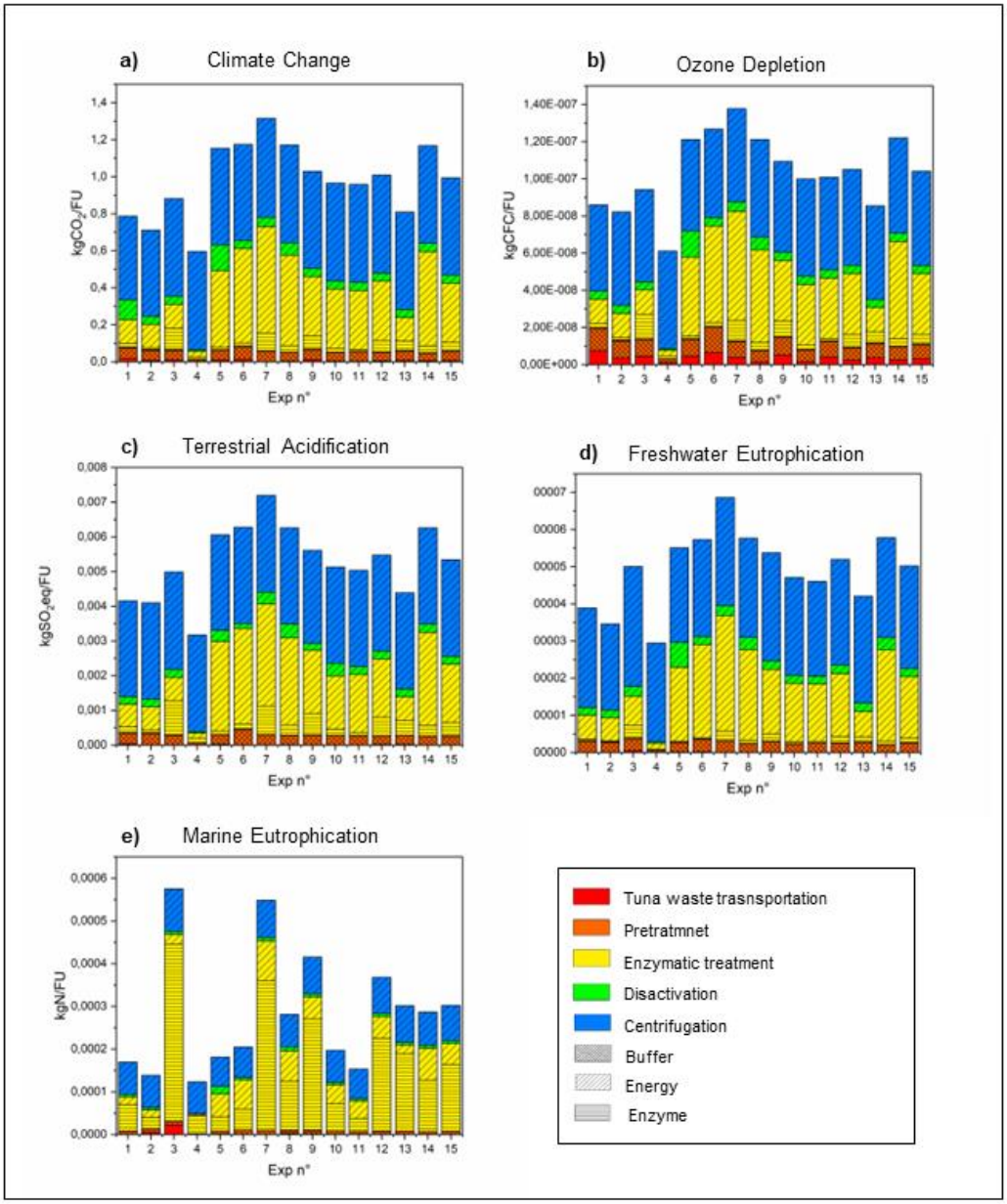
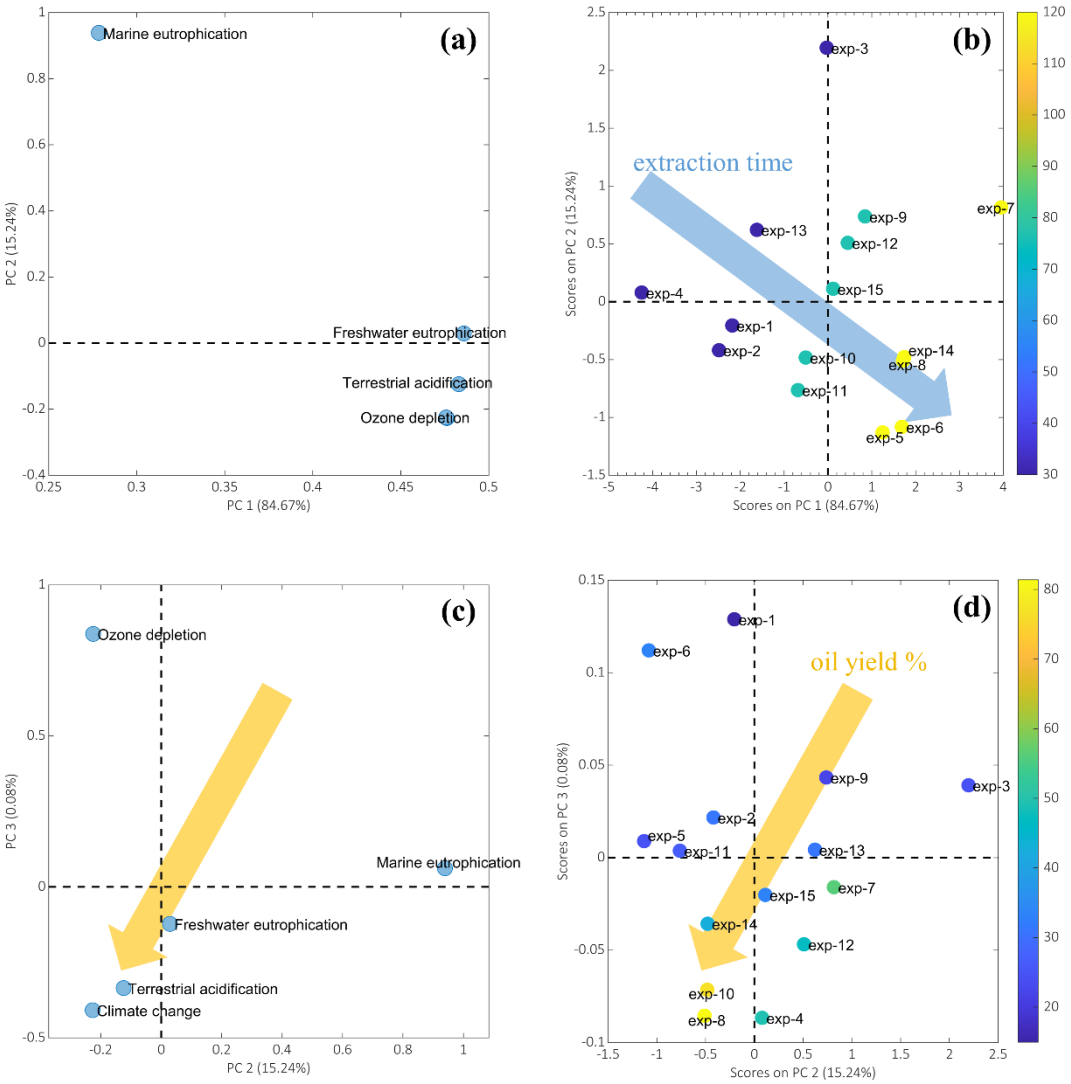

a) Climate Change
kgCO2/FU
Exp n°
b) Ozone Depletion
kgCFC/FU
Exp n°
c) Terrestrial Acidification
kgSO2eq/FU
Exp n°
d) Freshwater Eutrophication
Exp n°
e) Marine Eutrophication
kgN/FU
Exp n°
Tuna waste trasnsportation
Pretratmnet
Enzymatic treatment
Disactivation
Centrifugation
Buffer
Energy
Enzyme

Figure 6.


(a)
Marine eutrophication
Freshwater eutrophication
Terrestrial acidification
Ozone depletion
PC 2 (15.24%)
PC 1 (84.67%)
(b)
extraction time
exp-3
exp-9
exp-7
exp-13
exp-12
exp-15
exp-4
exp-1
exp-2
exp-10
exp-14
exp-8
exp-11
exp-6
exp-5
Scores on PC 2 (15.24%)
Scores on PC 1 (84.67%)
(c)
Ozone depletion
Marine eutrophication
Freshwater eutrophication
Terrestrial acidification
Climate change
PC 3 (0.08%)
PC 2 (15.24%)
(d)
oil yield %
exp-1
exp-6
exp-9
exp-3
exp-2
exp-5
exp-11
exp-13
exp-15
exp-7
exp-14
exp-12
exp-10
exp-8
exp-4
Scores on PC 3 (0.08%)
Scores on PC 2 (15.24%)

From tuna viscera to added-value products: A circular approach for fish-waste recovery by green enzymatic hydrolysis

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Abstract

Tuna represents one of the most consumed foods in many parts of the world. It is estimated that about 70% of the tuna's weight is discarded in the production of canned fillets. This work is focused on the optimization of production of an oil rich in omega-3 from the tuna viscera, provided by a canned tuna production company, using the Alcalase enzyme. Combined use of Design of experiments (DoE), life cycle analysis (LCA), and principal component analysis (PCA) on the collected analytical data made it possible to define the best combination of the values of pH, enzyme/substrate (E/S) ratio, and reaction time, respectively (pH=8.5), E/S (1%), and time (120 minutes), and to better understand the environmental bottleneck of the process. The outcomes of the study demonstrated that the duration of the hydrolysis and the associated electricity consumption is the factor that affects the most the environmental sustainability of the process.

