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Experimental study and sustainability assessment for fertilizers recovery from seafood waste

Supervisor

Prof. Ing. **Francesco Fatone**

Student

Claudia Pepi

Co-supervisors

Prof. Ing. **Anna Laura Eusebi**

Ing. **Josué Gonzalez Camejo**

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ABSTRACT

The increasing consumption of fish has led to an expansion of fish processing industry which in turn has resulted in increasing quantities of by-products, which may represent up to the 70% of processed fish and cause important management problems due to their susceptibility to rapid degradation. On the other hand, seafood non-edible residues contain a considerable number of biomolecules (proteins, polysaccharides etc.), and they can be used as starting products for the extraction of value-added chemicals, like nitrogen-derived compounds, representing a good opportunity to mitigate the environmental problems associated with their disposal.

In this context, under the SEA2LAND project, it has been studied the possibility to recover nutrients from mollusks and fish wastes, collected respectively from the local companies Co.Pe.Mo. and Ittica del Conero, in the form of protein hydrolysates (PHs) to be used as biostimulants for plants. In particular, the process of enzymatic hydrolysis as method of production has been experimentally studied at lab scale, taking into account the requirements provided by the reference legislation. Once the best conditions have been evaluated, the Life Cycle Assessment (LCA) methodology has been applied to determine the energy use and the potential environmental impacts associated with the generation of this kind of product in a hypothetical pilot-scale plant. In addition, an economic assessment has been also performed to determine the potential economic revenue, therefore the real convenience of this kind of industrial pathway.

With the aim of optimizing the process also from the point of view of environmental and economic efficiency, some operating conditions, although not the most optimal ones, have been preferred over others. For what concerns the environmental assessment, it has been found that the production of mollusks and/or fish based PHs has as most important impacts the fossil fuel depletion and the climate change, mostly related to the use of electricity and thermal energy during hydrolysis and final concentration. The impact on climate change resulted to be much lower in comparison with chemically produced PH from leather wastes. The solution studied seems to be also highly economically efficient, although uncertainties relating to investment costs should be taken into account. Finally, it can be concluded that mollusks are the best choice in terms of waste valorization, due to the lower environmental impact and, at the same time, very high the economic profit; however, for protein hydrolysate production it seems preferable to use the mix (fish plus mollusks), characterized by a medium environmental impact, and a profit closer to that of the mollusks.

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CHAPTER 1: INTRODUCTION

1.1 Statement of the problem

Global fish production is estimated to have reached about 179 million tons (Mt) in 2018 and is projected to reach 200 Mt by 2029 (OECD/FAO, 2020). Of the overall total, in 2018, 156 million tons were used for human consumption, equivalent to an estimated annual supply of 20.5 kg per capita per year, while the remaining 23 million tons were destined for non-food uses, mainly to produce fishmeal and fish oil (FAO, 2020).

The marine seafood includes finfish (pollock, tuna, herring, mackerel, whiting, and others), crustaceans such as shrimp, krill, crab, and lobster, bivalves including mussels, oysters, clams, and scallops, cephalopods such as squid and cuttlefish. The seafood industry comprises both capture fisheries and aquaculture (Venugopal & Sasidharan, 2021). Aquaculture production is projected to reach 105 Mt by 2029, 10 Mt more than the capture sector (OECD/FAO, 2020). Today aquaculture is by far the dominant source of bivalve mollusks: in 2018, shelled mollusks (17.3 Mt) represented 56.3% of the production of marine and coastal aquaculture, finfish (7.3 Mt) and crustaceans (5.7 Mt) taken together were responsible for 42.5%, while the rest consisted of other aquatic animals (FAO, 2020).

The increasing consumption of fish has led to an expansion of fish processing industry which in turn has resulted in increasing quantities of by-products, which may represent up to 70% of processed fish. These by-products are usually composed of heads (which represent 9-12% of total fish weight), viscera (12-18%), skin (1-3%), bones (9-15%) and scales (about 5%) (FAO, 2020). In the case of mollusks waste it includes mollusks not suitable for the market, since they are undersized, fouled with barnacles, broken shells or unwanted species.

The increase of fish by-products causes important management problems, since fish wastes generate an immediate need for disposal, due to their susceptibility to rapid degradation, which call for economic and logistic decisions. In fact, fishery wastes, unlike municipal wastes, have more proteins and other nitrogenous materials, responsible for rapid putrefaction (Venugopal & Sasidharan, 2021). The choice of a particular waste management option strongly depends on both the chemical composition and the mechanical properties of a datum residue. Typically, such biowastes are processed by paying a disposal cost to a third-party or specific waste management company.

The inadequate management of fish processing waste or by-products is one of the major problems that fish industry has to face nowadays. Traditionally, the practice of disposal of solid wastes encompasses dumping into land fill, its use in silage, fishmeal, and fertilizer (without adequate treatment) or as component of aqua- and poultry feeds (Venugopal & Sasidharan, 2021). These large quantities of fish by-product waste from fisheries would create serious pollution and disposal problems (Chalamaiah et al., 2012). For instance, the anaerobic decomposition of organic matter contained into fish leads to break down of proteins and other nitrogenous compounds, releasing carbon dioxide, methane, amines, diamines, ammonia (NH_3) and hydrogen sulfide (H_2S). In addition, there is an environmental concern associated with disposal of fish wastes into ocean waters, including reduced oxygen levels in the seawaters at the ocean bottom; burial or smothering of living organisms; and introduction of disease or non-native and invasive species to the ecosystem of the sea floor (Venugopal & Sasidharan, 2021).

However, seafood non-edible residues contain a considerable number of biomolecules such as proteins, polysaccharides, lipids, carotenoids, vitamins, minerals, and so on. Recovering these biomolecules can be an important way to improve global food security and mitigate environmental problems associated with seafood by-products/discards (Bruno et al., 2019). In this sense, biotechnological processes offer an economic and versatile way to concentrate and transform resources from waste/wastewater into valuable products. The sustainable processing of biomass (food, feed, chemicals, materials) and bioenergy into a spectrum of marketable products and energy is called “biorefining” (Venugopal & Sasidharan, 2021). Considering fish and mollusk wastes as important sources of biomaterials and converting it into an economic opportunity for biorefineries and similar industries, represents a possible solution to overcome the problem of fish waste management and is in line with circular economy principles.

It is now estimated that up to 25-35% of the total volume of fishmeal and fish oil is produced using these by-products, but regional differences exist (FAO, 2020). A more sustainable option is their use as starting products for the extraction of value-added chemicals (Nisticò, 2017). For instance, from seafood by-products and discards it is possible to recover nitrogen-derived compounds (muscle protein, collagen/gelatin, protein hydrolysate), seafood oil, chitin and chitosan, natural pigments, and minerals, all with multiple applications in pharmaceutical, cosmetics, and food (Bruno et al., 2019).

The valorization of seafood by-products in terms of value-added chemicals is the main goal of this work. As it will be deepened later, this will be achieved with the extraction of protein hydrolysate from the organic fraction of seafood by-products. In addition, it will be possible to address waste disposal issues caused by the slow natural degradation rate of their shells. For example, the shells of

bivalves can be turned into calcium carbonate or calcium oxide, two highly versatile chemical compounds with wide industrial applications (FAO, 2020).

1.2 Context of the study

The work carried out in this master thesis is part of the Project "SEA2LAND - Producing advanced bio-based fertilizers from fisheries wastes", a 4-year collaborative Innovation Action (IA) funded by the EU in the frame of the Horizon 2020 program. The general objective of the project is to provide solutions to help overcome future challenges related to food production, climate change and waste reuse. Based on the circular economy model, this project aims to meet this challenge by improving and adapting technologies for nutrient recovery to produce bio-based fertilizers (BBFs) and Tailor-Made fertilizers (TMFs) from fishery and aquaculture by-products generated in Europe. In this way it contributes to independence and security in the supply of nutrients to European agricultural systems, mitigating the existing nutrient imbalance in Europe. In the long term, this will encourage the production at large-scale of fertilizers in the EU from non-imported organic or secondary raw materials, on the side of the circular economy model, transforming by-products into nutrients for crops (https://sea2landproject.eu/).

The importance of this expected impact is clearer by knowing that the world's horticultural systems face a great balancing act between two needs: (1) raise the supply of food produced on the available farmland, since the global population will increase to more than 9.3 billion by 2050, and (2) reduce agriculture's impact on the environment and human health. Meeting these two targets presents a major sustainability challenge to scientists and producers (Colla et al., 2015). Therefore, with the rising demand to meet the nutritional requirements of a growing population and widespread consumer awareness of environmentally friendly agricultural practices as well as strict regulations on the use of chemical fertilizers, there is an urgent need to find alternative methods of sustainable horticultural production through technical and technological innovations. The use of plant biostimulants from fish by-products can improve nutrient uptake, nutritional efficiency, plant yields and the quality of products and is a promising alternative to conventional chemical fertilizer use (Madende & Hayes, 2020).

1.2.1 Adriatic Sea Pilot Case in SEA2LAND project

The project proposes the implementation of 9 technologies in 7 demonstration pilots in 6 areas representative of the fisheries sector (North, Baltic, Atlantic, Cantabrian, Mediterranean and

Adriatic). The role of UNIVPM in the Adriatic Sea Pilot Case is to develop a biorefinery fed by wastes from mollusk and fish industries. The goal of this refinery is to obtain protein hydrolysates for plant bio-stimulation, nitrogenous fertilizer (chitin), biochar-compost composite, and soil liming agent. From previous characterizations, as it will be described in Chapter 4, it was found that wastes from mollusks consist of around 20% of organic material (residual meat) and 80% of shells. Shells mainly are composed of inorganic material, and they are rich in $CaCO_3$ that will be used as a liming agent. The organic part, together with the fish wastes, is rich in protein (more than 50% on dry basis) and will be used to obtain:

1) protein hydrolysates through enzymatic extraction;
2) biochar-compost composite from the composting of solid residue of the hydrolysis step adding pyrolyzed leftovers (biochar).

To this purpose, mollusks and fish waste are recovered, respectively, from Co.Pe.Mo. Cooperative Company (Cooperativa Pescatori Molluschicoltori) and Ittica del Conero Soc. Coop.. Co.Pe.Mo. is considered as a point of reference for all Italian fishing cooperatives. They sell shellfish species from the Marche Region such as mussels, clams, and murex. From their activities, they produce around 500 $t \cdot y^{-1}$ of mollusk wastes. Ittica del Conero Soc. Coop. has been operating for several years both in the marketing and processing of fish products from the Adriatic Sea (tub gurnard, monkfish, hake, salmon, gilthead, cuttlefish, etc.) and disposes of around 80 tons of by-products (viscera, bones, heads, etc.) every year.

1.3 Aim of the thesis

This thesis is articulated according to two main objectives:

1) Experimental study and
2) Sustainability assessment

for fertilizers recovery from seafood waste.

1.3.1 Experimental study

The aim of this experimental study is to set up an optimized treatment scheme for seafood waste to produce protein hydrolysates through the process of enzymatic hydrolysis, at lab scale. The substrates under study, which will be presented in § 3.2.1, will be three: mollusks meat, fish waste, and a mixture of 50% mollusks meat and 50% fish. In addition to determining the best operating conditions for the

treatment of the three substrates, the experimental study aims to determine if there is an advantage from the use of the substrate mix compared to individual fish and mollusks.

The European regulatory framework will be analyzed for the definition of outcoming product which must match the minimum requirements provided by the reference regulation.

1.3.2 Sustainability assessment

Once the best conditions have been evaluated, the Life Cycle Assessment methodology will be applied in order to determine the potential effects associated with the production of protein hydrolysate in a pilot-scale plant, in terms of environmental impact. In addition, LCA represents a powerful tool for improving the scheme performance of the scheme, since it allows evaluating the treatment scheme to make it more sustainable. In particular, the three substrates introduced above will be compared at the same operating conditions, to determine which is the optimum one from an environmental point of view, both for what concerns the valorization of the three wastes and the production of protein hydrolysates.

An economic analysis will be also performed evaluating the CAPEX and OPEX, to determine the payback period and the potential profit of this kind of process.

CHAPTER 2: STATE OF THE ART

2.1 Regulatory framework

The current reference legislation includes:

- Regulation (EC) n. 1069/2009, the actual framework for the management of by-products from fishery industry, repealing Regulation (EC) n. 1774/2002;
- Legislative Decree n. 75/2010 "Riordino e revisione della disciplina in materia di fertilizzanti", and sequent modifications;
- Regulation (EU) n. 2019/1009, the new EU Fertilizer Regulation, repealing Regulation (EC) n. 2003/2003 from 16 July 2022.

2.1.1 Placement on the market of products of animal origin

According to the Italian Legislative Decree n. 75/2010, products listed in the relative Annexes, which use processed products of animal origin, may be placed on the market provided that the latter comply with the requirements and rules for processing laid down in Regulation (EC) No 1774/2002 of the European Parliament and of the Council. The present regulation is abrogated by the Regulation (EC) n. 1069/2009, laying down health rules as regards animal by-products and derived products not intended for human consumption.

Regulation 1069/2009 aims to adequately control the risk, either by directing such products towards safe means of disposal or by using them for different purposes, provided that strict conditions are applied which minimize the health risks involved. The regulatory framework already indicates waste valorization, but it is not mandatory. Indeed, the manufacturing of organic fertilizers or soil improvers is specifically designed by Regulation 1069/2009 as an option for the Category 3 materials.

More specifically, Article 7 states that Animal by-products shall be categorized into specific categories which reflect the level of risk to public and animal health arising from those animal by-products. Raw material at the base of this study falls into Category 3 material, which can comprise the following animal by products:

- aquatic animals, and parts of such animals, except sea mammals, which did not show any signs of disease communicable to humans or animals;
- animal by-products from aquatic animals originating from establishments or plants manufacturing products for human consumption;

- material originating from animals which did not show any signs of disease communicable through that material to humans or animals, like shells from shellfish with soft tissue or flesh.

More in detail, this regulation stats that Category 3 material shall be:

- processed, except in the case of material which has changed through decomposition or spoilage so as to present an unacceptable risk to public or animal health;
- used for the manufacturing of organic fertilizers or soil improvers, to be placed on the market in accordance with Article 32.

Article 32, providing rules for placing on the market and use, stats that organic fertilizers and soil improvers may be placed on the market and used if:

- they are derived from Category 2 or Category 3 material;
- they have been produced in accordance with the conditions for pressure sterilization or with other conditions to prevent risks arising to public and animal health, in accordance with the requirements laid down pursuant to Article 15 and any measures which have been laid down in accordance with paragraph 3 of this Article;
- they come from approved or registered establishments or plants, as applicable; and
- in the case of meat-and-bone meal derived from Category 2 material and processed animal proteins intended to be used as or in organic fertilizers and soil improvers, they have been mixed with a component to exclude the subsequent use of the mixture for feeding purposes and marked when required by measures adopted under paragraph 3.

2.1.2 Fertilizers' categories in Legislative Decree n. 75/2010

This decree is applied to:

a) products placed on the market as fertilizers CE, defined by the Regulation (EC) n. 2003/2003
b) national fertilizers, soil improvers, corrective products and related products placed on the market, defined, described, and classified in Annexes 1, 2, 3, 4, 5, 6 and 13.

The goal of this biorefinery is to obtain protein hydrolysates which can act as plant biostimulants. However, the present legislation includes several types of hydrolysates, vegetal- or animal-based, into different categories of fertilizers, depending on their minimum content of nitrogen and few other parameters. None of these categories comprises fish by-products as raw material, but as already discussed these are reported as Category 3 materials in the Regulation 1069/2009, and they may be

placed on the market as organic fertilizers and soil improvers. Therefore, the product at the base of this study cannot be directly compared with none of those categories, but it could be assimilated to both organic fertilizers and biostimulants, depending on the minimum requirements that is able to satisfy. For this reason, it is worth to examine more in deep the following categories of fertilizers:

- National organic fertilizers (Annex 1), comprising
  - Nitrogenous organic fertilizers, like Hydrolyzed animal epithelium, Hydrolyzed leather and skins, Amino acids and peptides;
  - Fluid nitrogenous organic fertilizers like Fluid hydrolyzed animal epithelium, fluid amino acids and peptides;
- Products with action on fertilizers, soil, plants (Annex 6) comprising
  - Biostimulants (on plants) like Alfalfa protein hydrolysate, Hydrolyzed animal epithelium (solid or fluid), Enzymatic hydrolysate of Fabaceae.

According to this decree, organic fertilizers are derived from organic materials of animal or vegetable origin, consisting of organic compounds to which the main elements of fertility are chemically bound in organic form or otherwise form an integral part of the matrix.

The main elements of fertility are:

1) «Main nutrients»: nitrogen, phosphorus, and potassium;
2) «Secondary nutrients»: calcium, magnesium, sodium, and sulfur.

Concerning the products with a specific action, these provide another fertilizer or soil or plants with substances which favor or regulate nutrients absorption or correct determined physiological anomalies.

2.1.2.1 Nitrogenous organic fertilizers (solid or fluid)

According to the present Legislative Decree, organic nitrogen stands for the nitrogen contained in chemical organic compounds of vegetal or animal origin or deriving from such products. The definition is further specified by the Method D1 reported in the “Gazzetta Ufficiale” of the 5/08/1986 n. 180, DM 24/03/86, applicable to all organic and mixed organic fertilizers in the presence or not of nitric nitrogen, according to which organic nitrogen is obtained by the difference between the total soluble and insoluble nitrogen and the sum of the various forms of nitrogen, other than organic nitrogen, present in the fertilizer.

The following types are investigated for comparison.