Keywords: Fish waste valorization, EPA and DHA, enzymatic extraction, design of experiments, life cycle analysis

1. Introduction

Tuna and particularly canned tuna is one of the most consumed fish globally; this seafood is fished and processed in more than 70 countries and represents an affordable and common source of protein worldwide (FAO, 2020). The main tuna species processed are skipjack (Katsuwonus pelamis), albacore (Thunnus alalonga), yellowfin (Thunnus albacares), bigeye (Thunnus obesus), and bluefin (Thunnus thynuss), which are mainly fished in the Pacific Ocean (Allain et al., 2016; FAO, 2020). Around 5.2 million metric tons of tuna fish have been captured in 2018, and at least three-quarters of all landed tuna have been canned (McKinney et al., 2020). Thailand is the leading country for tuna processing, while the largest consumers among the European countries are Spain, Italy, France, UK, and Portugal (Ababouch and Catarci, 2008; Allain et al., 2016; FAO, 2020)

Tuna, after capture, is frozen and transported to production plants where it is subjected to quality controls and then deprived of heads, viscera, and fins through mechanical sawing systems (Ababouch and Catarci, 2008). After that, the tuna is cooked in various ways that depend on the choice of industry. After the cooking step, the fillets are manually cleaned with knives, then they get canned to be finally sterilized (Hospido et al., 2006; Ababouch and Catarci, 2008; Ramakrishnan et al., 2013).

The principal product of the canned tuna industry is the fillet, consisting mainly of white muscle, while viscera, gills, dark flesh/muscle, head, bone, and skin are considered as waste or low-value by-products (Herpandi et al., 2011). These wastes can

reach up to 70 % of the total weight of the fish and still contain a high portion of valuable protein, lipid and nutritional components, which can represent a profitable resource for the nutraceutical, pharmaceutical and cosmetic industries (Araujo et al., 2021; Herpandi et al., 2011). With the increasingly problematic effects of climate change and the scarcity of natural resources, many researchers are now focusing on improving technologies to extract bioactive compounds from fish by-products. Two of the main products obtained from tuna waste are tuna oil and fish protein hydrolysates (Herpandi et al., 2011). There has been growing interest towards tuna oil because it is rich in polyunsaturated fatty acids (PUFAs), in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), omega-3 fatty acids, both responsible for preventing some cardiovascular and various inflammatory diseases (Calder, 2012). Fish protein hydrolyzates (FPH) is obtained by subjecting fish proteins to chemical or enzymatic hydrolysis, producing peptides and free amino acids. Several studies demonstrated that enzymatic hydrolysis, in an optimum combination of process conditions, is able to produce FPH with excellent properties, like antioxidant activity with high amounts of antihypertensive, anticancer, anti-anemia peptides (Herpandi et al., 2011). Moreover, enzymatic hydrolysis can separate at the same time the protein fraction, the oil fraction and the insoluble solid, reducing the use of toxic solvents and the production of chemical waste compared to other extraction processes (Araujo et al., 2021). These characteristics make the enzymatic hydrolysis process environmentally sustainable and an excellent candidate for producing high added-value products from canned tuna waste materials.

Several authors, in recent years, have dealt with the enhancement of tuna viscera through enzymatic hydrolysis. Je et al. (2009) tested the enzymatic hydrolysis of tuna using commercially available proteases such as Flavourzyme, Alcalase, Protamex, and Neutrase. Guerard et al. (2001), Ovissipour et al. (2012), and Klomklao et al. (2016)

instead tested only the alcalase focusing on the optimization of the reaction. All these authors have focused on the protein fraction as the only product of hydrolysis. Only De Oliveira et al. (2016) has focused on the possibility of extracting the oily fraction from yellowfin tuna head by enzymatic hydrolysis, making the enzymatic extraction of oil from tuna viscera still unexplored. This work aims to fill this gap, focusing on the optimization of the enzymatic extraction of oil from tuna viscera, through a design of experiments (DoE). The present work also, for the first time, analyzes the results of the DoE through a comparative study of life cycle assessment (LCA), in order to identify the environmental bottleneck of the process. To the best of the author's knowledge, there are no LCA works that deal with the enzymatic extraction of oil from tuna viscera. To compensate for the lack of literature data, and to have at the same time a critical analysis of the results obtained both from the experimental campaign and from the sustainability analysis, a chemometric study was applied through principal component analysis (PCA).

2. Material and methods

2.1 Raw materials and chemicals

Around 10 kg of frozen tuna (*Thunnus albacares*) waste materials were provided by the Company "Sebastiano DRAGO" (Siracusa, Italy), that produces canned tuna. The raw material was shipped and maintained at controlled temperatures of –20 °C until analysis and experiments. All reagents used in this work were high purity grade and provided by Sigma Aldrich-Merck (Germany). The enzyme applied for the hydrolysis was the Alcalase 2.4 L, an aqueous solution of Protease from *Bacillus licheniformis* with a declared specific activity ≥ 2.4 U/g.

2.2 Pretreatment of waste material

The frozen tuna waste material was thawed at room temperature and drained from the blood. After that, the viscera were manually separated from the bones and the skin using a sharp knife. To obtain a sample as homogeneous as possible, the viscera were blended and homogenized using a kitchen blender.

2.3 Protease characterization

The enzyme chosen for this study was Alcalase, a highly studied enzyme that has already been used to produce FPH from various fish species (Tacias-Pascacio et al., 2020). To choose the enzyme/substrate ratio and to monitor any degradation of the Alcalase during the time, an activity assay was conducted. First of all, it was necessary to measure the total amount of enzyme in the Alcalase solution, in terms of total proteins, through the Bradford assay using Bovine Serum Albumin (BSA) as standard protein (Kruger, 1994).

The Bradford assay is a protein determination method that involves binding Coomassie Brilliant Blue G-250 dye to proteins. When the dye binds to the protein, it is converted to a stable unprotonated blue form with the maximum absorbance at 595 nm.
Hence, 100 µL of diluted Alcalase was mixed with 1000 µL of the Bradford reagent into an Eppendorf tube. After 15 minutes the absorbance of the sample was measured at 565 nm through a spectrophotometer (Hach Lange DR5000). The protein amount was then determined by comparing the absorbance value with a calibration curve obtained using different concentrations of BSA (from 0.1 mg/mL to 0.01 mg/mL) mixed with the Bradford reagent. All the solutions were maintained at pH = 7 using a phosphate buffer. This assay allowed the correct choice of Alcalase dilution to apply in the activity assay.
A non-specific protease activity assay using casein as substrate (Cupp-enyard, 2008)

was chosen. The measurement of the activity was performed each day before of experiments and in order to avoid errors due to the degradation of the enzyme during time. In this assay, casein is digested by the Alcalase liberating tyrosine, which reacts with Folin & Ciocalteus reagent to produce a chromophore. This complex has a maximum absorbance in the blue region of the UV-Vis, and it is measurable with a spectrophotometer. Briefly, the diluted Alcalase was mixed with 0.65 % of casein solution and incubated at 37 °C for 10 minutes. After this time, Trichloroacetic Acid (TCA) was added to stop the enzymatic reaction, and more Alcalase was put in the solution and incubated for 30 minutes at 37 °C. Then the solution was filtered and was supplemented with sodium carbonate and Folin's reagent, mixed, and incubated again for 30 minutes at 37 °C. After the incubation time, the mixture was filtered, and the absorbance was measured at 660 nm. The µmol tyrosine equivalents released were obtained, by comparing the absorbance of the sample with a tyrosine calibration curve. Then the activity of the Acalase was calculated by the following equation (1):

$$\frac{Units}{mL\ enzyme} = \frac{\mu mol_{Teq}}{V_E \cdot t \cdot V_C} \quad (1)$$

where μmol_{Teq} are the micromoles of tyrosine equivalent obtained from the calibration curve, V_{tot} is the total volume of the assay (mL), t is the time (min), V_E is the volume of the enzyme (mL) and V_c is the volume of the cuvette (mL). It was possible to convert Units/mL in Units/mg of protein contained in the liquid enzyme by applying the following equation (2):

$$\frac{Units}{Protein\ (mg)} = \frac{\frac{Units}{Enzyme\ (mL)}}{\frac{protein\ (mg)}{Enzyme\ (mL)}} \quad (2)$$

2.4 Tuna viscera characterization

The total amount of lipids contained in the tuna viscera was determined using the Bligh and Dyer method (Bligh, E.G. and Dyer, 1959), originally developed to quantify the

total lipid in the fish muscle and later become the standard rapid method for fat determination in marine biological tissue (Bligh, E.G. and Dyer, 1959). In summary, 100 g of homogenized sample was mixed with 100 mL of chloroform and 200 mL of methanol. After that, 100 mL of chloroform and 100 mL of water were added to the mixture, agitated for 30 seconds, and then filtered. The liquid part was transferred into a graduated cylinder, where the separation between the polar and non-polar fraction of the solution occurred. The volume of chloroform was noted and the alcoholic fraction was removed using a glass pipette. The non-polar solution was evaporated through a Rotavapor (Labrota 4000, Heidolph) and the oily residue was weighed with an analytical balance. The measurement was performed in triplicate.

The total N content was first quantified through the Dumas or combustion method (Simonne, et al., 1997) utilizing an Elementar analyzer (Vario MACRO cube, Elementar). Then, the N total was multiplied for a nitrogen-to-protein conversion factor (equation 3). The factor chosen was 5.6, specific for fish protein, as suggested by Mariotti et al. 2008 (Mariotti et al., 2008) and not the standard 6.25 conversion factor, usually used for foodstuff.

$$Proteins\ \% = N \cdot 6.5 \quad (3)$$

The moisture and ash contents were determined following the "Rapporto ISTISAN 1996/34", and the total carbohydrate content was calculated by difference.

2.5 Experimental design and data modeling

To understand the effect of pH, enzyme/substrate ratio (E/S) and time on the amount of fish oil extracted and on the antioxidant activity of the fish protein hydrolysates, but also to optimize the extraction technique by minimizing and at the same time the number of experiments, a Design of Experiments (DoE) (Leardi, 2009) was put in

place. A three-factor and three-levels Central Composite Face Centered (CCF) design consisting of 15 experiments was employed, and 17 experimental runs, including three replicates at the center point, were performed. The design factors were pH (6.5, 7.5 and 8.5), E/S (0.1, 0.55 and 1) and time (30, 75 and 120 min), while the response variable was the yield of fish oil expressed in percentage (oil yield %). Based on literature information, the temperature was kept constant at 55 °C, which has been determined to be the optimal temperature for Alcalase (Li et al., 2018; Glowacz-Rozynska et al., 2016; Wang et al., 2019).

The design was created using MODDE 7 software (version 7.0.0.1), and the list of experimental runs, i.e., the experimental plan (with the original values of each factor) and matrix (coded version of the original values) are reported in Table 1. In the CCF design, the three levels are conventionally named as –1, 0, and +1, where –1 represents the lowest value of the factor range, +1 is the highest value and 0 is the central point (Taylor et al., 2021). In the presence of three factors, the experimental domain spanned by the CCF design can be represented in 3D space as a cube, like it is shown in Figure 1.

The data were analyzed using the R-based CAT (Chemometric Agile Tool, by Leardi R.) software, whose DoE modeling engine is based on the multiple linear regression (MLR) (Sergent, 1995) method. MLR is a regression method that allows modeling and inspecting each factor separately, but also in combination with one or more other factors. Its functioning provides enough flexibility to efficiently model the response under examination, while at the same time including or excluding the non-significant terms in the model equation, which can be represented as:

$$y = \beta_0 + \beta_A A + \beta_B B + \beta_C C + \beta_{AB} AB + \beta_{BC} BC + \beta_{AC} AC + \beta_{AA} AA + \beta_{BB} BB + \beta_{CC} CC \quad (4)$$

where y is the predicted response (oil yield %), ε represents the noise and I are the regression coefficients of each factor (A = pH, B = E/S and C = time), including the interaction between the factors (AB, AC and BC) and their quadratic terms (AA, BB and CC).

To better inspect the comparative LCA results (as described in Section 2.10) it was decided to study the obtained numerical values using the exploratory data analysis method principal component analysis (PCA, (Bro and Smilde, 2014). PCA is a decomposition method that allows obtaining clear representations of the samples' distributions, together with the correlation patterns among the variables by inspecting the so-called scores and loadings, respectively. In our case, the samples were the 15 experiments analyzed via LCA, and the variables were the five LCA impact categories.

2.6 Enzymatic extraction

The enzymatic extraction was conducted following the procedure of Ramakrishnan et al. (2013) with some modifications. Around 20 g of homogenized tuna viscera were put into a 50 mL falcon tube and heated at 90 °C for 10 minutes in a water bath, to deactivate any endogenous enzymes (Ramakrishnan et al., 2013). After cooling down at room temperature, a buffer was added to control the pH during the reaction in a ratio 1:1 (buffer:substrate). Three different buffers were used in different experiments of the DoE: phosphate buffer at different concentrations (pH = 6.5 and 7.5) and borate buffer (pH = 8.5). Then the mixture was thermostated at 55 °C for 30 minutes into an incubator with a shaking rate of 200 rpm (ES-20/60, Orbital Shaker- Incubator, Biosan). Different amounts of Alcalase were added to the mixture to obtain different E/S percentages (0.1, 0.55 and 1 %). The enzyme was then allowed to react for 30, 75 or 120 minutes. When the reaction time had elapsed, the falcon tube was put again into the water bath at 90 °C for ten minutes to stop the reaction. The mixture was cooled at room

temperature and centrifuged at 5000 rpm for 20 minutes with a SL 16R Centrifuge (Thermo Fisher Scientific).

2.7 Oil yields

The upper oil layer was collected using a plastic pipette and transferred into a glass sample holder. To make sure there was only fat in the sample, the oil was washed with a small amount of dichloromethane and left to evaporate under a chemical hood. The samples were then weighed on an analytical balance and the amount of oil was related to the total oil content present in the tuna viscera (see paragraph 2.4) to obtain the yield % according to the following equation (4).

$$oil\ yield\ \% = \frac{oil\ extracted\ (g)}{total\ oil\ in\ the\ sample\ (g)} \cdot 100 \qquad (5)$$

2.8 Omega-3 quantification

The omega-3 content in terms of EPA and DHA was determined according to UNI EN ISO 12966-2:2017 standard methods. Fatty acids were analyzed in the form of methyl esters (FAME) using a gas chromatograph equipped with a mass analyzer. To prepare the FA methyl esters, around 100 mg of sample was dissolved in 3 mL of methanolic KOH (0.6 M) in a falcon tube and stirred for 10 s under an N_2 flow to avoid oxidation of compounds. The solution was heated under agitation for 10 minutes at 70 °C using a water bath. After this time, and the complete dissolution of the oil, 3 mL of 5 % H_2SO_4 in methanol was added and the solution was heated at 70 °C for another 5 min. Then 2 mL of a saturated solution of NaCl and 2 mL of hexane were added, and the falcon was centrifuged at 4000 rpm for 10 minutes. The supernatant was collected, diluted 1:2, and transferred in a vial for the GC-MS analysis. The omega-3 analysis was conducted using a gas chromatograph (Agilent 7890A GC System) equipped with a ZB-FAME column (30 m x 0.25 mm ID x 20 μm; Zebron Phenomenex) and a quadrupole mass

detector (Agilent 5975 C VL MSD). Helium was used as carrier gas with a flow of 0.4 mL/min, the injection volume was 1 μL using the split mode with a split ratio of 20:1 at 240 °C. The column was initially maintained at 100 °C for 2 min and then the temperature was then increased to 240 °C at 5 °C/min and kept constant for 8 min (Brotas et al., 2020). The mass analyzer worked in electron ionization mode with ionization energy of 70 eV and in full scan mode between 50 and 600 a.m.u.. The temperatures of the source and quadrupole were maintained at 230 °C and 150 °C, respectively. Omega-3 quantification was determined through calibration curves prepared using a DHA and EPA certified standard.

2.9 Progress of protein hydrolysis

To understand the behavior of an enzyme-substrate system, at least five indicators must be considered: the substrate concentration (S), the enzyme/substrate ratio (E/S), the pH, the temperature (T) and the time (t). These indices can be substituted by the degree of hydrolysis (DH) because, as affirmed by Alder-Niessen (1982) (Alder-Niessen, 1982) in some conditions, the development of an enzymatic protein hydrolysis reaction depends only on the pH and DH. The DH is defined as the ratio of the number of peptide bonds broken by the total number of peptide bonds in the original protein (North and Zealand, 2010) and allows to obtain information on the progress of the reaction in real time. To measure the DH, the pH-stat method was employed. This method is based on the fact that, during the hydrolysis of the peptide bond, there is a pH decrease and, to maintain it constant, it is necessary to add some base. It is possible to relate the amount of added base to the DH by the following equation (6) (North and Zealand, 2010):

$$DH\% = \frac{B \cdot N_B}{\alpha \cdot m \cdot h_{tot}} \quad (6)$$

Where B is the base consumption (mL), N_B is the normality of the base, m is the mass of the protein of the sample, α is the average degree of dissociation of α-NH_2 groups in the protein of the sample and h_{tot} is the total number of peptide bonds per unit mass of protein. The working conditions chosen for this experiment were the optimal conditions obtained from the DoE experiments. The base used was NaOH 0.5 N. The term 1/α is defined as a calibration factor and depends on the temperature and pH, in these working conditions (55 °C and pH 8.5) it values 1.03 (Navarrete del Toro and García- Carreño, 2003). The term h_{tot} value depends on the source of protein, for fish, Navarrete et al. (2003) suggest using 8.6 m_{eq}/g. This experiment was conducted into a 1 Lbioreactor capable of automatically regulating the pH by a proportional integral derivative (PID) controller connected to a pH probe and a peristaltic pump. The reactor was also equipped with a heating jacket to keep a constant temperature of 55 °C, and Rushton impellers to stir the medium. The amount of base added over time was recorded by a computer. The model then was fitted to the experimental data using SigmaPlot (Version 10.0, Systat Software Inc., USA).