***Table 1**: Solid or fluid nitrogenous organic fertilizers selected for comparison from Legislative Decree n. 75/2010, Annex 1, and successive modifications.*

	N.	Type designation	Data on method of production and essential ingredients
Solid	16	Hydrolyzed animal epithelium	Residues of animal epithelium from tanneries and slaughterhouses, hydrolyzed with mineral acids
	18	Hydrolyzed leather and skins	Product obtained by hydrolysis under pressure of waste from processing of hides and skins
	22	Amino acids and peptides	A mixture of amino acids and peptides obtained by hydrolysis of animal epithelium by the method laid down in Reg. (EC) 142/2011, Annex X, Chapter II, Section 5, point D[1]
Fluid	6	Fluid hydrolyzed animal epithelium	Product obtained by enzymatic and/or chemical hydrolysis of animal epithelium
	9	Amino acids and peptides	A mixture of amino acids and peptides obtained by hydrolysis of animal epithelium by the method laid down in Reg. (EC) 142/2011, Annex X, Chapter II, Section 5, point D[1]

2.1.2.2 Biostimulants

For biostimulants it is mandatory to describe on the label doses of use and how to use them. The biostimulant activity must not result from the addition of substances with a phytohormonal action to the product.

***Table 2**: Biostimulants selected for comparison from Legislative Decree n. 75/2010, Annex 6, and successive modifications.*

	N.	Type designation	Data on method of production and essential ingredients
Biostimulants	1	Alfalfa protein hydrolysate	Product obtained by enzymatic hydrolysis of Alfalfa protein extract containing amino acids and peptides

[1] Preparation of raw Category 3 material by brining, liming, and intensive washing followed by exposure of the material to: (a) pH of more than 11 for more than three hours at temperature of more than 80 °C and subsequently by heat treatment at more than 140°c for 30 minutes at more than 3.6; or (b) a pH of 1 to 2, followed by a pH of more than 11, followed by heat treatment at 140 °C for 30 minutes at 3 bar.

	2	Hydrolyzed animal epithelium (solid or fluid)	Residues of animal epithelium from tanneries and slaughterhouses, hydrolyzed with mineral acids
	7	Enzymatic hydrolysate of Fabaceae	Product obtained by enzymatic hydrolysis of plant tissues belonging to the Fabaceae family

2.1.2.3 Requirements and tolerances

Table 3 reports the minimum requirements provided by the present Legislative Decree for the types selected in the previous paragraphs. Values for nitrogen and carbon are expressed as the percentage by weight of the product as marketed.

Table 4 reports the tolerance limits, provided in Annex 7 of the same decree, which must consider the variations in terms of production, sampling, and analysis; thus, they include measure uncertainties associated with analytical methods used for control. Tolerance values are expressed as absolute values in % by weight.

The parameters designated in Table 3 are different depending on the specific category. In general, for nitrogenous organic fertilizers the minimum requirements mainly concern organic nitrogen and soluble organic nitrogen. More specifically, amino acids and peptides have also to respect a minimum content of organic carbon and free amino acids, and a minimum molecular weight. Minimum requirements for biostimulants also depend on the raw material, but in general, the minimum content of total and free amino acids must be respected.

***Table 3**: Minimum requirements defined by the Legislative Decree n. 75/2010 for the selected types.*

Category	Type designation	N total	N organic	N organic soluble	C organic	Other	pH in water	Note
-	-	%	%	%	%	-	-	-
Nitrogenous organic fertilizers	Hydrolyzed animal epithelium	-	4	1 of N org	-	-	-	-
	Hydrolyzed leather and skins	-	10	-	-	C/N ≤ 4	-	-
	Amino acids and peptides	-	10	90 of N org	37	-	-	Molecular weight < 10000 Dalton Free amino acids > 8% w/w
Fluid nitrogenous organic fertilizers	Fluid hydrolyzed animal epithelium	8	-	90 of N total	20	-	4.5-6.5	-
	Fluid amino acids and peptides	-	10	90 of N org	37	-	-	Molecular weight < 10000 Dalton Free amino acids > 8% w/w
Biostimulants	Alfalfa protein hydrolysate	-	4.5	-	15	28% total amino acids 3.5% free amino acids	-	*(Alanine + Glycine)/(Proline + Gglutamic acid)* close to 1

	Hydrolyzed animal epithelium (solid or fluid)	-	4	1 of N org	15	C/N ≤ 6	-	*Glycine/(Proline+ Hydroxyproline)* = 1.1 Degree of hydrolysis dry matter > 330 Free amino acids > 10%
	Enzymatic hydrolysate of Fabaceae	-	-	-	-	5% total amino acids 1.5% free amino acids 30% degree of hydrolysis	-	Biostimulant activity. Content in Triacontanol of natural origin ≥ 6 mg/kg

***Table 4**: Tolerance limits according to the Legislative Decree n. 75/2010, Annex 7.*

Category	Type designation	C	C organic extractable/C organic	N	N organic soluble	
Nitrogenous organic fertilizers	Hydrolyzed animal epithelium	1.0	-	0.3	-	
	Hydrolyzed leather and skins	1.0	-	0.5	-	
	Amino acids and peptides	1.0	-	0.3	0.2	
Fluid nitrogenous organic fertilizers	Fluid hydrolyzed animal epithelium	1.0	-	0.3	0.2	
	Fluid amino acids and peptides	1.0	-	0.3	0.2	
Category	**Type designation**	**C organic**	**N organic**	**Free amino acids**	**Total amino acids**	**Triacontanol**
Biostimulants	Alfalfa protein hydrolysate	3.0	0.3	1.5	3	-
	Hydrolyzed animal epithelium (solid or fluid)	1.0	0.3	-	-	-
	Enzymatic hydrolysate of Fabaceae	-	-	-	-	5

2.1.2.4 Fertilizers allowed in organic production

All the typologies described are allowed in organic production if satisfying the additional requirement for admissibility in organic production and the conditions for use by the Reg. (EC) No. 889/2008, amended and corrected by Regulation (EU) No 354/2014. Despite the nutraceutical properties, the EU has banned the application of animal-derived protein hydrolysates on the edible parts of organic crops including fish protein hydrolysates and fish meal. However, animal-derived biostimulants can still be applied to no-edible parts of horticulture crops such as seeds during planting, roots and leaves to equally exert their biostimulant effect (Madende & Hayes, 2020).

In particular, as reported in the Lgs. Decree n. 75/2010, if leather is present (nitrogenous organic fertilizers n. 16, 18, 21, 5 fluid, biostimulant n. 2), the maximum concentration of chromium (VI) in mg/kg of dry matter must be "non detectable", and if the raw material is of animal origin the condition imposed for the use is that it is not applicable to edible parts of the crop.

***Table 5**: Additional requirements for admissibility in organic production according to Reg. (EC) n. 889/2008.*

	N.	**Type designation in accordance with this decree**	**Product designation in accordance with Reg. (CE) n. 889/2008**
Nitrogenous organic fertilizers	16	Hydrolyzed animal epithelium	Meat meal and/or leather Hydrolyzed proteins
	18	Hydrolyzed leather and skins	Leathers Hydrolyzed proteins
	22	Amino acids and peptides	Meat meal Hydrolyzed proteins
	5 (fluid)	Fluid hydrolyzed animal epithelium	Meat meal and/or leather Hydrolyzed proteins
	9 (fluid)	Fluid amino acids and peptides	Meat meal Hydrolyzed proteins
Biostimulants	1	Alfalfa protein hydrolysate	Products and sub-products of vegetal origin for fertilization
	2	Hydrolyzed animal epithelium (solid or fluid)	Meat meal and/or leather Hydrolyzed proteins
	7	Enzymatic hydrolysate of Fabaceae	Products and sub-products of vegetal origin for fertilization

2.1.3 Comparison with the new EU regulation of fertilizing products

Regulation (EU) 2019/1009 laying down rules on the making available on the market of EU fertilizing products, repealing Reg. (EC) N. 2003/2003, aims to identify the possible materials for use within the fertilizer market (biological or not), taking into account technical and legal requirements, considering the risk to human or animal health arising from them. This Regulation represents an important innovation in the field since the old one only considered the presence of mineral fertilizers.

Manufacturer of EC fertilizers who today refers to Reg. 2003/2003 and sell in Europe are obliged to apply the present regulation; manufacturers of standardized products from D. Lgs. n. 75/2010 that would like to sell in the rest of Europe can choose to apply the new regulation; manufacturer of products standardized by D. Lgs. n. 75/2010 that sells only in Italy are not obliged to apply it.

This regulation reports 11 Component Material Categories (CMCs) at the base of 7 Product Function Categories (PFCs) of EU fertilizing products.

In particular, it individuates as CMC 10 the derived products within the meaning of Regulation (EC) No 1069/2009 and states that an EU fertilizing product may contain derived products within the meaning of Regulation (EC) No 1069/2009 having reached the end point in the manufacturing chain as determined in accordance with that Regulation.

Regarding the PFCs, the attention can be focused on:

- PFC 1: Organic fertilizer (solid or liquid);
- PFC 6: Plant biostimulant.

2.1.3.1 PFC 1: Organic fertilizer

A fertilizer shall be an EU fertilizing product the function of which is to provide nutrients to plants or mushrooms. An organic fertilizer shall contain:

- Organic carbon (C org = organic matter x 0.56) and
- Nutrients

of solely biological origin.

A solid organic fertilizer shall contain at least one of the following declared primary nutrients: nitrogen (N), phosphorus pentoxide (P_2O_5) or potassium oxide (K_2O). Where a solid organic fertilizer contains only nitrogen, that content shall be at least 2.5% by mass of total nitrogen (N). Organic carbon (C org) content in a solid organic fertilizer shall be at least 15% by mass. The same is valid

for liquid ones, but the nitrogen content shall be at least 2% by mass of total nitrogen, and the organic carbon (C org) content shall be at least 5% by mass.

2.1.3.2 PFC 6: Plant biostimulant

A plant biostimulant shall be an EU fertilizing product the function of which is to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere:

- nutrient use efficiency,
- tolerance to abiotic stress,
- quality traits, or
- availability of confined nutrients in the soil or rhizosphere.

2.1.3.3 Requirements

For what concerns the requirements, both organic fertilizers and plant biostimulants must respect the limit values for metals reported in Table 6.

***Table 6**: Limit values for metals, according to the new Regulation (EU) 2019/1009.*

Cadmium (Cd)	1.5 mg/kg dry matter
Hexavalent chromium (Cr VI)	2 mg/kg dry matter
Mercury (Hg)	1 mg/kg dry matter
Nickel (Ni)	50 mg/kg dry matter
Lead (Pb)	120 mg/kg dry matter
Inorganic arsenic (As)	40 mg/kg dry matter

In addition, Biuret ($C_2H_5N_3O_2$) must not be present in organic fertilizer, and the copper (Cu) and zinc (Zn) content must not exceed respectively 300 and 800 mg/kg of dry matter. Instead, for biostimulants, the copper (Cu) and zinc (Zn) content must not exceed respectively 600 and 1500 mg/kg of dry matter. Limits for pathogens are provided too.

To sum up, the new European regulation does not provide the same category differentiation. Anyway, the categories reported in Table 7 can be assimilated.

***Table 7**: Category comparison between D. Lgs. n. 75/2010 and Reg. (EU) 2019/1009.*

D. Lgs. n. 75/2010	**Reg. (EU) 2019/1009**
Nitrogenous organic fertilizers (All. 1.5.1)	PFC 1(A)(I): Solid organic fertilizer
Fluid nitrogenous organic fertilizers (All. 1.5.1.1)	PFC 1(A)(II): Liquid organic fertilizer
Biostimulants (All. 6.4.1)	PFC 6(B): Non-microbial plant biostimulant

According to the Reg. EU 2019/1009, all the organic fertilizers and biostimulants must respect specific limits for metals and pathogens. These limits are not specified for the corresponding category in D. Lgs. n. 75/2010. Only for organic fertilizers the Total Pb must be below 30 mg/kg.

Instead, the Italian legislation is more completed for what concerns the minimum requirements that each type of product must present. The only parameters that can be compared with the new EU regulation are nitrogen and organic carbon. The requirements for PFC 1A (I) and (II) are much less restrictive than the ones for the corresponding product for the Italian legislation.

For biostimulants, the Italian legislation provides minimum requirements with variations depending on the type, at least for organic nitrogen and carbon, free and total amino acids, degree of hydrolysis, whereas the new EU regulation just specifies the limit values for metals as reported in Table 6. and for pathogens.

***Table 8**: Comparison between the minimum requirements (%) provided by D. Lgs. n. 75/2010 and Reg. (EU) 2019/1009, respectively for Nitrogenous organic fertilizers and Organic fertilizers.*

	Nitrogenous organic fertilizers (All. 1.5.1)	**PFC 1(A)(I): Solid organic fertilizer**
Total or organic Nitrogen	4 – 10 (organic)	2.5
Organic carbon	30 – 37	15
	Fluid nitrogenous organic fertilizers (All. 1.5.1.1)	**PFC 1(A)(II): Liquid organic fertilizer**
Total or organic Nitrogen	8 (total), 10 (organic)	2
Organic carbon	20 - 37	5

2.2 Commercially available products

It has not been possible to find hydrolyzed fish-based biostimulants commercialized in Italy, in fact, none of the categories listed in Annex 6 of the Legislative Decree n. 75/2010 report fish-based protein hydrolysates. However, fish-based biostimulants are available on the market in different European countries. Examples are the BioBooster Fish Hydrolysate by Symbio (UK), the C-BIO SeaActiv by C-BIO (Ireland), the STYM AMINO FISH by Orange Saft S.L (Spain), and many others. It is difficult to compare these products from a technical point of view, as they are marketed in different countries and not always technical sheets are available, but the main information in **Table 9** can be gathered for BioBooster Fish Hydrolysate and STYM AMINO FISH. Both the examples satisfy the nitrogen requirement of the Italian legislation, and surely the less restrictive requirements of the new European regulation.

***Table 9**: Examples of hydrolyzed fish-based biostimulants on the market.*

Name	Producer	Nitrogen	Amino acids	Organic carbon	State
BioBooster Fish Hydrolysate	Symbio	8% (total)	-	-	liquid
STYM AMINO FISH	Orange Saft S.L	6.30% (total) 5.75% (organic)	8.75% (free)	34.25% 50.00% total Organic Matter	Liquid

Furthermore, it is worth selecting some commercially available products on the Italian market to get an idea of the competitiveness of this product. For this purpose, different products belonging to the hydrolyzed animal epithelium (solid or fluid), used as biostimulants or nitrogenous organic fertilizers are selected from the Register of Fertilizers (DDG n.0214554 del 12/05/2022) and the relative prices at which they are available on the market, when declared, have been retrieved with a rapid internet research. Variation of the price depends on the size of the packaging: usually, the larger the package, the lower the price per kg of product.

***Table 10**: Average prices of hydrolyzed animal epithelium on the market.*

Commercial name	Producer	Technology	Type	Physical state	€/kg
AMINOSPRAY®	Pavoni&C S.p.a.	Hydrolysis	Fluid nitrogenous organic fertilizer Hydrolyzed animal epithelium	Liquid	5.00-7.00
LEAFEED®	Pavoni&C S.p.a.	Hydrolysis	Fluid nitrogenous organic fertilizer Hydrolyzed animal epithelium	Liquid	7.50-13.00
ETIXAMIN DF	ILSA S.p.A.	FCEH®	Nitrogenous organic fertilizer Hydrolyzed animal epithelium	Solid, water-soluble powder	5.40
ILSAMIN N90	ILSA S.p.A.	FCEH®	Biostimulant Hydrolyzed animal epithelium	Liquid	9.90

2.3 Literature review on fish-based protein hydrolysates

Protein hydrolysates (PHs) are an important group of plant biostimulants that have received increasing attention in recent years due to their positive effects on crop performances (Colla et al., 2015). Biostimulants are not fertilizers as they do not provide nutrients directly to plants but can facilitate the acquisition of nutrients by supporting metabolic processes in soil and plants (Madende & Hayes, 2020; Venugopal & Sasidharan, 2021). Protein hydrolysates can function as biostimulants for improvement of plant growth, and chlorophyll synthesis and to abate the negative effects of abiotic stresses in vegetables. The active ingredients in protein hydrolysates include various proteins, peptides, and free amino acids (Venugopal & Sasidharan, 2021). Fish by-products are enriched in proteins, fat, and amino acids following protease hydrolysis. Furthermore, they contain antioxidants, which are often suitable for food or feed applications. The above-mentioned nutritional qualities make fish protein hydrolysates excellent candidates for use in organic agriculture as biostimulants like other animal-derived protein hydrolysates (Madende & Hayes, 2020). The application of fish protein hydrolysates in horticultural practices has resulted in increased crop yields and better quality of fruit and vegetables compared to chemical fertilizers (Venugopal & Sasidharan, 2021).

Protein hydrolysates are classified according to whether they are of animal- or plant origin. Animal origin protein hydrolysates include leather by-products, blood meal, fish by-products, chicken feathers and casein, whereas plant-origin protein hydrolysates include legume seeds, alfalfa hay and vegetable by-products (Colla et al., 2015).

From a biochemical point of view, Protein hydrolysates are proteins broken into bioactive peptides that contain between 2 and 20 amino acids, that have been shown to have human health and animal feed supplementation benefits. In fact, during the hydrolysis process, the source not only maintains a high content of essential amino acids but also generates other activities with potential use as food additives such as antioxidant, antihypertensive, antithrombotic, immunomodulatory, and antimicrobial, among others. Non-hydrolyzed fish proteins do not possess these properties due to the poor accessibility to the functional peptide sequence (Zamora-Sillero et al., 2018). In a related manner, these bioactive peptides may have positive effects on plants when applied as biostimulants (Madende & Hayes, 2020).

2.3.1 Benefits and limitations in agriculture

PHs have been identified to improve the performance of several horticultural crops, including increased shoot, root biomass and productivity. Application of PHs to plant leaves and roots has been shown to increase Fe and N metabolism, nutrient uptake, and water and nutrient use efficiencies for both macro and microelements. The higher nutrient uptake in PH-treated plants has been attributed to several causes, including: the increase in soil microbial activity and soil enzymatic activities; the improvement of micronutrient mobility and solubility, in particular Fe, Zn, Mn and Cu; modifications in the root architecture of plants, in particular root length, density and number of lateral roots. PHs have been shown not only to improve plant nutrition but also the quality of fruits and vegetables in terms of phytochemicals (i.e., carotenoids, flavonoids, polyphenols), and they can reduce undesired compounds, such as nitrates. In addition, PH application has also been shown to avoid or reduce losses in production caused by unfavorable soil conditions and environmental stresses (thermal stress, salinity, drought, alkalinity, and nutrient deficiency (Colla et al., 2015).