2.10 Life Cycle Assessment

Life Cycle Assessment (LCA) was performed with SimaPro 9.0.4 software, database Ecoinvent 3.0. The goal of LCA was to compare all the experiments of the DoE, to choose the best extraction process in terms of environmental sustainability. To be consistent with the goal of the study and with the function to which the process system was designed for, the chosen functional unit was 1g of fish oil. The adopted approach was from grave to cradle. The definition of the boundary conditions of the study includes the transported tuna waste feed as feedstock for the process, then the tuna pre-treatment, the enzymatic extraction, the thermal treatment, and last the centrifugation to recover the extracted oil. The scheme of the boundary condition is drawn in Fig 1.

According to the zero-burden assumption, it was assumed that tuna waste did not contain any credits related to the impacts produced during the previous stages of its life cycle (Buttol et al., 2007). Furthermore, another assumption was the allocation criteria. To be more precautionary and avoided double discounting, all the environmental impacts and credit were allocated on the fish oil, which was the product target, and all the by-products and co-products generated in the studied system were considered as waste.~~The functional unit (FU) was 1 g of fish oil produced.~~

The life cycle inventory (LCI) defined all inputs and outputs involved in the processes. The primary data came from the present study, the produced emissions, the consumed material, and the required energy were referred to the chosen FU. The reagents which were not present in the Ecoinvent database were computed on a stoichiometric basis. The detailed inventory is provided in the supplementary materials. The secondary data, taken from Ecoinvent 3.0 were:

- Electricity, medium voltage {Row}| market for | Alloc Rec, S
- Tap Water from natural resource

Electricity RoW (rest of the world), was chosen to avoid geolocalizing the process, not being in the scope of the analysis.

Life cycle impact assessment (LCIA) was performed with the ReCIPE Midpoint (H) method. In the present study, the analyzed impact categories were: Climate change (kg CO_2 eq), Ozone depletion (kg CFC-11 eq), Freshwater eutrophication (kg P eq), Marine eutrophication (kg N eq) and Terrestrial acidification (kg SO_2 eq) because they were the most used impact categories in the fish processing industry (Avadí and Fréon, 2013; Ruiz-Salmón et al., 2021). The results of the LCA were also analyzed through a Principal Component Analysis (PCA) performed with Matlab (R2020a).

3. Results and discussion

3.1 Protease and Tuna viscera characterization

The commercial Alcalase solution shows an activity of 5.559 ± 0.004 (Units/mg protein).

The defrosted tuna was not deprived of blood and water, so the moisture content resulted quite high (77.5 ± 0.1 %). The carbohydrate content is not revealed (NR) and also the percentage of ashes resulted very low (2.22 ± 0.1 %). The most interesting values for this study are the percentage of proteins and lipids, respectively 17.70 ± 0.20 % and 2.58 ± 0.08 %.

These results are in line with other works investigating the composition of different body parts of several tuna species (Karunarathna and Attygalle, 2009; Vlieg and Murray, n.d.). Karunarathna and Attygalle (2009) affirm that the moisture percentage of tuna viscera is around 70 %, the protein content is between 16-19 %, the lipids percentage can vary between 0.9 and 1.34 % and ash content is around 1 % while the amount of carbohydrates is negligible in all tuna species. Slight variations are possible and depend on several factors, such as the species, diet, age and other parameters of the tuna (Karunarathna and Attygalle,2009).

3.2 Enzymatic extraction and interpretation of the Design of Experiments results

After extraction and centrifugation, the samples showed a four-layers separation with the oil floating on the top, followed by a light mixture of lipids and proteins, then by the FPH and a solid residue on the bottom of the falcon. The oil was collected using a plastic pipette, to be quantified and analyzed through a GC-MS instrument. The second and bottom layers were discarded, while the FPH layer was collected for future analysis.

Table 1 reports the results of the DoE in terms of oil yield %. The amount of extracted oil varies from 15 % up to 81.4 %. ~~To model the percentage oil yield, first a full MLR regression model was computed but, after a preliminary elaboration, the model was simplified excluding the non-significant terms. More specifically, the interaction terms and the quadratic terms, to model only the statistically significant terms~~. To model the percentage oil yield a full MLR regression model was first computed and after a preliminary elaboration, the model was simplified excluding the non-significant terms, i.e., the interaction and quadratic terms. Only the statistically significant terms were included, as described by equation 7. ~~The model equation was therefore reduced to the following (equation 7):~~

$$y = \beta_0 + \beta_{pH} pH + \beta_{E/S} E/S + \beta_t t + \varepsilon \quad (7)$$

The significant linear terms are reported in the plot of the coefficients (Figure 3a) and in Table 2, where the presence of * indicates the significance of the coefficients: ** = $p < 0.01$, *** = $p < 0.001$.

Time was the factor with the lowest influence on the extraction yield. The positive values of all coefficients indicate that they positively influence the response, i.e., the oil yield % increases with the increase of pH, E/S and time. These results are confirmed by the response surfaces reported in Figures 3b,c,d. These surfaces represent a three-dimensional view of the relationship between pairs of factors (reported on the x and y axes) and the response (plotted on the z axis), providing a clear picture of how the response is related to the factors.

In this model the response surface takes the shape of a tilted plane, where the yield increases when the factors increase. The flat look of the surface is due to the fact that all quadratic and interaction terms were excluded from the MLR model, therefore no terms describing possible curvatures of the response were included. According to the model, it

is possible to obtain the maximum oil yield % at maximum pH, E/S and time values (pH = 8.5; E/S % = 1; t = 120 min).

The influence of pH is one of the most important parameters to be analysed in enzymatic hydrolysis. Indeed, enzymes contain a catalytic active site with charger amino acids. The pH can affect the dissociation state of these amino acids changing the ionic bonds that maintain the three-dimensional shape of the protein. This may lead to alteration in protein function or inactivation of enzymes (Shu et al., 2016).

Alcalase was initially obtained from Bacillus subtilis and called "Subtilisin Carlsberg" (Tacias-Pascacio et al., 2020). Subtilisin, generally, work optimally in mildly alkaline. This kind of enzymes are serine proteases that contain catalytic triadin order of aspartic acid, histidine, and serine residues (Azrin et al., 2022) and in particular, Ser221, His64 and Asp32 (Wells and Estell 1988). The activity of subtilisin increases at alcaline pH because His64 is deprotonated. Therefore, by increasing the pH there is a greater activity of the enzyme which hydrolyzes more proteins, releasing larger quantities of oil and increasing the yield.

Results show a positive effect of the enzyme: substrate ratio on the enzymatich hydrolysis, the increase of the enzyme concentration resulted an higher oil yield.

At higher E/S, indeed, there are more enzyme active sites available to hydrolyze the substrate, resulting in a more effective cleavage of the peptide bonds, and consequently greater degradation of the proteins (Kurozawa et al, 2009).

To the best of the authors' knowledge, this is the first study regarding the enzymatic extraction of tuna oil using Alcalase applying DoE. These results are in line with Ramakrishnan et al. (2013), who investigated the effects of E/S and time, separately, on the oil extraction from mackerel waste material using Alcalase at 55 °C and pH = 8.5 (Ramakrishnan et al., 2013). They demonstrated that oil yield increases with the increase in the enzyme concentration (0.5, 1 and 2 %) and time (1, 2, 3 and 4 h),

reaching a plateau around an hour and a half of elapsed time. Qi-yuan et al. (2016) extracted oil from mackerel viscera applying a DoE to investigate how the extraction is affected by enzyme concentration, pH and temperature (Qi-Yuan et al., 2016). Their work confirms that as the E/S ratio increases, the oil yield also increases. In their study, De Oliveira et al. (2017) extracted oil from tuna by-product through enzymatic extraction using Alcalase and they affirmed that this technique allows obtaining an oil of better quality than other obtained with non-green extraction techniques (De Oliveira et al., 2017). However, there are very few literature studies in which oil is extracted from tuna waste, while there are many on enzymatic extractions from other fishes. Wang et al. (2019) affirmed that Alcalase (at 55 °C and pH = 8) could be used as a good protease for extracting a good quality Antarctic krill oil (Wang et al., 2019). The present work results are also in line with Głowacz-Rozynska et al. (2016) who tried to extract the oil from salmon waste, showing that it is possible to achieve yields of around 82 % using Alcalase at 55 °C and pH = 8 (Glowacz-Rozynska et al., 2016). Other authors effectively extracted good quality oil from fish waste such as catla, rohu, perch, copepod, tilapia or trout, using Alcalase (Babajafari et al., 2017; Li et al., 2018; Taylor et al., 2013; Vlieg and Murray, n.d.). Araujo et al. (2021) treated fish scraps from a market with Alacase al pH = 8, 50 °C and 180 min and deduced that the E/S ratio was the main factor controlling the hydrolysis (Araujo et al., 2021).

To evaluate the prediction power of the MLR model, two replicated experiments of the central point of the design (not included to compute the model) were performed. Table 3 reports the experimental values of the two experiments, the upper and lower values with their predicted values, and their residuals. The predictive capacity of the model is hence confirmed.

3.3 Progress of protein hydrolysis

To monitor the progress of the hydrolysis, the DH% as a function of time was measured using the pH-stat method in a 1 L Bioreactor, applying the optimum condition found with the DoE (55 °C, pH = 8.5 E/S = 1 %). As shown in the graph in Figure 4 DH% increases very quickly in the first hour of reaction and then stabilizes around 22 %. The experimental sets of (DH%, t) were fit to Eq. (9) using Sigma Plot and the results of the fitting procedure are listed in Figure 3 (Babajafari et al., 2017; Taylor et al., 2013). The squared points of Figure 3 represent the experimental values with their standard deviation and the continuous line the values obtained from the Equation 8. To evaluate the quality of the fitting, the correlation coefficient R, and the coefficient of determination R^2 were evaluated. These values, very close to 1, indicate that the regression predictions fit the data almost perfectly. The model passed also the Shapiro-Wilk test and the constant variance test with a significance level of 99 %.

$$DH\% = \frac{a + bt}{1 + ct} \tag{8}$$

These results are consistent with the work of Saidi et al. (2013), which hydrolyzed tuna by-products at the exact conditions of the present work and demonstrated that progress of hydrolysis increased roughly during the first hour and then no significant variation can be observed (Saidi et al., 2013). Ovissipour et al. (2010) obtained a trend in good agreement with our results, but the DH% stabilized around 16 %, probably because they did not work at controlled pH (Ovissipour et al., 2010). These results are also confirmed by Guerard et al. (2001) (Guérard et al., 2001). Klomklao and Benjakul (2016) obtained a curve with the same shape as the one in Figure 4, but their curve reached the plateau at an earlier time (20 min) and at higher DH% (35 %) (Klomklao and Benjakul, 2016). This is possibly caused by the fact that they used more severe T, pH and E/S conditions for the reaction compared to our experimental conditions. Valencia et al. (2014)

identified the mechanisms responsible for the shape of the Alcalase hydrolysis curve for fish proteins (Valencia et al., 2014). They demonstrated that these curves are characterized by an initial "hydrolysis phase" followed by a slowdown and stabilization of the degree of hydrolysis. The cause for this decrease in reaction rate could be associated with several factors, such as: a decrease in the concentration of peptide bonds of the substrate available for hydrolysis; a product inhibition caused by amino acids and soluble peptides released during the hydrolysis that act as an substrate competitor; and an enzyme deactivation (Klomklao and Benjakul, 2016; (Kurozawa et al, 2009).

3.4 Omega-3 quantification

The oil fraction was analyzed through GC-MS to quantify the omega-3 FA in terms of EPA and DHA; they represent 2,49 ± 0,32 % and 10,83 ± 1,13 % of the extracted oil. The percentages obtained in this work are lower than percentages achieved by De Oilivera et al. (2017). They extracted oil from tuna heads with Alcalase but applied different experimental conditions and reached 6 % of EPA and 27 % of DHA (De Oliveira et al., 2017). In general, the content of EPA and DHA in fish oil varies respectively from 5 to 26 %, and from 6 to 26 % of the total fatty acid, depending on fish species (Alkio et al., 2000). From the data found in the literature, tuna oil contains about 5 % of EPA and 25 % of DHA (Halldorsson et al., 2003; Zhang et al., 2017). In their work, Alkio et al. (2000) used tuna oil containing 4.6 % of EPA and 18.3 % of DHA, so these amounts are very variable (Alkio et al., 2000). Besides the study by De Oilivera et al. (2017), the tuna oil analyzed in these research works is never made from the tuna viscera, and the percentage composition of fatty acids can be very different. Moreover, even the extraction procedure is not consistent across the different studies, making the literature data hardly comparable to each other. In any case, the percentage

of omega-3 found in the oil extracted in this study makes it a product with excellent qualities. As a matter of fact, EPA and DHA contribute to preventing some cardiovascular and various inflammatory diseases such as hyperlipidemia, atherosclerosis, inflammation, and cancer. Moreover, they present some beneficial effects on the brain and nervous system (Alkio et al., 2000; Correa-Matos and Vaghefi, 2013; De Oliveira et al., 2017; Halldorsson et al., 2003; Zhang et al., 2017).

3.5 Life Cycle Assessment

The comparison of the environmental sustainability of the different extractions defined by the DoE was analyzed through Life Cycle Assessment (LCA).

Figure 4 shows the results of LCA among the 15 experiments of the DoE (the replicas of the central point were not considered) ~~in terms of~~ through five impact categories: Climate change (a, kg CO_2 eq), Ozone depletion (b, kg CFC-11 eq), Terrestrial acidification (c, kg SO_2 eq), Freshwater eutrophication (d, kg P eq) and Marine eutrophication (e, kg N eq). The analysis was limited to these five categories because, as indicated by Ruiz-Salmón et al. (2021), they are the most studied ones in LCA of fish and seafood processed products and, therefore, it would make the present study more comparable to what can be already found in the literature. By reviewing a considerable number of works on this topic, Ruiz-Salmón et al. (2021), highlighted that Ozone depletion was computed by 53 % of analyzed authors, followed by acidification (64 %) and eutrophication (66 %) (Ruiz-Salmón et al., 2021). However the study of Ruiz-Salmón et al. (2021) focused on the impact of landed fish, fish distribution and the process to make fish an edible product.

The aim of the selected five impact categories is following defined. Climate change is the indicator of the global warming potential (GWP) caused by the emissions of greenhouse gasses into the atmosphere. Ozone depletion indicates the emissions to the

atmosphere responsible for destroying the stratospheric ozone layer. Acidification is the indicator of the potential acidification of soils and water due to the release of gasses such as nitrogen oxides and sulfur oxides. The freshwater eutrophication indicator expresses the freshwater ecosystem's enrichment with nutritional elements attributable to the emission of compounds phosphor. Marine eutrophication is the indicator of enhancing the marine ecosystem with nutritive elements due to nitrogen-containing compounds (Jessen et al., 2015). To the best of the author's knowledge, ~~there are still no LCA studies on the enzymatic extraction of oil from tuna waste, so our discussion will be limited to the results obtained in this work.~~ in the literature, there are no available studies to compare the environmental impacts and credits of the process and substrate investigated in the present paper. For this reason, in the present work, the discussion of LCA results was done as a comparison between the 15 investigated configurations of the DoE.

Figure 5 reported the results of the 15 configurations for each impact category with the absolute value, where the contribution of each process step was referred as a percentage of the absolute value. Moreover, to underline the contribution of energy items in the total impact, it was highlighted in each process step in which it was required: pretreatment, enzymatic extraction phases, disactivation unit and oil recovery through centrifuge equipment.

Figure 5 proved that for all the tested configurations, the main contribution to the total environmental impact among the five investigated impact categories was the consumed energy and in particular the energy required for the enzymatic extraction and centrifugation phases.

As shown in Figure 5a-e, experiment n° 4 presented the lower contributions in all the impact categories analyzed in this work. This experiment was conducted at pH of 8.5 with an E/S of 1 % and for 30 mins. It is important to notice that, according to the DoE,

this experiment does not correspond to the highest obtainable oil yield but with the same quantity of oil produced, it is the most sustainable combination of experimental conditions, from an environmental point of view. By analyzing Figure 5, it is possible to note ~~notice~~ that all the experiments with a reaction time of 30 minutes, which correspond to the lowest level of the time factor analyzed in the DoE, had minor impacts. It could be deduced that the duration of the reaction greatly influences the sustainability of this process. Indeed, for the climate change category, the experiments with the longest enzyme extraction step (120 min) reached the highest energy contribution in that phase, which ranged between 30-52 % of the total impact in all the considered impact categories.

Furthermore, in all the considered impacts categories, the recovery of the oil is one of the highest energy consumer items, hence the present study underlined the importance of optimizing the downstream technology to recover the product target.

Another attentioned parameter in the present study of sustainability is the role of the enzyme. In the marine eutrophication the effect of the enzyme was highlighted, and it reached the highest contribution in the experiment in which the dose of the Alcalase was the highest one (1 mL) and the yield of fish oil was not the optimal one compared to the amount of tuna treated (0,2 g/ g of tuna).

Among the investigated configurations, the one, reaching the highest impact of enzyme in marine eutrophication, was the experiment n°3 with a value equal to 72 % of the total enzymatic pretreatments (made up by the sum of the enzyme contribution and energy required to carry out the enzymatic extraction phase).