Phytotoxic effects and growth depression of fruiting crops have been also reported after repeated applications of animal-derived PH products. The detrimental effect of some animal derived-PHs on plant growth can be attributed to an unbalanced amino acid composition, higher concentration of free amino acids and high salinity. Besides phytotoxicity effects, there is an increased concern on the use of animal derived PHs in terms of food safety, as demonstrated by the European Regulation No. 354/2014, which prohibited the application of these products on the edible parts of organic crops.

However, Colla et al. (2015) concluded that, evaluating safety and fertilizer efficacy of animal-derived PHs, PHs did not negatively affect eukaryotic cells and soil ecosystems, and PHs can be used in conventional and organic farming without posing harm to human health and the environment (Colla et al., 2015).

2.3.2 PH Production methods

Fish protein hydrolysates are ordinarily derived from fish skins and other by-products such as heads, muscle, viscera, bone, frames, and roe (Chalamaiah et al., 2012). In order to recovery protein and peptides from fish by-products several methods such as chemical or biochemical hydrolysis have been developed (Zamora-Sillero et al., 2018). These methods are summarized in Figure 1.

Chemical methods involve acid and alkaline hydrolysis. Since these are methods relatively inexpensive and easy to operate, they have been the preferred practices to produce protein hydrolysates at industrial scale. However, chemical hydrolysis is difficult to control due to its harsh reaction and unspecific peptide bonds cleaving, giving a heterogeneous yield of peptides and reduces the nutritional quality of products (Zamora-Sillero et al., 2018). One other critical aspect of chemical hydrolysis is the conversion of free amino acids from the L-form to D-form (racemization). Since the amino acids in proteins of living organisms are only in the L-form, plants cannot directly use D-amino acids in their metabolism, making the PH less effective or even potentially toxic for plants. Finally, the large use of acids/alkalis during chemical hydrolysis led to an increase in salinity of PHs (Colla et al., 2015).

Biochemical methods include autolysis and enzymatic hydrolysis. The autolysis process involves the action of endogenous proteolytic enzymes on animal proteins. There are no enzyme costs involved in this kind of method, and it is simpler to manage. On the other hand, the main limitation is the reduced functionality and the difficulty in obtaining a homogenous hydrolysate. Instead, the hydrolysis of proteins by exogenous enzymes or enzymatic hydrolysis allows a better control of the hydrolysis process and the resulting product. Therefore, enzymatic hydrolysis is considered the most effective way to obtain protein hydrolysates with bioactive properties (Zamora-Sillero et al., 2018).

Due to the disadvantages of chemical hydrolysis, enzymatic hydrolysis was chosen as the method of production of protein hydrolysates in this experimental study. Therefore, the next paragraphs focus on this method of production.

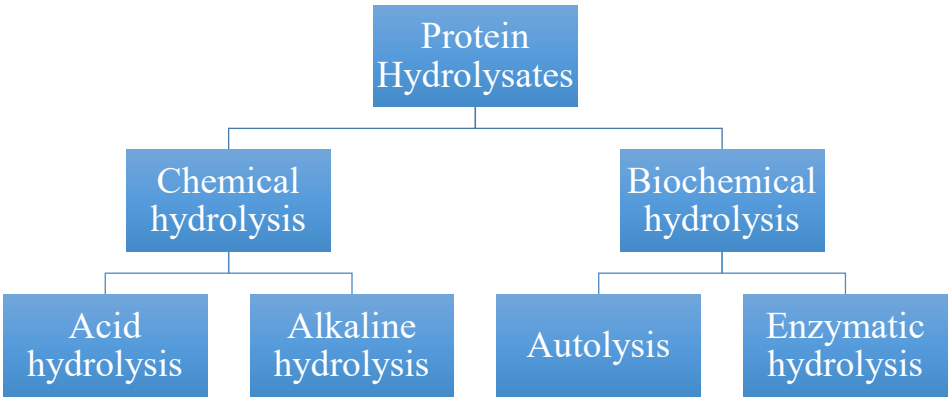


***Figure 1**: Classification of PH production methods.*

2.4 Enzymatic hydrolysis as method of production

A typical technological scheme of FPH manufacturing is presented in Figure 2. The process starts with the takeover of raw materials. Pre-treatment of raw material consists mainly of mincing and dilution, but in many cases, a defatting procedure is applied. This is followed by solubilization with water. In this process, several proteolytic enzymes are commonly used to hydrolyze the proteins and convert them into high added-value products with functional, biological, and nutritional properties (Zamora-Sillero et al., 2018; Kristinsson and Rasco, 2000). The selected enzyme is mixed homogeneously with the mince–water slurry. Physicochemical conditions of the hydrolysis reaction such as temperature, pH and enzyme to substrate ratio must be adjusted to optimize the activity of the proteolytic enzyme (Zamora-Sillero et al., 2018). Generally, enzymatic hydrolysis is carried out from one to several hours under mild conditions: slightly elevated temperatures (generally around 35–65°C) and a certain pH according to the optimal requirements of the used enzymatic systems (Petrova et al., 2018).

After a certain time, when a certain desired extent of hydrolysis is achieved, there is a need to stop the hydrolyzation process by thermal treatments according to the hydrolysis method. After termination of hydrolysis, the protein mixture is delivered to solid separation where liquid part is separated from the solids. In some cases when the final product must have a certain quality, for example, to be used for biochemical purposes as a microbiological media, removal of salt can be provided. After that, the protein mixture can be concentrated to remove some water before the drying procedure. Liquid FPH slurry is then delivered for drying and dried FPH is sent for packaging and transportation (Petrova et al., 2018; He et al. 2013).

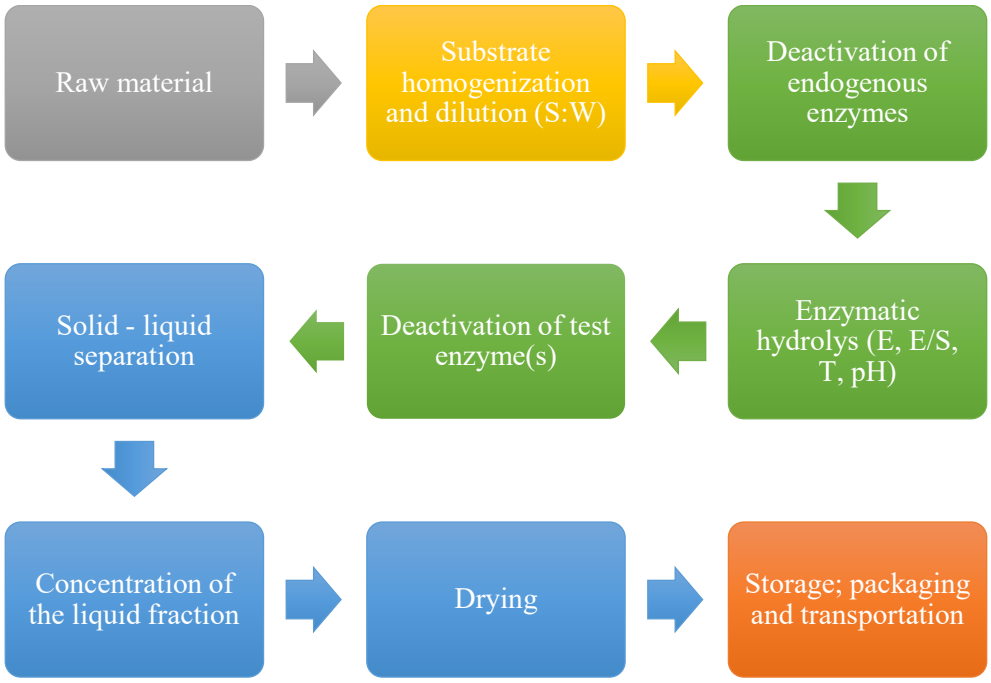


***Figure 2**: Principal steps of protein hydrolysate production.*

To sum up, any hydrolysis process involves at least five independent variables (Zamora-Sillero et al., 2018):

1) protein substrate (S) concentration,
2) enzyme-substrate ratio (E:S),
3) pH,
4) temperature, and
5) time.

All the steps involved in the process and the present variables are discussed hereafter, reporting several literature case studies. Most of the studies analyzed concern the study and optimization of operating conditions for laboratory scale hydrolysis. However, for the possibility of developing a pilot scale system, some pilot study cases have been reported, in particular with regard to the optimization of energy consumption and costs and the equipment which could be used.

2.4.1 Degree of hydrolysis

The effectiveness of the process is typically expressed in terms of the Degree of Hydrolysis (DH). The DH is defined as the proportion of the total number of peptide bonds that are cleaved during hydrolysis (Adler- Nissen, 1986) and is calculated as follows:

$$DH(\%) = \frac{h}{h_{tot}} * 100$$

Where h is the number of hydrolyzed peptide bonds, and h_{tot} is the total number of peptide bonds.

Therefore, the higher the DH, the greater number of peptides would be produced in the solution that will result in an increase in protein solubility and the possibility to recover the protein (Zamora-Sillero et al., 2018) to be used as a fertilizer or biostimulant.

The degree of hydrolysis achieved in the hydrolysis is determined by the conditions used in the process such as type of substrate, substrate concentration, E:S ratio, incubation time as well as the physicochemical conditions such as pH and temperature. Moreover, another factor that will determine the degree of hydrolysis is the nature of the enzyme, characterized by its specific activity and type of activity. The nature of the enzyme used will not only influence the degree of hydrolysis but also in the type of peptides produced (Zamora-Sillero et al., 2018).

There is no standard method for determining DH. Instead, a number of methods have been developed and are commonly used to determine the DH of protein hydrolysates. These include the pH-stat, osmometric, soluble nitrogen after trichloroacetic acid precipitation (SN-TCA), 2,4,6-trinitrobenzenesulfonic acid (TNBS), o-phthaldialdehyde (OPA), amino acid nitrogen, and formol titration methods (Rutherfurd, 2010). The pH-stat method is the most used by different authors (Table 14). However, the "Gazzetta Ufficiale del 26/01/01 n.21", DM 21/12/00, Suppl. n.8 reports the OPA method for the determination of the degree of hydrolysis in protein hydrolysate – based fertilizers.

The process effectiveness may be evaluated also in terms of nitrogen recovery, defined as:

$$NR\ (\%) = \frac{Total\ nitrogen\ in\ the\ hydrolysate\ (mg)}{Total\ nitrogen\ in\ the\ substrate\ (mg)}$$

Benjakul and Morrissey (1997) found that a linear correlation is present between the nitrogen recovery and the degree of hydrolysis (Figure 3).

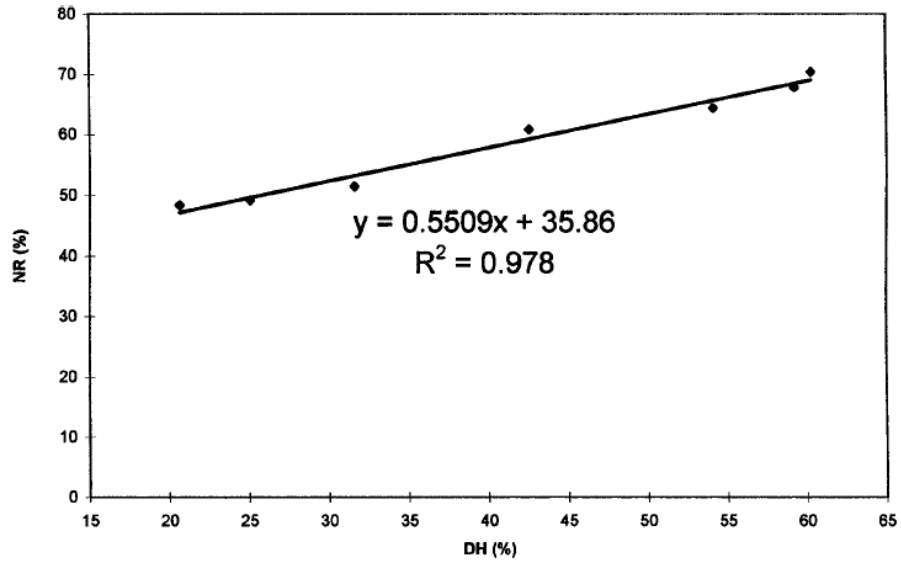


***Figure 3**: Correlation between nitrogen recovery and degree of hydrolysis (Benjakul and Morrissey, 1997).*

2.4.2 Pre – treatment

The purpose of the pre-treatment step is to prepare homogenized water–mince mixtures with low fat content for the subsequent step of hydrolyzation (He et al., 2013).

In some cases (Chi et al., 2015; Dai et al., 2012), lyophilized samples were used as starting materials for enzymatic hydrolysis.

2.4.2.1 Substrate concentration

Different substrate to water ratios have been used in literature. In some cases, fish co-products are minced mixed with equal amounts (w/w) of water to form a homogeneous water–mince slurry. It has been observed that increasing the amount of water does not increase the protein recovery of protein hydrolysates but reducing water decreases it (He et al., 2013; Benjakul & Morrissey, 1997). In other cases, substrate to water ratios 1:2 and 1:3 (w:v, kg:l) led to higher degrees of hydrolysis than 1:1 and 1:1.5 ratios (Vázquez et al., 2020) (Figure 4).

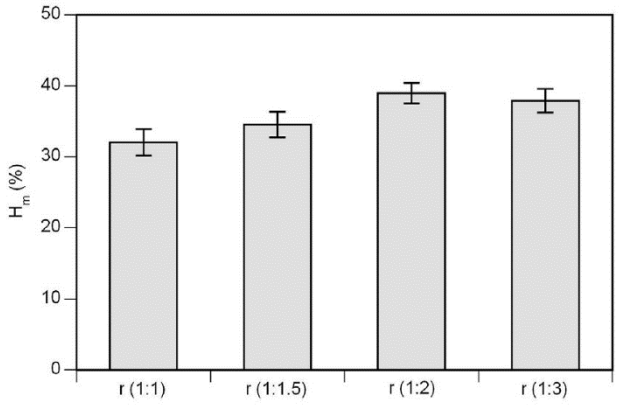


***Figure 4**: Optimization studies of Alcalase hydrolysis of blue whiting discards. Individual effect of S:L ratio over Hm (maximum degree of hydrolysis) (Vázquez et al., 2020).*

In some cases, a buffer solution is added to the minced fish, for example, phosphate buffer (Chi et al., 2015). Anyway, the presence of buffer salts may affect the final properties of the hydrolysates (Kristinsson & Rasco, 2000).

Producers should evaluate the amount of added water for the hydrolyzation step in order to provide a suitable extent of the protein breakdown and at the same time avoid extra costs for dehydration of unnecessary water fraction from FPH on the step of drying (Petrova et al., 2018). In addition, more concentrated products are usually more valuable in the market than diluted ones, since, as it will be better discussed in § 2.4.4.3, they are more stable, therefore they have a longer shelf-life, and lighter with the same nitrogen content, therefore they are easier to store and less expensive to transport.

2.4.2.2 Defatting

When dealing with fish, more than mollusks, it is necessary to pay attention on the fat content, since several types of fish are rich in lipid fraction. The fat content of fish protein hydrolysates needs to be well-controlled when the product is intended for human consumption. In this case, The Food and Agriculture Organization (FAO) of the United Nations (UN) set up a standard that the fat content of fish protein hydrolysates has to be below 0.5% (w/w), in order to avoid the influence of lipid changes on the quality of final FPH during shelf-life. A higher fat content darkens the final products, due to the release of brown pigments from lipid oxidation (He et al., 2013; Kristinsson & Rasco, 2000).

Neither European nor Italian regulations provide a fat content limit for agricultural applications. However, some authors indicated that lower fat contents favor nutrient and peptides absorption (Tejada et al., 2011). These authors determined the fat content of four different biostimulants

produced through enzymatic hydrolysis variable between 2.0 and 6.5 g/kg, and the soil enzymatic activities were higher in soil amended with the product characterized by a lower fat content.

To obtain a product of a lipid content not exceeding 0.5% by weight, Quaglia & Orban (1987) defatted ground sardines by extraction with isopropanol three times (solvent: substrate ratio 1:1) at 46°C for 30 min, and then homogenized the mixture with water. Hoyle & Merritt (1994) used an ethanol (90%) extraction directly on minced herring at the fish/ethanol ratio of 1:2 at 70°C for 30 min, then mixed with equal volume of water, hydrolyzed the mixture, then spray dried it.

However, when comparing DH produced from whole mince and defatted mince, the hydrolysates obtained from mince possessed a higher DH than those derived from defatted mince did (Klompong at al., 2007). This result was in accordance with Hoyle & Merritt (1994) who found that protein hydrolysates produced from defatted herring had a lower DH than those from the original herring. The proteins in defatted mince were most likely denatured, and Hoyle & Merritt (1994) found that denatured fish protein possessed poor wettability, and hence accessibility of enzyme to the substrate. In addition, the high temperatures used in the defatting process might inactivate endogenous enzymes and hence reduce the rate of hydrolysis (Klompong at al., 2007).