Among the five impact categories, the pretreatment unit, consisting in pH regulation with the addition of a buffer, had environmental impacts detectable in terrestrial acidification and freshwater eutrophication due to the adopted chemicals. Specifically, the configurations n° 1, 2 and 6 exhibited pretreatment unit impact in terrestrial

acidification and freshwater eutrophication ranging between 6,5-8 and 6-8,10 % of the total environmental impacts in that categories.

The transport of tuna exhibited a considerable impact in the ozone depletion category due to the necessity of transporting the feedstock with a refrigerated lorry for a distance of 1524 km (from the south of Italy to the north of Italy plant) in accordance with the study of (Khoo, et al 2019). The contribution of tuna transport in ozone depletion decreased by decreasing the amount of tuna, because according to the chosen FU (1g of fish oil), it means that the process is high performing.

According to the study of (Sillero et al,2021) about LCA of biorefinery systems in general and not specific for substrate or type of process, the enzyme application impacts on marine eutrophication due to its origin, whereas pretreatment impacts on terrestrial acidification and freshwater eutrophication due to the use of chemical and their post consume, whereas the product recovery exhibits great impacts on climate changes and ozone depletion due to the energy requirement.

3.6 Evaluation of LCA results through PCA

To better compare the LCA results, it was decided to organize the values reported in Figure 4 into a data matrix of dimensions 15 × 5 (one column for each impact category) and to analyze it using PCA. Prior to analysis, the data table was scaled to unit variance and mean centered. The PCA results are reported in Figure 6. As already highlighted in Figure 5, Climate change (a), Ozone depletion (b), Terrestrial acidification (c) and Freshwater eutrophication (d) share the same overall trend, while Marine eutrophication (e) shows a rather different pattern. This aspect is confirmed by the PC1-PC2 loadings plot (Figure 6a), as all impact categories appear to be highly correlated, since they are located at positive PC1 values. PC2 accounts for the difference between Marine eutrophication and the other four impact categories. Regarding the DoE experiments'

distribution in the scores plot of Figure 6b, it can be noticed a clear trend related to the extraction time (blue arrow and coloring). Moreover, from this plot it becomes clear that experiment n°4 has the lowest impact in all categories, while experiment n°7 has the largest impact in all categories.

The pattern of correlations among the impact categories described by PC2-PC3 (Figure 6a,b) seems to be related to the response, as the experiments' distribution colored according to the oil yield % follow a rather clear trend (yellow arrow). The experiments with the highest response also have higher impacts in the Climate change and Terrestrial acidification categories, while the same experiments show much lower impacts in the Ozone depletion and Marine eutrophication categories.

~~In order to better understand the reason for this statement, it is necessary to analyze the so-called Sankey Diagram (Figure 6), whose concept was developed by Riall Sankey (Jessen et al., 2015) with the aim of analyzing the thermal efficiency of steam engines. These diagrams are now extensively used to depict quantitative flows in several areas of application. In this work, the Sankey Diagrams were used to visualize the contribution of single steps to the total impact of the process. Figure 6b-f reports the Sankey Diagrams of the five impact categories analyzed for experiment n°4. Each box represents one component of the process, as described in figure 6a where the boundary conditions are illustrated: "1" is the pretreatment step, "2" is the extraction step, "3" is the deactivation step, "4" is the centrifugation step, "Electricity" is the electricity needed to operate the equipment and "Enzyme" is the Alcalase used for the hydrolysis These boxes are connected in order by arrows whose size is proportional to the normalized impact of that component. The centrifugation step (4) is the last one and it contains the impacts of all the previous steps; therefore, it represents 100 % of the impact (Jessen et al., 2015). Components that contribute less than 1 % of the total have not been shown in the graph. As shown in Figure 6, almost all impact is caused by~~

~~electricity in all four categories.~~ Considering the results of the LCA expressed in figure 5 and of the PCA represented in figure 6, the following considerations can be made. Almost all impact is caused by electricity in all four categories. In this LCA study, non-geolocalized medium voltage electricity was used, referring to the whole world (Simapro). This electricity represents an average of all types of electricity used globally, from renewable and non-renewable sources (coal, oil, gas, biomass, nuclear, hydro, wind, geothermal, solar marine) (Schmidt et al., 2011). The production of this electricity causes emissions of greenhouse gasses, nitrogen oxides, and sulfur oxides, substances responsible for the depletion of the ozone layer and compounds rich in phosphorus and nitrogen, damaging the various ecosystems (Kalender and Alkan, 2019). For this reason, the DoE experiments with the shortest time are also those with the lowest environmental impact.

The only impact category where the enzyme also contributes in large quantities to the total is Marine eutrophication. This LCA study assumed that the Alcalase is produced from potato starch along with a bacterial strain, as suggested by Rosa et al. (2020) (Rosa et al., 2020). It is possible that the cultivation of potatoes and the use of fertilizers greatly influence this impact category, increasing the emission of substances rich in nitrogen into rivers or coastal areas or the nitrogen applications to the soil

These results are in line with the results obtained from the DoE. Indeed, the best experimental configuration is the one that uses a buffer at pH 8.5 and an E/S of 1 %. The duration of the process seems to be most responsible for the environmental impact, but time seems to be the least significant factor among the variables analyzed using the DoE. Moreover, the study of hydrolysis kinetics has shown that the DH% stabilizes around a plateau after one hour. Therefore, it would be possible to reduce reaction times by reducing the environmental impacts without losing much in oil yield. Another way to reduce the contribution of electricity could be to geolocate the process and use

renewable resources. For what it concerns the contribution due to the use of the enzyme, new scenarios could be envisaged, changing the type of enzyme or its way of production.

4. Conclusions

~~The enzymatic hydrolysis of tuna waste represents an innovative technology to exploit canned tuna processing wastes. This study has shown that it is possible to obtain good quality tuna oil rich in omega-3 fatty acids through enzymatic hydrolysis of tuna viscera with Alcalase. For the first time, LCA and PCA analysis, combined with a DoE approach, demonstrated that the duration of the hydrolysis and the associated electricity consumption is the variable that affects the most the environmental sustainability of the process.~~

The enzymatic hydrolysis of tuna waste represents an innovative technology to exploit canned tuna processing wastes to obtain products with high added value, such as tuna oil and FPH. This study has shown that it is possible to obtain good quality tuna oil rich in omega-3 fatty acids through enzymatic hydrolysis of tuna viscera derived by a canned tuna production company with commercial enzyme Alcalase.

The DoE made it possible to optimize oil extraction and identify the best pH (8.5 °C), E/S (1%), and time (120 minutes). It was possible to obtain a model able to compute the yield as a function of the three-factor analyzed. The study of the progress of the DH% over time showed that after one hour, the reaction slows down. The experiments of the DoE were compared to each other through an LCA analysis, and the results were investigated with a PCA. This innovative approach allowed to demonstrate that the duration of the hydrolysis and the associated electricity consumption is the variable that most affects the environmental sustainability of the process.

Further studies would be needed to reduce the time of hydrolysis, analyze in more depth the FPH obtained and its proprieties and design a possible scale-up in order to evaluate the technical, economic and environmental feasibility of this process at an industrial level.

Acknowledgments

We thank "Ditta Drago Sebastiano di Giuseppe Drago" (Siracusa) for sending the tuna waste materials from their production chain.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Figure Captions:

Figure 1. A representation of the three-factors, three-levels, Central Composite Face Centered (CCF) experimental domain. To model and explain the results of the DoE, a multiple linear regression (MLR) was applied, as described in Section 2.5.

Figure 2. Scheme of the LCA boundary conditions. For each process, input and output of matter and energy are illustrated.

Figure ~~2~~ 3. (a) The coefficients plot of the MLR model (**= $p < 0.01$, *** = $p < 0.001$) and the response surfaces for oil yield %: (b) interaction between E/S and pH, (c) interaction between time and pH, (d) interaction between time and E/S.

Figure ~~3~~ 4. Enzymatic extraction progress curve and results of the fitting procedure Black squares represent the experimental values. Each experiment was conducted in triplicate, and the error bars correspond to standard error. The continuous line represents the modeled values (eq. 9). a, b and c are the coefficients of equation 9. R and R^2 represent the quality of the fitting.

Figure ~~4~~ 5 . Results of comparative LCA between the 15 experiments of the DoE in terms of five impact categories: (a) Climate change (kg CO_2 eq), (b) Ozone depletion (kg CFC-11 eq), (c) Terrestrial acidification (kg SO_2 eq), (d) Freshwater eutrophication (kg P eq) and (e) Marine eutrophication (kg N eq).

Figure ~~5~~ 6. PCA scores and loadings plots of the comparative LCA results. Only the most informative combinations of principal components are reported: PC1-PC2

loadings (a) and scores (b) plots; PC2-PC3 loadings (c) and scores (d) plots. The coloring in (b) corresponds to the extraction time, while in (d) it corresponds to the oil yield % (the response of the DoE). The coloring range values can be interpreted using the color bars on the right-hand side.

Tables:

Table 1 The experimental plan (i.e., the factors described with their original values) and matrix (i.e., the coded version of the factors' values) for tuna oil extraction optimization, whose yield values are reported in the last column on the right.

Experiment N°	pH (coded)	E/S (coded)	time (coded)	pH (original)	E/S (%) (original)	time (min) (original)	oil yield % (response)
1	–1	–1	–1	6.5	0.1	30	15.0
2	1	–1	–1	8.5	0.1	30	31.7
3	–1	1	–1	6.5	1	30	25.0
4	1	1	–1	8.5	1	30	49.3
5	–1	–1	1	6.5	0.1	120	24.1
6	1	–1	1	8.5	0.1	120	33.5
7	–1	1	1	6.5	1	120	56.0
8	1	1	1	8.5	1	120	81.4
9	–1	0	0	6.5	0.55	75	21.9
10	1	0	0	8.5	0.55	75	76.6
11	0	–1	0	7.5	0.1	75	27.7
12	0	1	0	7.5	1	75	47.0
13	0	0	–1	7.5	0.55	30	31.3
14	0	0	1	7.5	0.55	120	43.0
15	0	0	0	7.5	0.55	75	34.8
16	0	0	0	7.5	0.55	75	38.7
17	0	0	0	7.5	0.55	75	42.5

Table 2. Coefficients of the MLR model and their significance with the explained variance of the data. *= p < 0,05 **= p < 0.01, *** = p < 0.001

Coefficients				Explained Variance %
β_0	β_{pH}	$\beta_{E/S}$	β_t	
39.968	13.043	12.665	6.575	71.58
0.0000***	0.00009***	0.0011**	0.0148*	

Table 3. Prediction results of experiments 18 and 19.

Exp N°	Experimental value	Lower	Predicted value	Upper	Residuals
18	24.16	34.90	39.97	45.04	–2.20
19	39.25	34.90	39.97	45.04	0.71

Figures:

Figure 1.

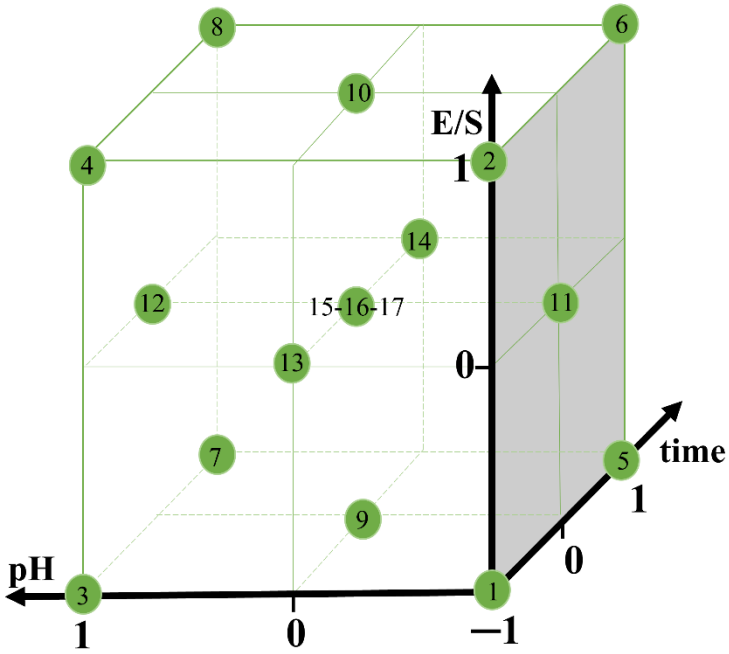


Figure 2.

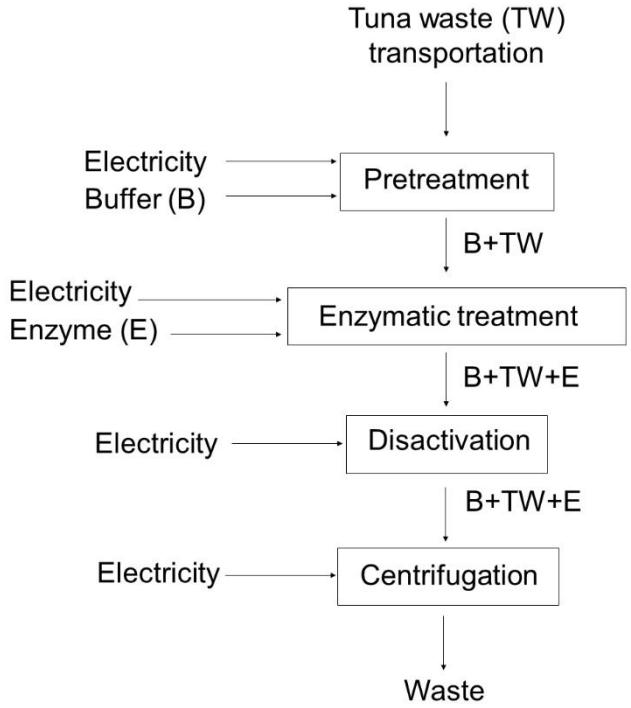

Tuna waste (TW)
transportation
Electricity
Buffer (B)
Pretreatment
B+TW
Electricity
Enzyme (E)
Enzymatic treatment
B+TW+E
Electricity
Disactivation
B+TW+E
Electricity
Centrifugation
Waste

Figure ~~2.~~ 3.

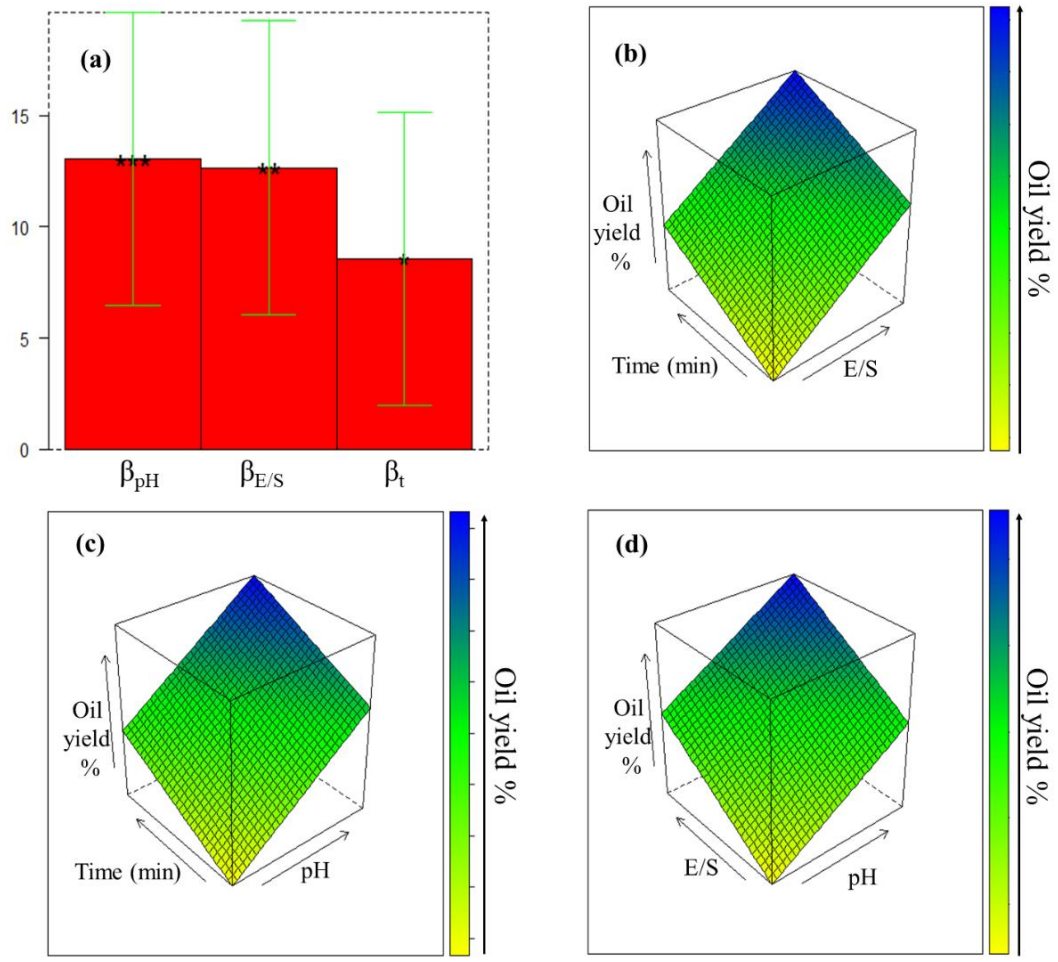


Figure ~~3~~ 4.

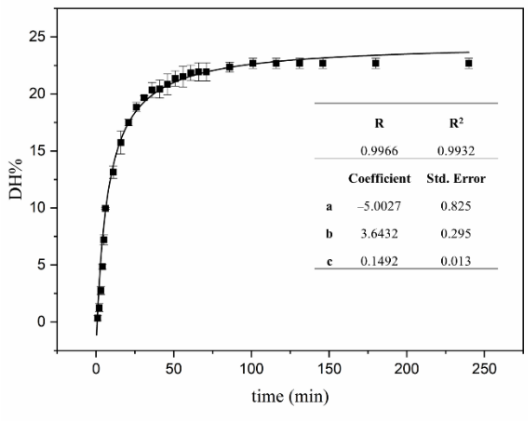


Figure-4 5.