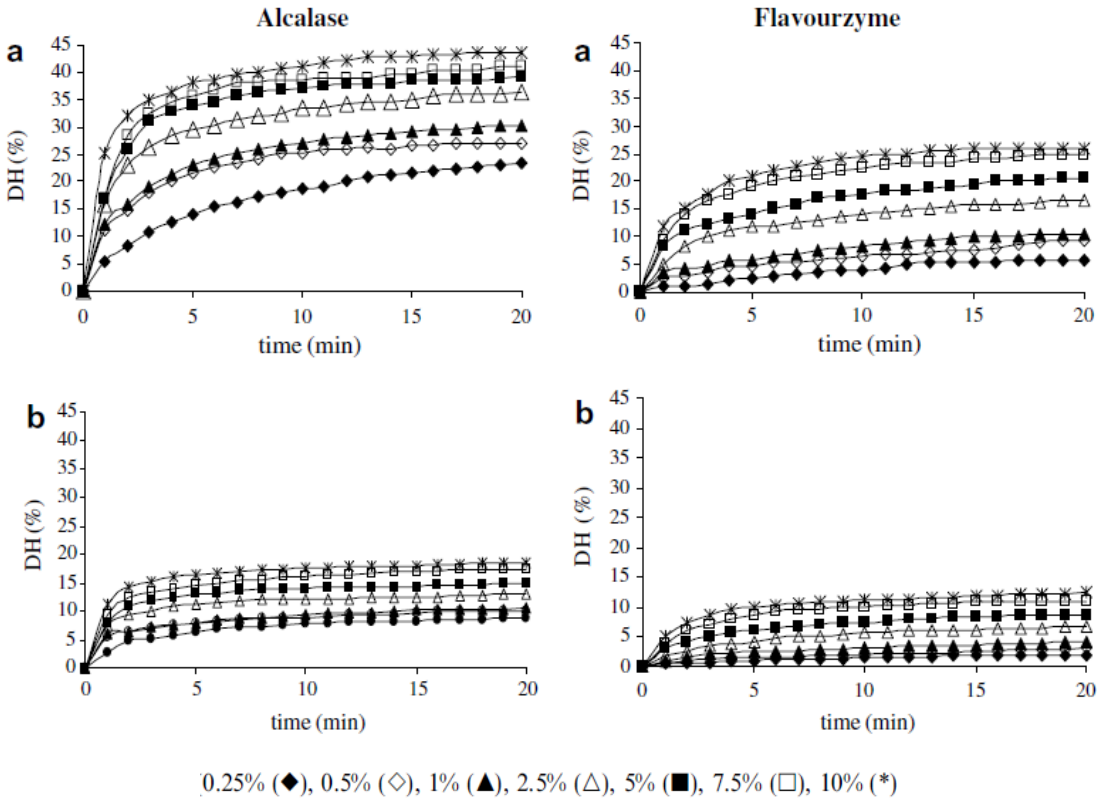


***Figure 5**: Degree of hydrolysis (DH) of yellow stripe trevally mince (a) and defatted mince (b) during hydrolysis, with Alcalase and Flavourzyme at different concentrations (Klompong at al., 2007).*

2.4.3 Hydrolysis

Once the substrate has been prepared, the mixture is added to a reaction vessel where the hydrolysis takes place.

Often a flask, ranging from 0.5 to 3 L with a closefitting multi-socket lid, has been used. The sockets in the lid usually carry: a stirrer, driven by an overhead variable speed motor to ensure adequate mixing of the system, a thermometer to monitor temperature, a pH electrode to monitor pH, and a "pH-stat" device, where acid or base is added to maintain a constant pH (Kristinsson & Rasco, 2000). Typically, at lab scale, a water bath is used to maintain the temperature of the reaction vessel constant, whereas, at pilot and industrial scales hydrolysis is carried out in thermostatically stirred-batch reactor (Wang et al., 2010; Chen et al., 2017), where the mixture can be heated to deactivate the exogenous enzyme at the end of the process. Usually, these are equipped with temperature and pH control, reagent addition, and an internal filter to separate large solids before centrifugation (Vázquez et al., 2020; Pérez-Martín et al., 2020).

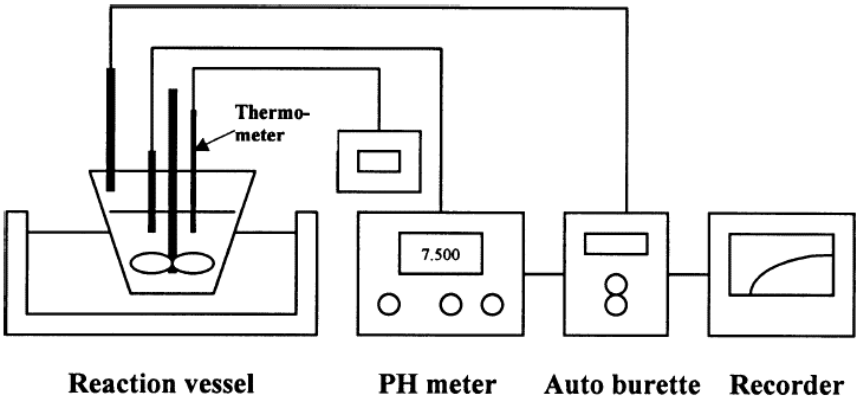


***Figure 6**: A typical enzymatic hydrolysis reaction system in the laboratory (Kristinsson & Rasco, 2000)*

Concerning the stirring rate, generally, it varies between more than 100 and 350 rpm (Naik et al., 2020; Vázquez et al., 2020; Sierra-Lopera & Zapata-Montoya, 2021). In particular, Vázquez found the agitation for optimum digestion to be 200 rpm.

2.4.3.1 Endogenous enzymes

Even though the focus of this work is the production of fish and/or mollusks-based PH with enzymatic hydrolysis for the advantages discussed above, it is worth knowing more in depth also the effect of endogenous enzymes.

Unfortunately, literature evidence on the use of endogenous enzymes, as anticipated, has shown several limitations due to their use instead of exogenous enzymes. The digestive enzymes involved

in the autolytic process are a very complex mixture, all with different activity, which result in end-products of different molecular profiles. They are primarily the serine proteases trypsin and chymotrypsin, and the thiol protease pepsin, all major enzymes of fish viscera and digestive tract, in addition lysosomal proteases, or catheptic enzymes, present in fish muscle cells also contribute to proteolytic break down to some extent. Another complication is that the presence of certain digestive enzymes and their concentration may be highly seasonal, gender and age specific, and can vary tremendously within a species as well as between species. These variations make it very hard to control the hydrolytic process and direct the production of hydrolysates with specific molecular properties (Kristinsson & Rasco, 2000). Furthermore, Shahidi et al. (1995) hydrolyzed ground capelin by endogenous enzymes and found the protein recovery of hydrolysates produced considerably lower compared with commercial enzymes, 22.9% compared with 70.6% with Alcalase (Kristinsson & Rasco, 2000); Ovissipour et al. (2013) found that FPH yield, and oil recovery were the lowest with autolysis (respectively 63.64% and 6.41%), in comparison with the highest yield obtained with Alcalase® (82.3%), and the highest oil recovery observed with bromelain (6.41%). However, Aspmo et al. (2005) discovered that by combining the action of endogenous viscera enzymes with commercial enzymes, 95% solubilization of matter (based on concentration) can be accomplished and that the difference between no added enzyme and added enzyme needed for maximum yield however is less than 20%.

On the other hand, it is important to point out that addition of exogenous enzymes is one of the main contributors to the cost of the process, therefore minimal enzyme consumption is desirable when the goal is to make a profitable business from fish hydrolysates (Aspmo et al., 2005). This is the reason way, usually, at pilot scale endogenous enzymes are not deactivated in order to reduce the energy consumption for heating a relevant quantity of hydrolyzing mixture and to improve the performance of the exogenous enzyme, whose dose should be as low as possible.

2.4.3.2 Enzyme and E:S ratio

At lab scale, when the purpose of the study is to select the best exogenous enzyme for hydrolysis or to define the optimal conditions for the selected enzyme, at the beginning of the process the mixture is often heated to about 85–95 °C for 5–20 min to terminate the endogenous enzyme activity (Zamora-Sillero et al., 2018) so that effects from commercial enzymes on resultant hydrolysates can be investigated and to facilitate the removal of the fat present in the material (Bhaskar et al., 2008).

The choice of the enzyme is usually determined by a balance between efficacy and cost-effectiveness. Enzyme selection is essential for the preparation of the functional properties of protein hydrolysates.

In fact, protease specificity affects the size, the amount, the composition of free amino acid and peptides and their amino acid sequence which influences the bioactivity of the obtained hydrolysate. Some industrial food-grade proteinases derived from microorganisms have been used to produce bioactive peptides by enzymatic hydrolysis such as Alcalase®, Flavourzyme®, and Protamex®, as well as enzymes from plants such as papain or bromelain and animal sources such as pepsin and trypsin (Zamora-Sillero et al., 2018).

Enzymes with an optimal working pH in the acidic range such as pepsin were preferred in earlier times as the low pH could also inhibit microbial growth. However, the acidic pH atmosphere also led to low protein recoveries, low nutritional values due to the destruction of the essential amino acid tryptophan and low functionalities due to excess hydrolyzation (He et al., 2013; Kristinsson & Rasco, 2000). Therefore, enzymes with an optimal reaction pH close to neutral, such as Alcalase, Neutrase and Flavourzyme, are now used more extensively (He et al., 2013).

In comparison with animal or plant-derived enzymes, microbial enzymes have several advantages, including greater pH and temperature stabilities and they have been reported to be most efficient in the hydrolyzation of fish proteins (He et al., 2013). This has been confirmed also by Dai et al. (2012) for mussel protein hydrolysate: he found that the DH of mussel protein hydrolysate treated with Alcalase was significantly higher than others treated with Protamex, Trypsin, Neutrase, Papain, Flavourzyme. On the other hand, it has been observed that thirty minutes of hydrolysis using pepsin produced large amounts of small-molecular-weight peptides with MW around 5 kDa and small amounts of peptides in the range 97-14 kDa; meanwhile, Alcalase produced large amounts of peptides in the high-molecular-weight range and very low amounts of short peptide fragments with MW around 5 kDa (Jayaprakash and Perera, 2020).

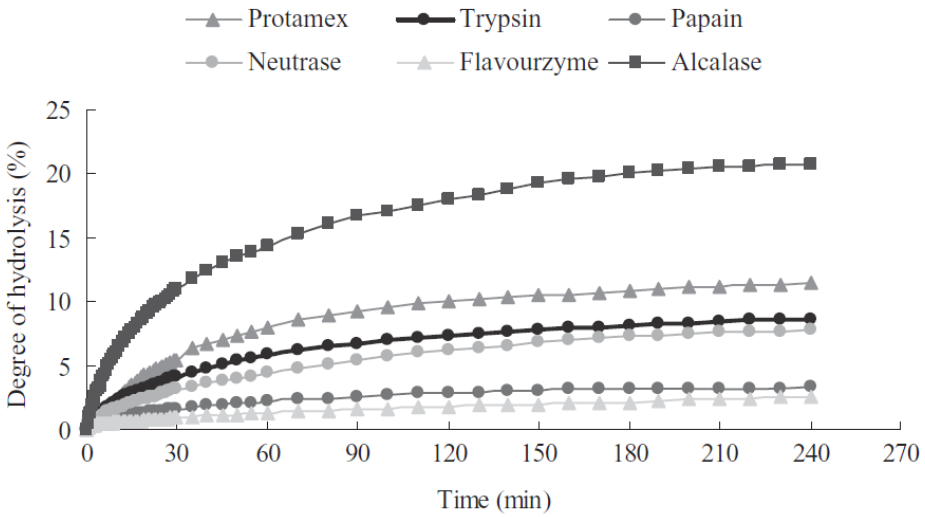


***Figure 7**: Enzymatic hydrolysis of mussel protein with different proteases at E:S of 1.5/100 (w/w) and mussel protein concentration at 5% (Dai et al., 2012).*

The E:S ratio and processing time are set up according to desired functionalities and protein recovery of the final protein hydrolysates (He et al., 2013). In general, several authors showed that the increase in the enzyme concentration corresponds to an increase in the DH (Quaglia & Orban, 1987; Benjakul & Morrissey, 1997; Klompong at al., 2007; Vázquez et al., 2020) and nitrogen recovery (Benjakul & Morrissey, 1997). However, a reduction in rate of DH and NR with increasing enzyme concentration was observed by Benjakul & Morrissey, 1997.

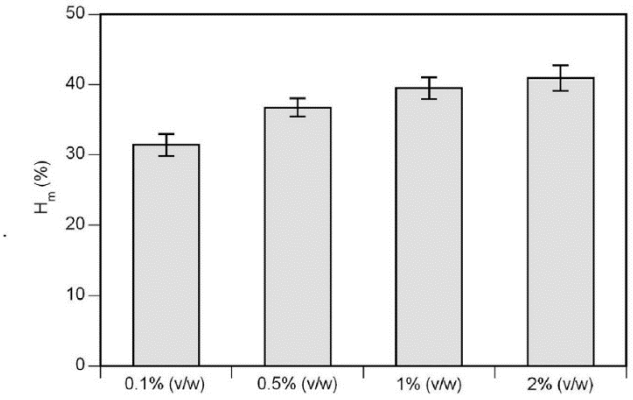


***Figure 8**: Optimization studies of Alcalase hydrolysis of blue whiting discards. Individual effect of Alcalase concentration over the maximum degree of hydrolysis (Vázquez et al., 2020).*

2.4.3.3 pH and temperature

As already seen, processing temperature and pH are adjusted to the optimal values of the selected enzyme. Each type of enzyme has determined optimum usage conditions declared by the provider. On the other hand, different authors determined more specific values coming from the optimization

of enzymatic hydrolysis with fish or mollusks as raw materials. In general, it can be summarized that Alcalase is more effective at temperatures between 50 and 60°C and pH between 8 and 9.5; Protamex is more effective at temperatures between 40 and 50 and neutral pH; for Neutrase that works at neutral pH the optimum temperature is 50-55°C; for Flavourzyme the best temperature is between 50 and less than 55 and the optimum pH is around 7 and 8.

***Table 11**: Optimum working range for the most used enzymes. AU = Anson Units, measure of the activity of the enzyme.*

Enzyme	Provider	T (°C)	pH	Reference
Alcalase® 2.4 L (2.4 AU/g)	Novozymes	50	8	Camargo et al., 2021
	Novozymes	58.5	8.1	Sierra-Lopera & Zapata-Montoya, 2021
	Novozymes	60	8.65	Vázquez et al., 2020
Alcalase 2.4L	Novo Nordisk's Enzyme Business	60	9	Dai et al., 2012
Alcalase® (0.6AU/g)	Novo industry	50	8.5	Bhaskar et al., 2008
Alcalase 2.4 L	Novo Nordisk Biochem North America	60	9.5	Benjakul & Morrissey, 1997
Protamex®	Sigma Aldrich	50	7	Camargo et al., 2021
Protamex	Wuxi Enzymes Co.	40	7	Dai et al., 2012
Protamex®	Novozymes	51	6.85	Silva et al., 2010
Neutrase 1.5 L	Novo Nordisk's Enzyme Business	50	7	Dai et al., 2012
Neutrase 0.5 L	Novo Nordisk Biochem North America	55	7	Benjakul & Morrissey, 1997
Flavourzyme 500L	Sigma Aldrich	53.8	8.3	Sierra-Lopera & Zapata-Montoya, 2021
Flavourzyme	Wuxi Enzymes Co.	50	7	Dai et al., 2012

As already stated, at lab scale, the optimum temperature for the selected enzyme is typically reached by placing the vessels in a thermoregulated environment, like a water bath, while at pilot and industrial scales thermostatically stirred-batch reactors are used. However, the achievement and maintenance of the predetermined temperature have a high energy demand, that results on high environmental impacts related to power consumption, especially when a relatively high operating temperature is used.

For controlling the pH, the following reagents are typically used: sodium hydroxide (NaOH), and hydrochloric acid (HCl) (Jayaprakash and Perera, 2020), depending on whether the purpose is to increase or decrease it, in order to obtain the optimal value for the selected enzyme. However, it should be noted that the addition of NaOH or HCl to control the pH contributes to additional costs for chemicals and this could be even more relevant in processes at pilot and industrial scale. Furthermore, some authors found a relatively high ash content of fish protein hydrolysates (up to 27% of total composition of dried product) due to usage of added acid or base for adjustment of pH (Chalamaiah et al., 2012), which corresponds to a high inorganic content.

2.4.3.4 Time

Time of hydrolysis reported by different authors ranges from less than 1 hour to 6 hours (Table 13

Table 13). However, Jayaprakash and Perera (2020) considered that within 30 min, most of the proteins were hydrolyzed by the pepsin and Alcalase enzymes. The results obtained were similar to those obtained by Dai et al. (2012) with Mytilus edulis (blue mussel). Using yellow stripe trevally, Klompong et al. (2007) found that DH (%) gradually increased when hydrolyzed for 5 to 20 min and reached a steady phase within 20 min, after which no further hydrolysis took place. Instead, Camargo et al. (2021) reported that the action of the hydrolysis of fish muscle and skin showed a high rate for the first 100 min and a slower rate of hydrolysis after 150 min until reaching a steady state.

Also, the conditions needed to terminate hydrolysis must be defined. Typically, the reaction is terminated by a sudden inactivation of the enzyme. Typically, for thermosensitive enzymes, the inactivation is accomplished by the elevated temperatures of around 75–100 °C applied for 5–30 min, depending on the type of enzyme (Petrova et al., 2018). Typically, water bath or hot plates are used to this purpose. However, terminating the reaction by thermal means is undesirable because of the

effects of heat denaturation on the protein that leads to exposure of hydrophobic residues and subsequently protein aggregation. Denaturation is usually undesirable because it results in altered physicochemical properties, particularly a loss in protein solubility and functionality (Kristinsson & Rasco, 2000).

2.4.4 Final treatment

2.4.4.1 Solid – liquid separation

The next step is to separate the solubilized protein from the non-hydrolyzed material (sludge of solids and non-soluble proteins). The solids are separated from the liquid protein mixture mainly in order to purify the hydrolysates from insoluble while the fish oil fraction is removed to avoid undesirable fat oxidation processes in the final FPH.

Centrifugation or filtration are commonly used to this purpose. Quite often filtration precedes centrifugation for the removal of gross material like bones (Naik et al., 2020; Vázquez et al., 2020; Nam et al., 2020; Bhaskar et al., 2008).

Centrifugation separates the mixture into three fractions: sludge of solids and non-soluble proteins at the bottom, aqueous layer at the middle and lipid phase at the top. The oil phase over the aqueous phase should be removed and the soluble fraction collected (Zamora-Sillero et al., 2018; Kristinsson and Rasco, 2000). Centrifugation in the processing of FPH can be done in one or several steps according to the desired quality of FPH. He et al. (2013) reported that the centrifugation at 4000g for at least 20 min is effective in separating the mixture into the three fractions, but several authors used different speed (Tab. 17).

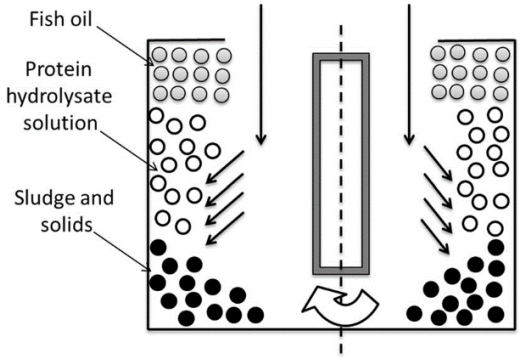


***Figure 9**: Principal scheme of simplest centrifuge (Petrova et al., 2018).*

In more advanced technologies, when a certain quality of final FPH is needed, the filtration of small chemical particles from the hydrolyzation mixture can be provided (microfiltration, ultrafiltration and nanofiltration from bigger to smaller particle separation correspondingly) (Petrova et al., 2018).

Italian legislation provides a maximum molecular weight of 10 kDa for amino acids and peptides falling into the nitrogenous organic fertilizers. In this case, UF membranes of intermediate molecular weight cut-offs (MWCO) (about 4-8 kDa), allow hydrolysate fractionation for the enrichment of specific molecular weights. Lower molecular weight FPHs demonstrates higher antioxidant activity (Halim et al., 2016).

At pilot scale, vertical centrifuges (Pérez-Martín et al., 2020), decanters (Beaulieu et al., 2013) or tricanters (Vázquez et al., 2020) are typically used for solid-liquid separation. Several technology providers suggest using a tricanter as first stage, optionally followed by a disk stack centrifuge for further oil removal, obtaining high purity of final product (Flottweg; Gea).

2.4.4.2 Concentration

Concentration is used to reduce water content in the protein mixtures before drying and, thus, reduce the energy costs of the drying step. However, at the same time, preliminary water removal by concentration needs additional equipment and energy to provide the dehydration process, thus, the need of such an extra treatment is decided by a producer for each certain case.

Concentrators are apparatuses where the liquid solution to be concentrated goes through a large specific surface which is heated up. Thus, going through the heated surfaces of concentrators, moisture is evaporated and removed to the atmosphere by vapor.

The temperatures used for evaporation and evaporation rate vary for different FPH depending on the composition of the mixture and other properties influencing the boiling point such as DH, protein source or the choice of enzyme.

Mostly used modifications of concentrators are falling and rising film evaporators which differ from each other by the way of product supply and, correspondingly, by the construction (Petrova et al., 2018).

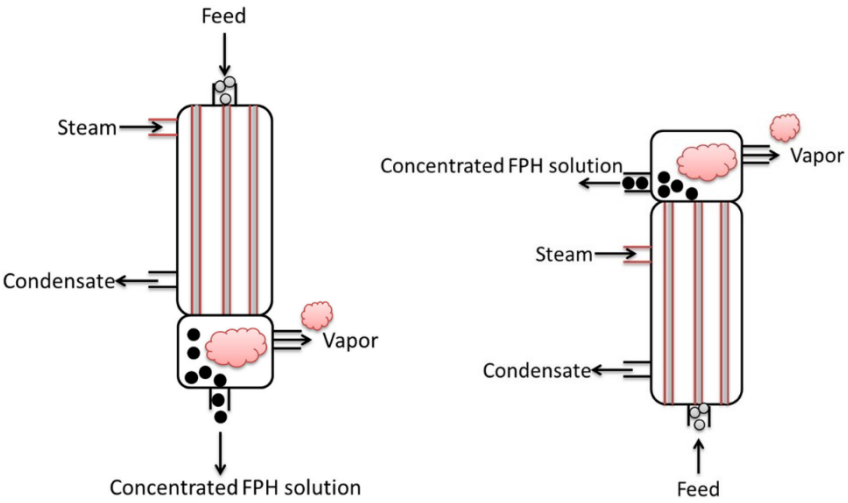


***Figure 10**: Principal scheme of falling film evaporator (left) and rising film evaporator (right) (Petrova et al., 2018).*

For concentration at pilot scale, several types of evaporators can be employed, among all ball vacuum concentration tanker (Wang et al., 2010; Chen et al., 2017), and mechanical vapor recompression evaporator (Colantoni et al., 2017).

2.4.4.3 Drying

FPH are marketed in two forms: liquid and dried. Liquid FPH is a watering mixture of hydrolyzed proteins, which contains up to 90% of moisture if not previously concentrated. FPH in a liquid form is highly unstable for a long-term storage and, moreover, it is difficult to be transported (Petrova et al., 2018). In fact, liquid forms of fish protein hydrolysates can spoil quickly due to the high water content and the ease with which bacteria utilize proteins as substrates (He et al., 2013). Thus, dried FPH is preferable due to a longer shelf-life, easier storage and transportation. Nevertheless, at the same time, the removal of such a big amount of water from liquid FPH is a difficult and costly task, which is one of the challenges of dried FPH production (Petrova et al., 2018).

The drying process of FPH is usually accomplished by a limited number of drying apparatuses: spray dryers which provide convectional drying, and vacuum freeze dryers and roller drum dryers which utilize contact heat supply. The choice of drying method at laboratory scale generally depends on the availability of drying equipment at a certain laboratory and not depends on the purposes of the research or the properties of studied FPH. Generally, the mostly used drying equipment for research purposes is spray and freeze dryers (Petrova et al., 2018).

At large scale, from the experience, the authors suggest convection spray dryers as the most used drying systems due to a satisfactory productivity, reasonable quality of the final FPH and a relative

easiness of operation. Spray driers with capacity 10 kg/h were used for drying the final product by Wang et al. (2010) and Chen et al. (2017).

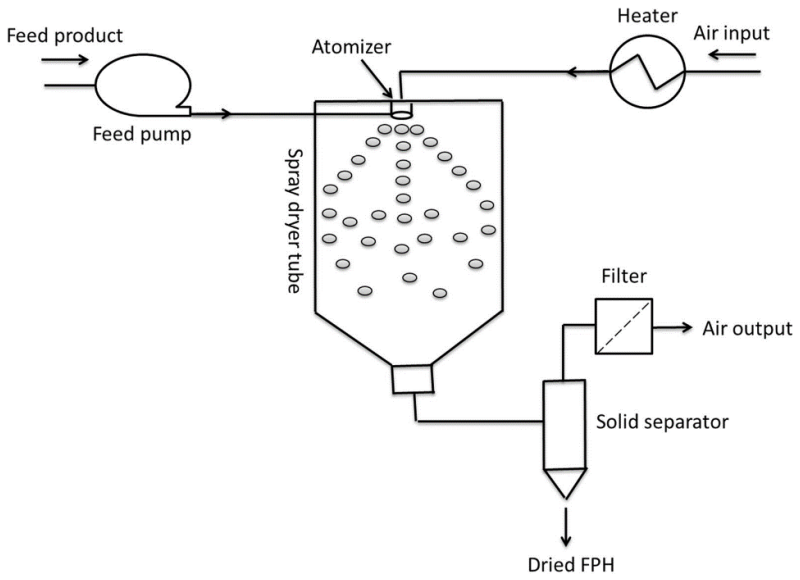


***Figure 11**: Principal scheme of spray dryer (Petrova et al., 2018).*

After this step, the hydrolysates are normally stored at 4°C or lower, in some cases with vacuum packaging mainly to prevent lipid oxidation (Petrova et al., 2018; He et al. 2013) until further analysis or application.

2.4.5 Summary of literature case studies

Table 12, Table 13, and Table 14 respectively provide a comparison between the pre-treatment procedures, the optimal conditions for enzymatic hydrolysis from DH and/or nitrogen recovery point of view, and the final treatment of PH determined by different authors. In many cases, different conditions were tested in order to optimize the process, but only the optimal ones are presented for synthesis. Case studies are divided according to the scale of the process. Laboratory-scale studies are analyzed in terms of optimal operating conditions, while pilot-scale studies serve as examples for the biorefinery covered by the SEA2LAND project.

Production of PH at pilot or industrial scale is usually performed by using the optimal parameters for enzymatic hydrolysis selected from laboratory tests, with small variations aimed to make the process more sustainable from environmental and economical point of view.

in summary, it can be concluded that typically the same amount of substrate and water is used, since it should be removed at the end of the process by concentration or drying, the main energy consumer step in the technological chain (Petrova et al., 2018). In fact, due to huge energy supply to the drying,

energy saving in the drying process should become an important issue of FPH production. Some pilot models of spray dryers have specific energy consumption in the range between 3000 and 5500 kJ/kg of evaporated water, but the real heat consumption by industrial models of spray dryers is much higher (Petrova et al., 2018). Usually, endogenous enzymes are not deactivated mainly to reduce the energy consumption for heating the hydrolyzing mixture and to improve the performance of the exogenous enzyme, whose dose should be as low as possible. In addition, from an industrial point of view, hydrolysis carried out without any pH adjustment would be economically desirable.

***Table 12**: Synthesis of pre-treatments by different authors.*

Ref.	**Source**	**Pre-treatment**		
-	-	**Homog.**	**Other**	**S:W**
Lab scale				
Jayaprakash & Perera, 2020 (1)	Green-lipped mussel meat	Homogenizer, 1500 rpm, 5 min	-	1:10 (w:v)
Naik et al., 2020 (2)	Mussel	Blender, 1 min	-	1:2 (g:ml)
Silva et al., 2010 (3)	Mussel meat	Manual blender	-	1:2 (w:w)
Dai et al., 2012 (4)	Mussel meat	Homogenizer	Minced, freeze drying, defatting, then vacuum-dried and sieving	5:100 (w:v)
Nisov et al., 2022 (5)	Baltic herring (BH), descaled roach (DR)	Kitchen mixer, 60 s	-	1:1 (w:w)
Camargo et al., 2021 (6)	Whitemouth croaker (WC) and Banded croaker (BC) (muscle and skin)	Blending	-	-
Sierra-Lopera & Zapata-Montoya, 2021 (7)	Red tilapia scales	Endless screw mill to 850 µm after drying	Disinfection, frozen, dried at 60°C for 24 h	45 g/L
Vázquez et al., 2020 (8)	Galician fishing fleet	Grinding	-	1:2 (w:v)

Nam et al., 2020 (9)	Catfish by-products	Grinding to 1 cm	-	1:1 (g:ml)
Egerton et al., 2018 (10)	Blue whiting	Robot Coupe blixer 2	-	1:2 (w:v)
Bhaskar et al., 2008 (11)	Indian major carp	Waring blender	Centrifugation for oil separation; extraction with distilled water (1:1 w/v; 3 times); centrifugation	
Klompong at al., 2007 (12)	Yellow stripe trevally	Grinding, 0.4 cm holes	Defatting	1:20.43 (g:ml)
Šližyte et al., 2005 (13)	Cod by-products	Manual mincer 10 mm holes	-	1:1 (g:ml)
Benjakul & Morrissey, 1997 (14)	Pacific whiting solid processing wastes	Grinding	-	1:1 (w:v buffer)
Pilot scale				
Pérez-Martín et al., 2020; Vázquez et al., 2017 (1)	Discards and fishery by-products	Grinding	Separation of viscera, heads, skins, and bones from muscle	1:5
Vázquez et al., 2020 (2)	Five fish discard species	-	-	1:2 (w:v
Chen et al., 2017 (3)	Fish skin	Mincing	-	1:1 (w:w)
Beaulieu et al., 2013 (4)	Blue mussels waste	-	-	1:1.6 (w:w)
Wang et al., 2010 (5)	Oyster	Mincing	-	1:1 (w:w

***Table 13**: Synthesis of hydrolysis parameters by different authors.*

Ref.	**Hydrolysis**						
-	**End. E deact.**	**E; E:S**	**°C**	**pH**	**rpm**	**h**	**Ex. E deact.**
Lab scale							
1	100°C, 10 min	Alcalase; 1:10 (w:w S protein)	60	8.5	-	4	100°C, 10 min
2	80°C, 10 min	Protamex; 1:50 (w:v)	35	7	130	1.5	95°C, 10 min
3	-	Protamex; 4.5:100	51	6.85	-	3	85°C, 10 min
4	-	Alcalase 2.4L; 1.64:100	57	9.12	-	4	95°C, 15 min
5	-	Protamex, Neutrase, Corolase; 1.4 nkat/ground fish	50	-	-	0.5	75°C, 15 min
6	80°C, 20 min	Alcalase 2.4L; 2:100 w:w	50	8	-	5	80°C, 20 min
7	-	Alcalase 2.4L; 4.4 g/L	58.5	8.1	350	3	90°C, 10 min
8	-	Alcalase 2.4L; 1:100 (v:w)	60	8.65	200	4	90°C, 15 min
9	-	Alcalase; 0.4:100 (ml:g)	50	-	-	3	-
10	-	Flavourzyme® 500L (6176.00 AzU/mL); 39054 AzU	50	7	300	3	90°C, 20 min
11	85°C, 20 min	Alcalase 0.6L; 1:100 (v:w)	55	8	-	2	85°C, 20 min
12	-	Alcalase; 10:100 w:w	60	8.5	-	20 min	90°C, 15 min

13	Microwave, 900W, 95°C, 5 min	Flavourzyme; 0.1:100 w:w	50	Nat.	150	1	Microwave 90°C, 5 min
14	-	Alcalase 2.4L; 20AU/kg waste	60	9.5	-	1	90°C, 5 min
Pilot scale							
1	-	Esperase (E) or Alcalase (A) 1:100 (v:w)	60.8 (E) 64.6 (A)	8.9 (E) 9.4 (A)	200	6	90°C, 15 min
2	-	Alcalase 2.4L; 1:100 (v:w)	60	8.65	200	4	90°C, 15 min
3	-	20000 U/kg fresh protein substrate	40	8	150	4	90°C, 15 min
4	-	Protamex; 1:100	45	-	-	1	90°C
5	-	400000 U/kg fresh protein substrate	50	7.5	200	5	90°C, 15 min

***Table 14**: Synthesis of final treatments by different authors.*

Ref.	**Final treatment**		**Results**	
-	**Solid-Liquid separation**	**Concentration and drying**	**DH (%)**	**N/protein recovery**
Lab scale				
1	Centrifuge 1200xg, 10 min	Freeze-drying	8-10% (OPA)	-
2	Filtration	Freeze-drying	2.41-7.55% (pH-stat)	-
3	Centrifuge 3500 rpm, 20 min	-	26.5% (pH-stat)	P: 65%

4	Centrifuge 4000xg, 20 min	Freeze-drying	20% (pH-stat)	P: 85.77%
5	Centrifuge 4000xg, 15 min (twice)	Freeze-drying	11.7-16.5% DR, 6.8-8.4% BH (OPA)	-
6	-	Freeze-drying	40.9% BC, 42.8% WC (pH-stat)	-
7	Centrifuge 1160g, 20 min, 20°C	-	15.65% (pH-stat)	-
8	Filtration 100 µm; centrifuge 15000g, 20 min (pre and post sterilization)	-	23-47% (pH-stat)	-
9	Sieving; centrifuge 5000 rpm, 15 min, 4°C	-	30.69% (DNFB)	N: 82.24%
10	Centrifuge 4566g, 0°C, 20 min; filtration Whatman paper No. 2	Freeze-drying	41.47% (TNBS)	-
11	Filtration, centrifugation	Spray-drying	51.44% (SN-TCA)	N: >71%
12	Centrifuge 2000g, 10 min	Freeze-drying	40% (pH-stat)	-
13	Centrifuge 2250g, 30 min	-	19.5-33.7% depending on the fraction (Formol titration)	-
14	Centrifuge 3000g, 10 min; lipid layer skimming with 2-layers of cheese cloth	Freeze-drying	-	N: 65%
Pilot scale				
1	Filtration; centrifugation	Spray drying	>30% (pH stat)	-
2	Filtration;	-	23-47%	-

	centrifugation (tricanter)		(pH-stat)	
3	Plate-frame pressure filtration	Ball vacuum concentration; spray drying	-	-
4	Clarifying decanter 3500g; centrifuge 11000g; nano-filtration of supernatant	-	-	-
5	Centrifuge 9000g, 20 min	Ball vacuum concentration; spray drying	-	-

2.4.6 Protein Hydrolysates composition

Several research documents have been reported the chemical composition of fish protein hydrolysates that are prepared from various fish protein sources. Differences in the composition depend mainly on the original substrate and enzyme used, the possible pretreatments such as fat removal, the addition of chemicals for pH control and the physical state of the product (spray/freeze dried or liquid PH).

Many researchers reported the protein content of fish protein hydrolysates ranged between 60% to 90% of total composition of dried FPH (Chalamaiah et al., 2012), specifying that this high protein content is due to the solubilization of proteins during hydrolysis and removal of insoluble solid matter by centrifugation. Vázquez et al. (2020) reported the concentration of total soluble protein determined by Lowry method slightly lower than the total protein determined as total nitrogen x 6.26, ranging from 38.1 to 55.4 g/L on fresh basis depending on the specie. For mussel PHs, Naik et al. (2020) found that the protein content was around 30% and 40% (w:w) for most of the samples.

Several studies reported the fat content for various fish protein hydrolysates were below 5% of the total composition of dried product. Only few reports described the fat content above 5% level for fish protein hydrolysates. Also in this case, the low-fat content of fish protein hydrolysates is because of removal of lipids with insoluble protein fractions by centrifugation (Chalamaiah et al., 2012). Concerning mollusks based PHs, the lipid content of mussel meat hydrolysates was between 0.97% and 2% of dried product in Naik et al. (2020).