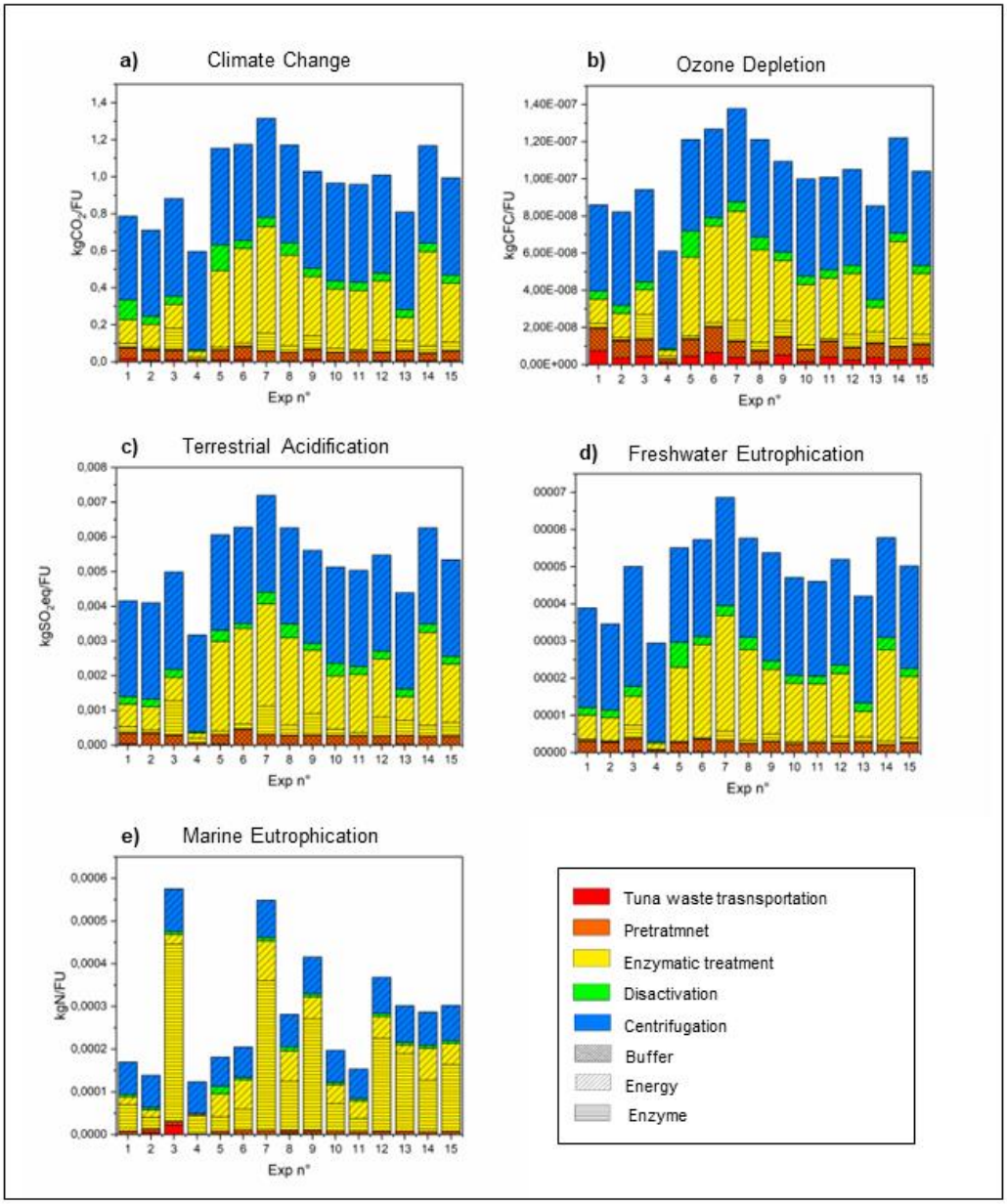
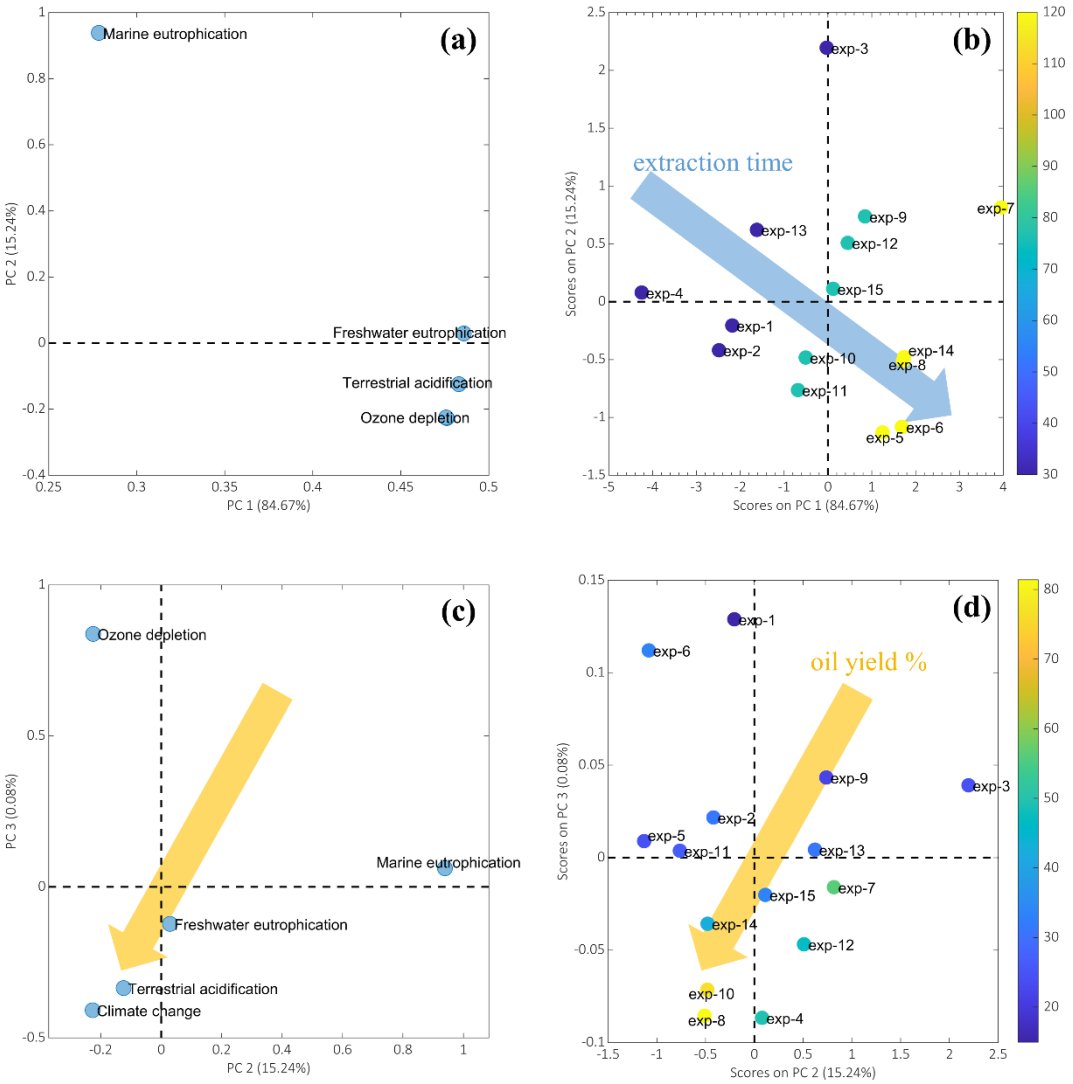

a) Climate Change
kgCO2/FU
Exp n°
b) Ozone Depletion
kgCFC/FU
Exp n°
c) Terrestrial Acidification
kgSO2eq/FU
Exp n°
d) Freshwater Eutrophication
Exp n°
e) Marine Eutrophication
kgN/FU
Exp n°
Tuna waste trasnsportation
Pretratmnet
Enzymatic treatment
Disactivation
Centrifugation
Buffer
Energy
Enzyme

Figure ~~5~~ 6.


(a)
Marine eutrophication
Freshwater eutrophication
Terrestrial acidification
Ozone depletion
PC 2 (15.24%)
PC 1 (84.67%)
(b)
extraction time
exp-3
exp-9
exp-7
exp-13
exp-12
exp-15
exp-4
exp-1
exp-2
exp-10
exp-14
exp-8
exp-11
exp-6
exp-5
Scores on PC 2 (15.24%)
Scores on PC 1 (84.67%)
(c)
Ozone depletion
Marine eutrophication
Freshwater eutrophication
Terrestrial acidification
Climate change
PC 3 (0.08%)
PC 2 (15.24%)
(d)
oil yield %
exp-1
exp-6
exp-9
exp-3
exp-2
exp-5
exp-11
exp-13
exp-15
exp-7
exp-14
exp-12
exp-10
exp-8
exp-4
Scores on PC 3 (0.08%)
Scores on PC 2 (15.24%)

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

04/09/2022