Most of the studies demonstrated that protein hydrolysates from various fish proteins contain moisture below 10% of the total dried product. The low moisture content of protein hydrolysates is related to the type of sample and to the higher temperatures employed during the process of evaporation and

spray drying (Chalamaiah et al., 2012). When concentration and/or spray drying is not applied, the humidity results to be above 90% on fresh basis (Vázquez et al., 2020).

The ash content of fish protein hydrolysates was reported by many studies ranged between 0.45% to 27% of total composition. The relatively high ash content of fish protein hydrolysates is due to usage of added acid or base for adjustment of pH of medium (Chalamaiah et al., 2012).

The percentage of organic matter reported by Vázquez et al., 2020 on liquid hydrolysates from different fish species treated with Alcalase is between 4.8 and 6.4% on fresh basis.

Italian legislation provides also minimum requirements for free amino acids content of at least 8% (w:w) for amino acids and peptides (solid or fluid) and at least 10% in case of biostimulant produced from hydrolyzed animal epithelium. Egerton et al. (2018) reported the total and free amino acids (g/100g protein) of blue whiting protein hydrolysates with different commercial proteases. Free amino acids ranged from 10.63 with Alcalase, 12.1 with Protamex, 19.44 with Savinase, and 43.63 with Flavourzyme, whereas total amino acids were between 68.15 with Flavourzyme, 103.16 with Protamex, 108.67 with Savinase, and 111.63 with Alcalase. The total amino acid for mussel meat protein hydrolysates produced with Protamex were 66.76 g/100g protein in Silva et al. (2010); 55.57 g/100 g dried hydrolysate in Dai et al. (2012).

***Table 15**: Protein Hydrolysates composition from literature.*

Composition		**Product**	**Reference**
Protein content	60-90%	Dried FPH	Chalamaiah et al., 2012
	38.1-55.4 g/L	Liquid FPH	Vázquez et al., 2020
	30-40%	Dried mussel PH	Naik et al., 2020
Fat content	<5%	Dried FPH	Chalamaiah et al., 2012
	<1-2%	Dried mussel PH	Naik et al., 2020
Moisture	<10%	Dried FPH	Chalamaiah et al., 2012
	>90%	Liquid FPH	Vázquez et al., 2020
Ash content	0.45-27%	Dried FPH	Chalamaiah et al., 2012
Organic matter	4.8-6.4%	Liquid FPH	Vázquez et al., 2020
Free amino acids	10.63-43.63 g/100g protein	Dried FPH	Egerton et al., 2018
Total amino acids	68.15-111.63 g/100g protein	Dried FPH	Egerton et al., 2018

	66.76 g/100g protein	Liquid mussel PH	Silva et al., 2010
	55.57 g/100g mussel PH	Dried mussel PH	Dai et al., 2012

2.5 Life Cycle Assessment

According to the standard ISO 14040:2006, "Life Cycle Assessment (LCA) is a technique to quantify the environmental aspects and potential impacts associated with a product: by the collection of an inventory with the flows in and out the system, the environmental assessment of the impacts liked to these flows, and the interpretation of the results in relation with the objectives of the study."

This tool applies the principle of the Life Cycle Thinking, with which companies are able to identify the multiple environmental, economic, and social issues across the entire life cycle of a product, or system in general. The final aim of this kind of approach is the sustainable development, production, and consumption, which means being able to meet the needs of the present without compromising the ability of future generations to meet their needs. Sustainability is achieved only by finding a balance between the environmental, economic, and social domains, creating an environmental effective, economic affordable and socially acceptable system.

From technical point of view, the LCA represents an important tool for the quantitative comparison of two or more technical systems (scenarios), in terms of environmental impact. In this sense it can drive the decision making, the strategic planning, and the public policy making towards a more sustainable development and improvement of processes and products.

To this purpose, fundamentals of the ISO 14040 and 14044 must be implemented. The LCA framework operates with four separate phases: The definition of the objectives and scope of the study, the compilation of an inventory of all inputs and outputs of a product system, the evaluation of the potential environmental impacts and the interpretation of the results in relation to the objectives of the study (Hauschild, Rosenbaum and Olsen 2018). The relationship between the phases is illustrated in Figure 12.

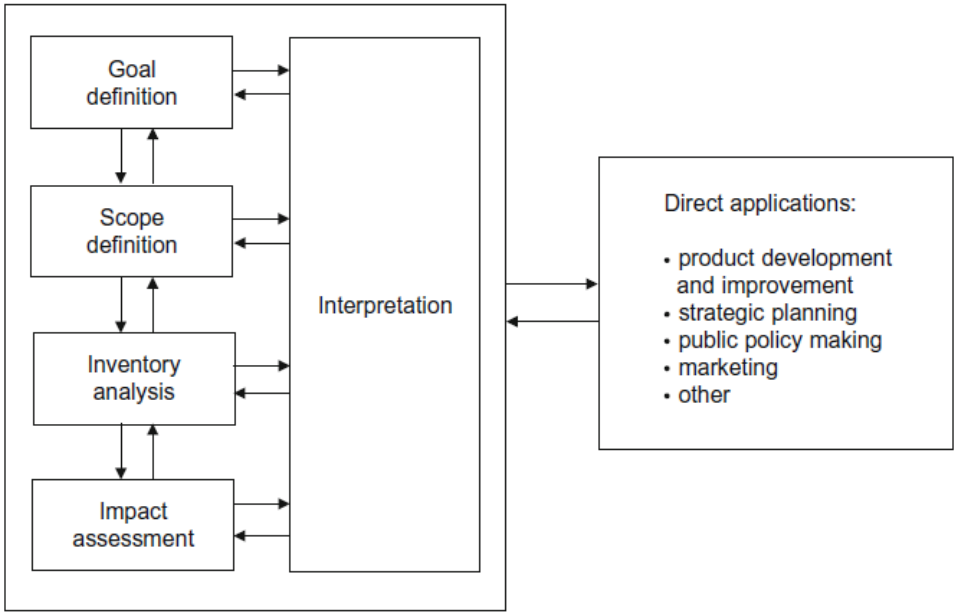


***Figure 12**: Framework of LCA modified from ISO 14040 standard.*

Hence, the first step consists of the goal definition, that sets the context of the LCA study and is the basis of the scope that includes the definition of the:

- system, group of processes that working together allow to create a product with a determined function, defining the flow diagram of the process being studied;
- system function, the product application and thus it will determine partially the functional unit of the study;
- functional unit (FU), that identifies the qualitative and quantitative aspects of the function and is the reference unit for the calculations of the flows (outgoing and incoming) of material and energy in the system and allows the comparison with the possible alternatives;
- system boundaries, that means deciding which activities and processes belong to the life cycle of the product under study.

LCA divides the world into Technosphere and Ecosphere (Figure 13). The Technosphere can be understood as everything that is intentionally “manmade” and also includes processes that are natural in origin but manipulated by humans. All unit processes of an LCI model belong to the Technosphere. The ecosphere is sometimes referred to as “the environment” or “nature” and can be understood as everything which is not intentionally “man-made”. Elementary flows are per definition the only flows that go across the boundary between the Technosphere and the Ecosphere (Hauschild, Rosenbaum and Olsen 2018).

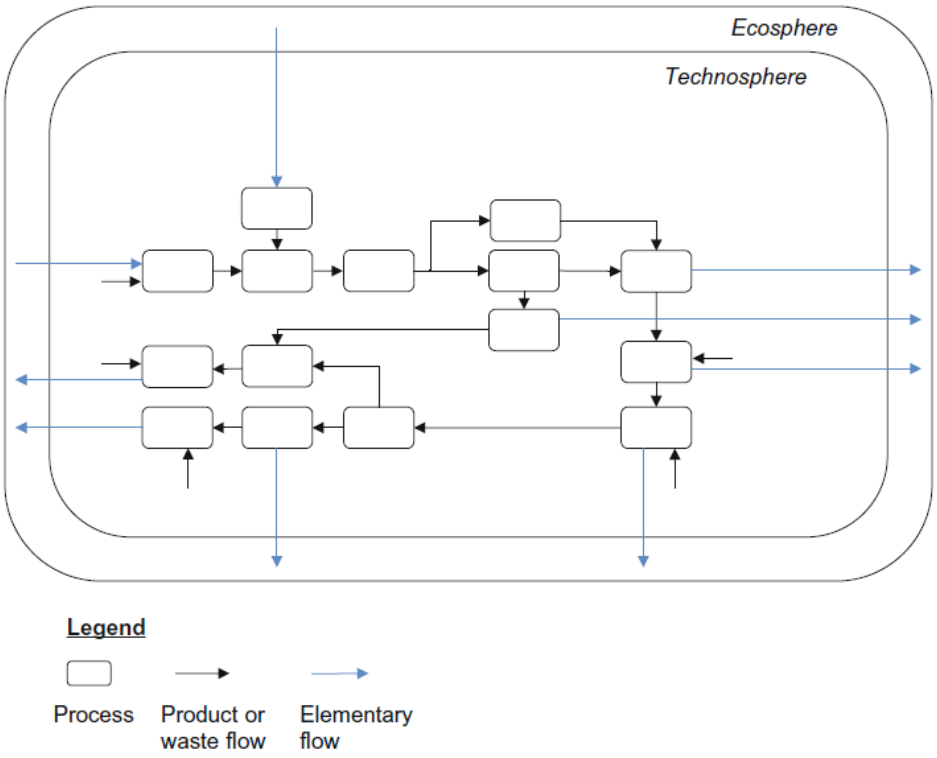


***Figure 13**: Division between ecosphere and Technosphere for a generic product system. Elementary flows are represented by blue arrows, while flows within the Technosphere are in black (Hauschild, Rosenbaum and Olsen 2018).*

Concerning the system boundaries, defined within the Technosphere, according to the purpose of the study, it is possible to distinguish between three different approaches:

- gate to gate, if the LCA is related only to the production phase;
- cradle to gate, if the LCA also involves the unit processes upstream of the production phase, like the extraction and preparation of raw materials and resources exploited;
- cradle to grave, if the LCA involves all the life cycle chain of the product, starting from the extraction of resources to the final disposal.

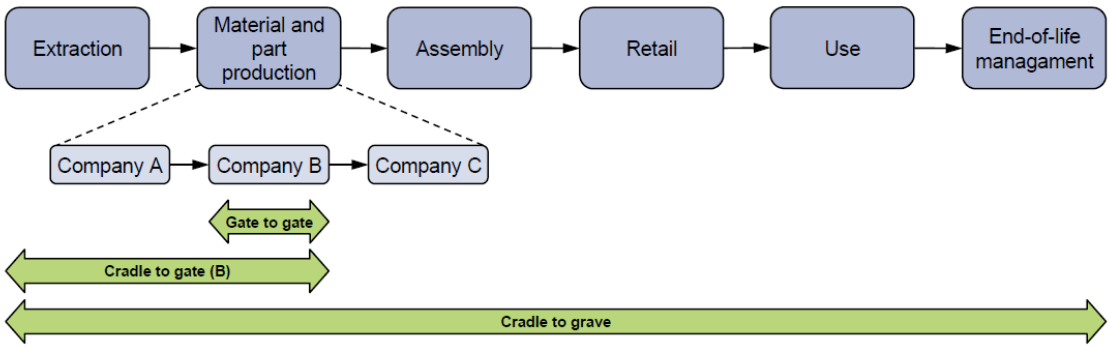


***Figure 14**: Cradle to grave, cradle to gate and gate to gate data sets as parts of the complete life cycle (JRC, EU 2010).*

Besides, it is useful to distinguish between unit processes belonging to the foreground and background system. The foreground system is commonly defined as comprising those processes of a product system that are specific to it. The foreground system is largely modelled using primary data, i.e., data collected first-hand by the LCA practitioner, e.g., obtained through the commissioner of the study. The background system, in contrast, is commonly defined as those processes of a system that are not specific to it. Such processes take part in numerous product systems besides the one studied. Examples are energy, heat, and chemicals production and transport, or the waste management systems. The background system is typically modelled using LCI databases, which contain average industry data representing the process in specific nations or regions. Several LCI databases are available to use in LCA software.

The purpose of the second step, the inventory analysis (LCI), is to highlight all the physical flows referable to the various phases relating to the product, in terms of input of resources, materials, semi-products and products and the output of emissions, waste, and valuable products for the product system. In this way, important elements are identified to be taken into consideration for the creation of a real inventory of substances and weights (inventory table). In particular, resources and emissions, since not exchanged between unit processes, are referred to as elementary flows.

The result of LCI is an inventory of the aggregated quantities of elementary flows, separated into resources and emissions, from all the unit processes within the system boundary.

Once finalized the inventory, the impact assessment translates the physical flows and interventions of the product system into impacts on the environment using knowledge and models from environmental science. Impact assessment turns a Life Cycle Inventory into a Life Cycle Assessment. It consists of five elements of which the first three are mandatory according to the ISO 14040 standard:

1) Selection of impact categories representative of the assessment parameters that were chosen as part of the scope definition. Which impacts do I need to assess?
2) Classification of elementary flows from the inventory by assigning them to impact categories according to their ability to contribute by impacting the chosen indicator. Which impact(s) does each LCI result contribute to?
3) Characterization using environmental models for the impact category to quantify the ability of each of the assigned elementary flows to impact the indicator of the category. The resulting characterized impact scores are expressed in a common metric for the impact category. How much does each LCI result contribute?
4) Normalization, e.g., expressing LCIA results relative to those of a reference system. Is that much?
5) Grouping or weighting, e.g., aggregating several impact indicators results into a group.

As the final step, the interpretation phase considers both outcomes of the inventory analysis and the impact assessment elements characterization and, possibly, normalization and weighting. The interpretation must be done with the goal and scope definition in mind and respect the restrictions that the scoping choices impose on a meaningful interpretation of the results, e.g., due to geographical, temporal or technological assumptions. The effects of the interpretation may lead to a new iteration round of the study, including a possible adjustment of the original goal.

2.5.1 LCA for protein hydrolysis

For sustainability assessment, LCA methodology has been applied in this study to evaluate the energy use and the environmental impact of the production process of enzymatically produced protein hydrolysates. In particular, it provides a methodology for comparing the energy use, greenhouse gas emissions, and water use associated with protein hydrolysate production starting from different substrates, under the same operating conditions, and it would be a valuable tool for understanding the real convenience of a potential industrial pathway. Specifically, this methodology will help to understand which are the most relevant impact categories, and which are the main contributors (electricity, heat, water use…) and the contributions of each life cycle stage to each impact category. In this way, LCA also allows to identify the main hotspots in the production chain to propose improvement options (García-Santiago et al., 2020). In addition, it is possible to assess the effect of plausible parameter variability, like the technical efficiency in recovering nitrogen, the use of a particular type of equipment for concentration and so on (sensitivity analysis).

Unfortunately, LCA studies on the environmental impact of production processes of protein hydrolysate-based biostimulants are rarely available in literature, and each study presents its own boundaries, more or less extended, depending on the goal.

For example, García-Santiago et al. (2020) studied the environmental impacts of different valorization schemes implemented and tested on a fully operative real marine biorefinery located in an important Spanish fishing port, using a gate-to-gate perspective. These authors used the characterization factors reported by the ReCiPe Midpoint (H) method (Goedkoop et al., 2013), and the environmental categories included in the LCA analysis were: climate change; terrestrial acidification; freshwater and marine eutrophication; human toxicity; freshwater and marine ecotoxicity; water depletion; fossil depletion, and the normalized index. These categories were identified as the most representative for the valorization of marine biomass. In particular the most relevant categories were climate change (115.23 kg CO_2-eq), fossil depletion (30.04 kg oil eq), and human toxicity (26.99 kg 1.4-DB eq), with respect 100 kg of whole fish processed. Electricity consumption was identified as the main environmental hotspot, with reaction and drying (stages of high energy demand) being the units that generate the largest environmental impacts.

Colantoni et al. (2017) analyzed the environmental impact of chemically-produced PH from leather waste vs. enzymatically-produced PH from legume grains. These authors also identified the hydrolysis as the most impacting stage of the process. The results obtained in terms of CO_2 emissions, fossil energy consumption and water use through the application of LCA showed that the production process of the animal-derived protein hydrolysate was characterized by higher energy use (+26%) and environmental impact (+57% of CO_2 emissions) in comparison with the enzymatic production process of lupine-derived protein hydrolysate. The result is mainly due to the different processes of hydrolysis, which requires higher temperature, pressure, and chemical inputs in chemical hydrolysis in comparison to an enzymatic hydrolysis process. In particular, Colantoni et al. (2017) for PH production from lupine grains determined an impact on climate change equal to 586.9 g CO_2-Eq/kg of PH, excluding the agricultural phase necessary to the growth of lupine grains, and 743.2 g CO_2-Eq/kg of PH considering the whole process. In any case, the calculated impacts are much lower than the ones which the same authors retrieved for PH production from leather wastes through chemical hydrolysis (1532.2 g CO_2-Eq/kg of PH), only at industrial scale because the collagen is considered a waste product of the leather industry.

2.6 Economic assessment

Economic performance metrics can be divided into three categories based on life cycle phases:

1) project initiation and construction,
2) operation and maintenance (O&M),
3) end-life costs.

Project initiation and construction costs include not only capital expenditure (CAPEX) for infrastructure and equipment, but also area requirements (e.g., land footprint may affect land purchase costs), energy and labor costs during construction and external costs associated with construction. Operation and maintenance (O&M) costs include chemicals and other consumables, equipment maintenance, licensing fees, administration, and training and labor requirements. Energy requirements also represent a significant operational cost. The end-life costs are related to the disposal of the equipment at the end of the lifetime.

2.6.1 Economic assessment for protein hydrolysis

The major aspects which affect the economic performance of enzymatic hydrolysis are:

- the quality, application, and commercialization of final products, influencing the revenues which can be obtained by their placing on the market;
- the operating and investment costs of the technology itself.

There are several factors (variables) affecting the economy of the process, many of them antagonistic, such as the price of the enzyme vs. its concentration vs. reaction rate, the overall time of the process and the resulting yield vs. the maximum performance of the entire technological equipment (Pecha et al., 2021).

Pecha et al. (2021) studied the technological-economic optimization of enzymatic hydrolysis used for the processing of chrome-tanned leather waste, but the same concepts are valid also for our case.

In particular, the authors subdivided the total costs related to enzymatic hydrolysis in:

- total capital investment, including the delivered equipment, the equipment installation, the instrumentation and control, piping, electrical systems, engineering and supervision, and construction expenses;

- total manufacturing costs, including the total operating costs (chemicals, electrical energy, thermal energy, waste stream processing), the maintenance and other fixed costs, and the labor.

The optimization of the entire production process shall be addressed if the overall plant economic performance is to be optimized. However, during plant lifetime (20–30 years) many factors like prices of chemicals and feedstock or utilities do change which affects specific values of optimal technology conditions. In other words, one can expect a shift in the optimal conditions during long-term operation of plant (Pecha et al., 2021).

CHAPTER 3: MATERIALS AND METHODS

This section reports all the information on the raw materials and chemicals used for the process, the workflow with specifications on how the single phases have been carried out at lab scale, the analytical methods used for functional characterization of raw materials and for controlling the process, and how the methodology has been set-up. Finally, the specifications on how the LCA methodology has been implemented, and the software used to analyze data are described.

3.1 Raw materials and chemicals

3.1.1 Shellfish by-products and fish waste

Raw materials consist of shellfish by-products generated by Co.Pe.Mo. (Cooperativa Pescatori Molluschicoltori), and fish waste from Ittica del Conero Soc.Coop., both located in Ancona.

The production activity of Co.Pe.Mo. is articulated through different steps: purification by washing, selection and sorting on special machines controlled by qualified personnel, and packaging. There are three production lines with annual productivity of 9830 tons of shellfish commercialized in 2020. In particular, 7809 tons of clams, 1236 tons of mussels and 785 tons of murex were processed. Clams cover 79.5% of the total final product, followed by mussel (12.5%) and murex (8%). During the selection and sorting phase, there is a generation of by-products mainly composed of mollusk not suitable for the market since they are undersized, fouled with barnacles, broken shells or unwanted species. Approximately 521 tons of shellfish by-products were produced in 2020, resulting in 5.30% of the total processed volume.

Fish waste is provided by Ittica del Conero Soc. Coop., operating in the marketing and processing of fish products from the Adriatic Sea. It disposes around 80 tons of by-products every year, including tub gurnard, monkfish, hake, salmon, gilthead, cuttlefish, etc.

First both products have been characterized in terms of chemical-physical composition, contaminants, and microbiology, to understand if they were suitable for the process of enzymatic hydrolysis for recovery of protein hydrolysates to be used as biostimulants and/or fertilizers. To this purpose, several sampling campaigns have been conducted.

3.1.1.1 Sampling protocol for functional characterization

Four different side-streams were identified (Figure 15):

a) Clam (*Chamelea gallina*) by-products,
b) Mussel (*Mytilus galloprovincialis*) by-products,
c) Murex (*Bolinus brandaris*) by-products,
d) Mixed shellfish by-products.

***Figure 15**: Clam (A), Mussel (B), Murex (C), Mixed shellfish (D).*

The first three samples were collected directly from each production line. Workers remove the not-market valuable product and throw it in 100 L bins close to them. Once fulfilled the bin, the workers empty it into a bigger one located in a cool room where by-products from each line are mixed all together. Specifically, 2 kg of SFB were collected from the 100 L bin and stored separately to the other coming from distinct packaging lines. Another 2 kg were also collected from one 500 L bin in the cool room as a sample of mixed waste.

Following the same procedure, three sampling campaigns were conducted. The first in March, the second in April and the last one in May 2021 to see if any variability would occur. Low variability was expected on the separately collected samples (mussel, clam, and murex) except for the biological cycle of mollusks. For instance, the spawning or the reproductive period for Mytilus edulis is spring to summer, whereas gametogenesis occurs during the winter season when the mussels get bigger before spawning. Depending on when the mussels are harvested, the size, meat yield and composition vary. A high meat yield, protein, lipid, and pigment content (e.g., carotenoid) are often associated with the gametogenesis phase of maturation in mussels (Naik et al., 2020).

Higher variability was instead expected on the mixed sample since strongly influenced by the productivity of each packaging line. Therefore, also the sampling time (morning or afternoon) affects the composition of the mixed by-products.

Four sampling campaign have been conducted to collect fish waste from Ittica del Conero in Ancona, between June and November 2021.

In particular, 2 kg of fish waste were collected from the cooling room: cuttlefish have been mainly collected in June, cuttlefish with some fish, fish heads and combined waste have been analyzed in July, and combined waste has been sampled in September and November. Samples were shredded by manual mincer before analysis.

Fish waste collected in the last two campaigns was used as substrate for enzymatic hydrolysis. In these last two campaigns part of the samples were frozen before analyzes, in order to check the effect of freezing as method of substrate conservation.

Figure 16: Combined fish waste.

3.1.1.2 Sampling protocol for experimental study

Raw materials already described have been used as substrates for the hydrolysis once their composition was known. To this purpose, they were sampled from the respective industry as it was described before, during the days of the tests, or the day before. In the last case they were previously pre-treated, as it will be discussed afterwards, and stored in the fridge. In some cases, pre-treated fish was frozen and used some weeks later to prepare the mix.

3.1.2 Chemicals

The commercial enzymes that have been tested are Alcalase® Pure 4.0 L and Protana Prime provided by Novozymes. For Alcalase the key enzyme activity is provided by serine endoprotease that hydrolyses internal peptide bonds. It is produced by the fermentation of a microorganism (*Bacillus licheniformis*) that is not present in the final product. In Protana the key enzyme activity is provided by exopeptidase that liberates amino acids by hydrolysis of the N-terminal peptide bond. Also, this enzyme is manufactured by fermentation of a microorganism (*Aspergillus oryzae)* that is not present in the final product. Table 16 summarizes the main characteristics of the two enzymes.

***Table 16**: Enzymes' characteristics. AU (Anson Units), LAPU (Leucine Amino Peptidase Units), CPDU (Carboxypeptidase Units) are different ways to express enzyme activity.*

	Alcalase® Pure 4.0 L	**Protana Prime**
Component name	Protease (Subtilisin)	Aminopeptidase Carboxypeptidase
Activity	4 AU-A/g	970 LAPU/g 890 CPDU-A/g
Color	Brown	Brown
Physical form	Liquid	Liquid
Approximate density (g/ml)	1.17	1.27
Protein (g/100g)	17	10

For the test at controlled pH, NaOH 3M has been used to increase the pH values of the single substrates under hydrolysis up to the stabilized value for the test.

3.2 Workflow

The specific workflow depends on the different test conditions. Anyway, the following main steps can be identified, as discussed in the state-of-the-art section and illustrated in Figure 17: pre-treatment of the substrates (preparation, dilution, homogenization), deactivation of endogenous enzymes (optional), enzymatic hydrolysis, deactivation of test enzyme, solid-liquid separation, concentration.

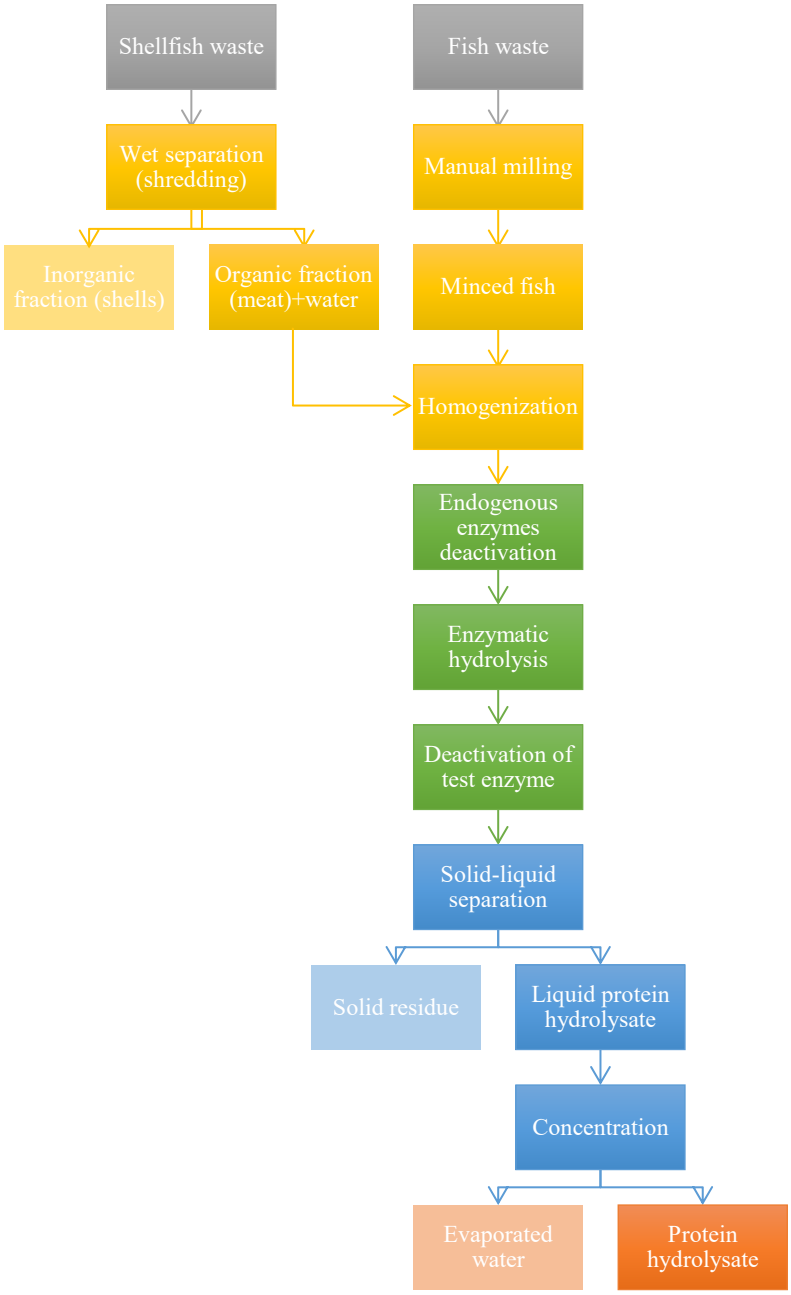


***Figure 17**: Workflow. Raw material in grey, pre-treatments in yellow, enzymatic hydrolysis and deactivation of enzymes in green, post-treatments (solid-liquid separation and concentration) in blue, final product in red.*

3.2.1 Pre-treatments

Pre-treatment of shellfish waste from Co.Pe.Mo. consists of the wet separation of the meat (organic fraction), from the shell (inorganic fraction). Wet separation was obtained with the use of a shredding pump, more commonly used with livestock waste to be fed in the anaerobic digester. Specifically, the workflow was: loading of the shredding tank with shellfish waste and tap water, running of the shredding pump, unloading of the liquid fraction enriched by lighter organic residue, and unloading of settled shredded shells. Since raw shellfish waste is composed, on average, of 80% of shell and 20% of meat, to obtain a liquid fraction with meat to water ratio of 1:2 (as required by the following hydrolysis step), 1 kg of water was added per 3 kg of waste to be shredded. Unfortunately, it was not possible to achieve a lower meat-to-water ratio since the shredding pump requires a minimum water content to work.

***Figure 18**: Meat-shell wet separation. Water addition (a), during shredding (b), separated shells (c).*

Fish waste from Ittica del Conero was minced in the lab by using manual milling, without water addition.

In the case of fish waste, the selected amount of substrate was weighted in a 1-liter borosilicate becher and tap water was added to dilute the mixture until the stabilized substrate to water ratio was achieved. In case of mollusk waste, the water dilution step was already performed in the previous pretreatment. The mix substrate was prepared by mixing 50% of minced fish and 50% of mollusk meat, always considering that mollusks were already diluted. For example, 600 g of mixture with a substrate to water ratio equal to 1:2 is prepared by weighing 100 g of fish, 300 g of mollusks-water mixture, 200 g of water to globally have 200 g of meat and 400 g of water.

Except for the tests n. 1 and 2 (§ 3.4), the mixture (substrate + water) was at this point homogenized with the use of a kitchen blender for about 1 minute.

When dealing with fish waste, in particular in presence of fatty fish like salmon, fat content can be relevant. However, no defatting procedure was applied at this stage of experimentation, in order to determine the behavior of the substrate as it is during hydrolysis. In addition, a defatting procedure would provide additional expenses for the chemicals needed.

At the end of pre-treatments, substrates available for the test were:

a) homogenized fish waste (F),
b) shredded mollusks with water (M),
c) mixture of 50% a) and 50% b) (MIX),

3.2.2 Deactivation of endogenous enzymes

Endogenous enzymes may be at this point deactivated in order to test the efficiency of the commercial enzyme, or not if the combined effect of endogenous and exogenous enzymes must be expected. When the deactivation of endogenous enzymes had to be provided, this was obtained by heating the becher containing the homogenous mixture on a hot plate at the temperature of 100°C for 10 minutes, with occasional manual mixing to facilitate the homogenous distribution of heat (Figure 19).

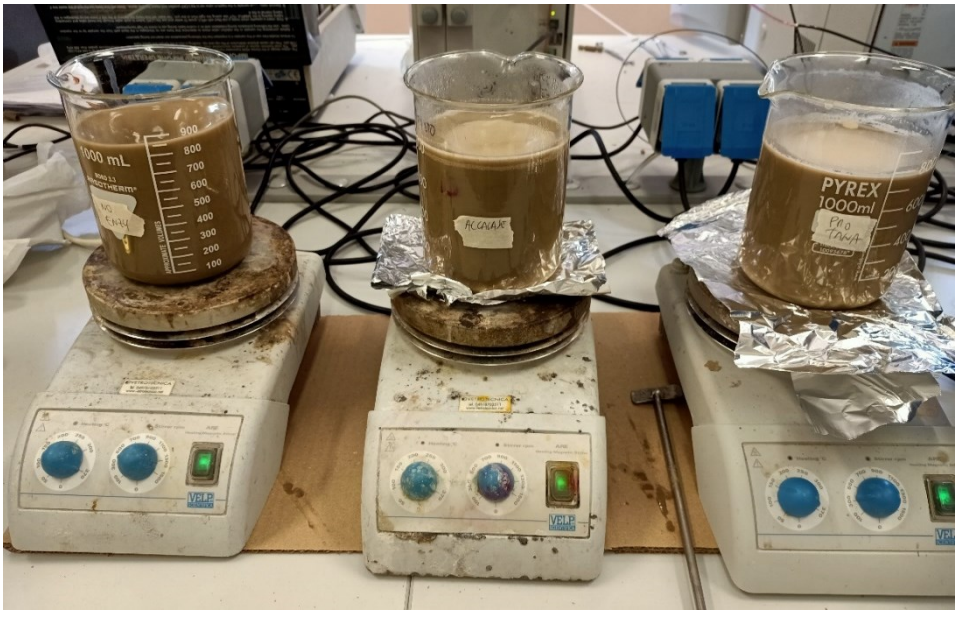


***Figure 19**: Deactivation of endogenous enzymes over the hot plate.*

3.2.3 Enzymatic hydrolysis

The test becher was placed in a water bath that creates a thermoregulated environment for the process, set-up at the selected temperature for the test (Figure 20).

An overhead stirrer was used to keep the mixture continuously stirred. If the test was conducted under closed conditions, the cup of the becher was obtained with an aluminum tray, with a hole on the top for the accommodation of the overhead stirrer.

Enzymes were dosed at the specified enzyme to substrate ratio (v:w) by using a glass pipette. For the test at controlled pH, NaOH 3 M was manually dosed with a glass pipette in order to keep constant the stabilized pH value that was monitored with a pH probe each 10-15 min.

Sampling was manually done by taking about 25 ml of the mixture under hydrolysis with a plastic container.

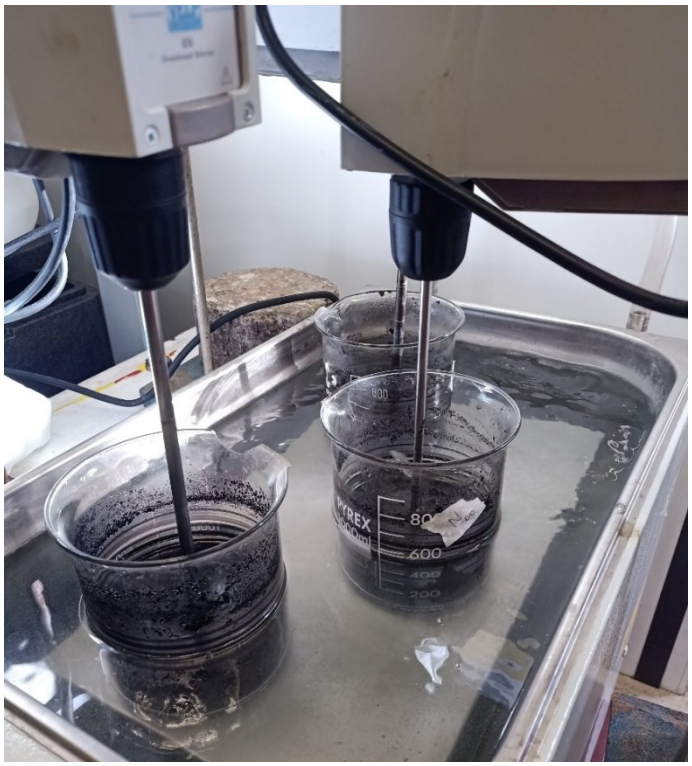


***Figure 20**: Hydrolyzing mixture in the water bath.*

3.2.4 Deactivation of test enzyme

The enzymes in the samples were then deactivated to avoid the process to continue under uncontrolled conditions, causing wrong measures. This was done by spilling the sample into a glass flask for thermal deactivation at 100°C for 10 minutes, over the hot plate previously used for endogenous

enzyme deactivation. From test n. 4 onwards (§ 3.4), also glass flask containing the samples under deactivation was closed with an aluminum cup, to avoid water evaporation that would lead to mixture concentration.

At the end of the process, the becher was reported at room temperature and the enzymes were deactivated immediately, or the becher was kept mixed for 10-24 hours before deactivation, depending on the time management.

3.2.5 Solid-liquid separation

At this point, the liquid fraction containing soluble proteins was separated from the solid one, in order to extract the liquid fraction containing hydrolyzed peptides (Figure 21).

Samples taken during the process were directly centrifuged at 6000-7000 rpm for 20-30 minutes and analyzed as soon as possible.

Instead, having a bigger volume to manage, residues of the test were separated first by sieving at 355 micrometers, followed by centrifugation for the passing fraction under the same conditions of the samples.

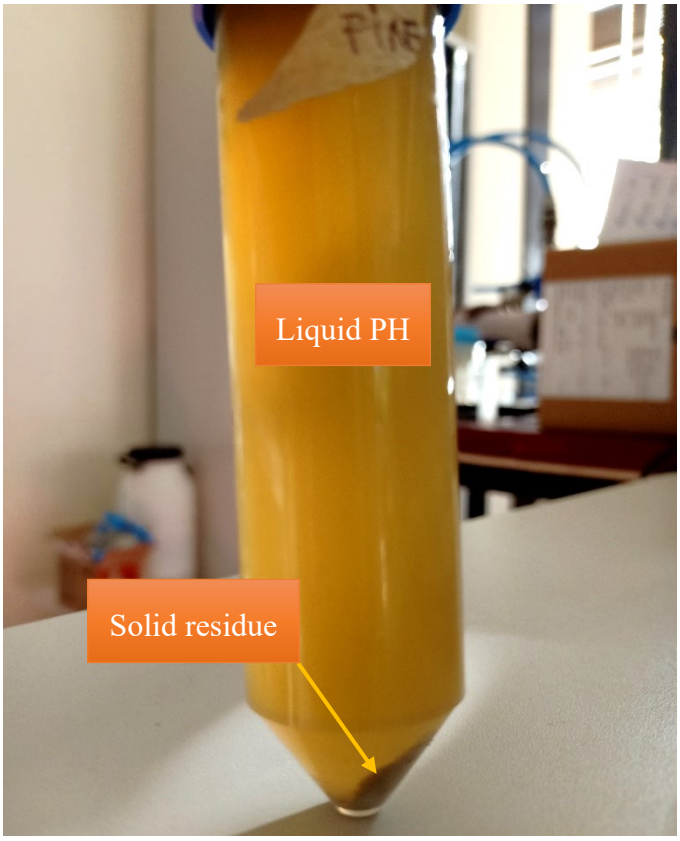


Figure 21: Result of centrifugation step.

In any case, especially in the case of fish and, when the fish was particularly fatty, also for the mix, fat caused problems in liquid-solid separation and it was not possible to achieve a complete three-phase separation, as described by Petrova et al. (2018) and reported in Figure 9. In particular, with centrifugation, fats have partially acquired a semi-solid structure, not adhering to the walls of the test tube, and therefore moving with the liquid PH during the spilling of the liquid fraction, and stored with it

3.2.6 Concentration

To align the final product with law requirements, in particular the minimum nitrogen content (4% by weight), it may be necessary to concentrate the liquid hydrolysate outcoming from the centrifugation step. This was done just in a few cases on the final hydrolysate by using a laboratory rotavapor (Figure 22).

Figure 22: Lab-scale rotavapor.

3.3 Analytical methods

3.3.1 Functional characterization of seafood waste

The functional characterization of seafood waste was not the object of this thesis, therefore the analytical methods applied, and the main results previously obtained will be just discussed.

Analytical parameters for seafood waste characterization were selected following what the European and National regulations on fertilizers establish for the target products to be obtained by the proposed technologies.

For the characterization of the shellfish by-products, ad hoc methods for numerous parameters do not exist. Therefore, methods already used for the characterization of other biomasses were adopted and/or minimally modified to describe shellfish by-product properties, useful to predict their recovery as fertilizers. The analytical procedures which will be described below, refer to internationally recognized methods. All analyzes were performed in duplicate, and the average values are obtained from three different sets of samples.

For fish waste characterization, average values have been obtained from the different samples, without distinguishing between the single species. Therefore, fish waste will be considered combined waste.

3.3.1.1 Chemical-physical composition

In the case of mollusk waste, a representative quantity of each sample was manually separated and weighed (more or less 1 kg for shells and 200 gr for meat) to initially assess the yield of meat compared to the shell in terms of fresh weight (FW). Afterwards, all samples as well as fish waste were dried for 24 h at 105 °C and then shredded in a blender to pass through a 1 mm mesh (APHA, 1998, Method 209 A).

For both shellfish by-products and fish waste, volatile solids (VS) and ashes were determined by weighing the sample after calcination in a furnace overnight at 550 °C (APHA, 1998, Method 209D).

pH values were determined by a pH-meter after water extraction (ratio 1/5 w/w) (ISO 10390, 2021) and, on the same extract, electrical conductivity was determined by a conductivity meter (EN 13038, 2011).

Total nitrogen (TN) was determined by N Elementar Analyzer (ISO-13878, 1998) and protein content was calculated by using a conversion factor of 6.25.

Phosphorous (P) and potassium (K), sodium (Na), magnesium (Mg) and calcium (Ca) were determined by ICP-MS after acid digestion (EPA, 1998).

On the meat fraction of the shellfish by-products samples, the fats content was also assessed, as suggested by Adani et al., (1995). In brief, a gravimetric method was applied based on two successive extractions in Soxhlet using a hexane mixture: ethanol (2:1 vol/vol) and then only ethanol.

From the Ca content, total carbonates were calculated for the shell fraction of the shellfish by-products samples. Anion, chlorides (Cl^-) and sulphates (SO_4^{2-}) were quantified on water extracts of the samples by using, respectively, the following methods: Diagnostic Test Nanocolor Chlorid 200 Analogous to EPA 325.1 and Diagnostic Test Nanocolor Sulfat HR 1000 Analogous to APHA 4500.

3.3.1.2 Contaminants

Heavy metals contents were determined by inductively coupled plasma mass spectrometry -ICP-MS- (Varian, Fort Collins, USA), preceded by microwave acid digestion (EPA, 1998, Method 3051) of the samples. Standard samples (National Institute of Standards and Technology, Gaithersburg, MD, USA) were run with all samples to ensure precision in the analysis.

3.3.1.3 Microbiology

On shellfish by-products, Salmonella and E. coli contamination were determined on the whole fresh samples without separation between meat and shell adopting the methods proposed by the U.S. Composting Council (1997). For Salmonella spp. Method 10.03 was applied, based on a multiple-tube enrichment technique. The most probable number was calculated to approximate the inoculum in the original sample. For E. coli the Method 10.02 was adopted. It is based on a membrane filter technique followed by a colony forming units (CFU) count and the results are expressed as CFU per mass of the original sample.

3.3.2 Experimental study

3.3.2.1 Substrates

Substrates for the hydrolysis were analyzed after being pre-treated: fish has been tested as it was after manual milling; mollusk as it was after shredding, hence already comprising water; mixed substrate was recreated by mixing fish and shredded mollusks with the correct amount of water.

Substrates were characterized by measuring the following parameters:

- Total Solids (TS, %) (and humidity), measure of dry matter content (UNI EN 13040:2002);
- Total Volatile Solids (TVS, % dry matter), measure of the organic matter content (APHA, 1998, Method 209D);
- Total Kjeldahl Nitrogen (TKN, g N/kg dry matter), measure of the total concentration of organic nitrogen and ammonia (UNI EN 15604).

Analyses for substrates characterization were performed in duplicate. For TS measurement, a certain substrate content was weighted in a crucible previously heated to remove the residual humidity ($W_{empty\ crucible}$) and then heated at 105°C into the oven until a constant weight was achieved. Therefore, TS was defined as the ratio of the final weight of the crucible with substrate content at 105°C ($W_{105°C}$) and the initial weight (W_{in}). Humidity was retrieved by difference.

$$TS(\%) = \frac{W_{105°C}}{W_{in}} * 100$$

TVS test was conducted by weighing the same crucible outcoming from the oven after calcination in the muffle overnight at 550°C, as described in 3.3.1.1. Hence, it was calculated as:

$$TVS(\%) = \frac{W_{105°C} - W_{550°C}}{W_{in} - W_{empty\ crucible}} * 100$$

From the measure of TVS it is possible to assess the content of organic carbon (C%TS) as TVS/1.83, as described by Barrington et al. (2002).

Nitrogen content of each substrate was determined by Kjeldahl method on dry matter. Basically, this method consists of three main steps:

1) digestion of the sample at high temperatures for 7 hours with strong sulfuric acid and mercury as catalysts, resulting in an ammonium sulphate solution;
2) distillation by adding a base to the ammonium sulphate solution to convert NH_4^+ to NH_3, followed by boiling and condensation of the ammonia NH_3 gas in a receiving solution made of boric acid;
3) titration to quantify the amount of ammonia in the receiving solution.

Thus, the amount of nitrogen in the sample can be calculated from the following relations respectively for solid and liquid samples:

$$TKN\left(\frac{gN}{kgTS}\right) = \frac{(A-B)*N*C}{W} \quad ,or \quad TKN\left(\frac{mgN}{l}\right) = \frac{(A-B)*N*C*1000}{V}$$

Where:

- A is the ml of HCl solution used in titrating the sample,
- B is the ml of HCl solution used in titrating the blank,
- N is the normality of HCl solution,
- C is the milliequivalent weight to nitrogen (14 mg),
- W is the g of sample digested,
- V is the milliliters of sample digested.

From this measure it was possible to obtain the crude protein content by multiplying the measure by a conversion factor. The original, and still frequently used, conversion factor 6.25 is based on an assumption that the general nitrogen content in food proteins is 16% and that all nitrogen in foods is protein-bound. These are, however, quite rough assumptions as the relative nitrogen content varies between amino acids and amino acid composition varies between food proteins (Sosulski & Imafidon, 1990).

The Kjeldahl method was chosen since it is still recognized as the official method for food protein determination by the AOAC International. Furthermore, several attempts have been made using the colorimetric method described by Lowry et al. (1951) for the measurement of total soluble protein (g P/kg TS) contained in the dried substrate. For this purpose, the salt/alkaline extraction described by Mæhre et al. (2016), was previously performed to extract proteins from the dried substrate. Then, the measures of the absorbance outcoming from the Lowry method were reported on a calibration curve to obtain the protein concentration.

3.3.2.2 Samples and hydrolysates

For process monitoring, the temperature and pH of the mixture under hydrolysis were measured each 1-2 hours by using a specific probe, and samples of about 25 ml were taken at the same time to be analyzed after the enzyme deactivation. Measures of pH and temperature were taken again once the hydrolyzed mixture was reported at room temperature.

The procedures for measuring TS, humidity and TVS were applied for the characterization of the final liquid hydrolysates and solid residues, as done for the substrates.

Measures of weight (g) and volume (ml) of liquid and solid fractions (samples and hydrolysates) resulting from centrifugation allow to calculate their density (kg/l). In addition, with weight measurements the yields (Y, %) of liquid and solid mass for the samples and hydrolysates were calculated as:

$$Y(\%) = \frac{W_{liquid\ fraction}}{W_{liquid\ fraction} + W_{solid\ fraction}} * 100$$

Where W (g) represents the weight of liquid and solid fractions.

The liquid fractions of samples and hydrolysates were analyzed by measuring the conductivity (mS) using a EC probe, the nitrogen content with Kjeldahl method (g N/l), and the total soluble protein (g P/l) with the colorimetric method described by Lowry et al. (1951), whereas the solid residues were analyzed for TS and TVS content and TKN after drying. When possible, Lowry method was performed in duplicate, differently from Kjeldahl method which requires much more time.

Besides characterizing the product to make it comparable with law requirements, TKN and protein measurement on the samples help to assess the performance of the process. The effectiveness of the process was evaluated in terms of Nitrogen Recovery (NR, %), and rate of hydrolysis (g crude protein/l*h). Nitrogen recovery has been calculated as done by Benjakul and Morrissey (1997):

$$NR\left(\%\frac{g}{g}\right) = \frac{TKN_{liquid}}{TKN_{substrate}} * 100$$

Where:

- TKN_{liquid} was calculated by multiplying the concentration of TKN in the liquid (samples or hydrolysates) by the weight of the mixture under hydrolysis, taking into account also the contribution of the enzyme, and the yield of liquid;
- $TKN_{substrate}$ was calculated by multiplying the concentration of TKN in the substrate by the weight of the mixture.

The rate of hydrolysis stands for the rate with which the hydrolyzing mixture achieves the final crude protein at the end of the process. They have been calculated as the slope of the linear regression line using the values of TKN*6.25 (g crude protein/l) during the time of hydrolysis (h).

Table 17 reports a summary of the analyzes carried out on substrates, samples, and final residue.

***Table 17**: Summary of the analyzes carried out during the experimental study.*

<table>
<tr><th>Substrates</th><th colspan="2">Samples</th><th colspan="2">Post-hydrolysis</th></tr>
<tr><td rowspan="6">TS and humidity
TVS
TKN</td><td colspan="2">pH (during process)</td><td colspan="2">pH</td></tr>
<tr><td colspan="2">Temperature (during process)</td><td colspan="2">Temperature</td></tr>
<tr><td colspan="2">Weight (total and tare)</td><td colspan="2">Weight (total and tare)</td></tr>
<tr><td colspan="2">Volume</td><td colspan="2">Volume</td></tr>
<tr><td>Liquid</td><td>Solid</td><td>Liquid hydrolysate</td><td>Solid residue</td></tr>
<tr><td>Volume
Conductivity
TKN
Total soluble protein (Lowry)</td><td>Weight</td><td>Volume
Weight
Conductivity
TS and humidity
TVS
TKN
Total soluble protein (Lowry)</td><td>TS and humidity
TVS
TKN</td></tr>
</table>

3.4 Methodology set-up

The experimental strategy adopted in this work was based on the variation of one parameter controlling the hydrolysis per test. Specifically, the first three tests have been executed to set-up the basic operating conditions (enzyme, temperature, pH) and the accommodation of the vessel, with which the following tests would be performed.

The following parameters are defined as variables affecting the hydrolysis process:

- substrate (g),
- temperature (°C),
- type of enzyme,
- pH,

- substrate to water ratio (S:W),
- enzyme to substrate ratio (E:S),

Table 18 reports the operating conditions of the tests, carried out in chronological order from the top to the bottom. The variables are discussed in the following paragraphs.

***Table 18**: Test conditions. The conditions under exam in each test are highlighted in yellow.*

Test	**Substrate**	**T**	**Enzyme**	**pH**	**Evaporation**	**S:W**	**E:S**	**Duration**
n.	-	*°C*	-	-	-	*w:w*	*v:w*	*h*
1	Fish Mollusks Mix	28 40 60	Alcalase Protana blank	Natural	Yes	1:2	1:100	4
2	Fish Mollusks Mix	60	Alcalase	Natural 7.5 8.5	Yes	1:2	1:100	4
3	Fish	40 60	Alcalase	Natural	Yes No	1:2	1:100	4
4	Fish Mix	60	Alcalase	Natural	No	1:1 1:2	1:100	4
5	Fish Mix	60	Endogenous E + A blank	Natural	No	1:1	1:100	6
6	Fish Mix	60	Endogenous E+A	Natural	No	1:1	1:100 1:200	6
7	Fish Mollusks Mix	28 40 60	E+A	Natural	No	1:2	1:100	6

3.4.1 Enzyme

The test n. 1 was carried out by testing the three different substrates under the same condition of dilution (S:W=1:2). The aim was to determine the best enzyme for each substrate and temperature, to be used for the following tests. Specifically, it has been chosen to test two commercial enzymes

(Alcalase, Protana) at a E:S ratio of 1:100, at three temperatures (28, 40, 60 °C), without pH modification for 4 hours of duration.

To determine the effects arising from the addition of commercial enzymes, the endogenous ones were previously deactivated.

It must be also specified that these tests were conducted with open vessel, since the first idea was that water should be anyway removed at the end of the process, therefore there was no need to consider the evaporation. Furthermore, the hydrolyzing mixture in test n. 1 with mix substrate was previously homogenized with the use of the blender, differently from the other two substrates. The better results of mix substrate in comparison with the other suggested the necessity to homogenize the mixture with the blender in the following tests.

3.4.2 pH

The aim of the test n. 2 was to determine if the pH control, realized with chemicals addition, is necessary to achieve good results in terms of rate of hydrolysis and/or nitrogen recovery. Therefore, during this test the three substrates, diluted 1:2, were tested with Alcalase 1:100 at 60°C at three different pH: the natural one (without any adjustment), 7.5 and 8.5 pH. The choice of that values was done according to literature evidence on the use of Alcalase, which suggests that the best pH was above 8. The pH 7.5 was added as intermediate value between the 8.5 and the natural one, that was around 6.6 for mollusks, 6.4 for mix substrate, and 6.1 for fish.

3.4.3 Evaporation

Analyzing the results of mass balances carried out for this test and for the previous one, it was observed that the evaporation was relevant and should be accounted. It is particularly important if a reliable comparison between tests at different temperature must be elaborated. In fact, the evaporation concentrates the mixture during hydrolysis, therefore results of 60°C tests could be overestimated in terms of nitrogen and proteins recovery and hydrolysis rate.

The results made necessary to determine a trend and quantify the effect of evaporation, hour by hour, during the hydrolysis. This was done in the test n. 3, by testing the fish substrate (the easiest and fastest to be pre-processed) at the temperatures of 40°C and 60°C in both open and closed vessels. In order to determine the evaporation trend, the weight of the becher containing the hydrolyzing mixture has been recorded during all the steps which required a thermal treatment, therefore pre and post the

deactivation of endogenous enzymes, sampling, deactivation of test enzyme, and the difference between the points have been calculated, in order to obtain the cumulative evaporation.

Furthermore, the effect of evaporation during the deactivation of enzymes occurring for samples on the hot plate was also accounted. To this purpose TKN and protein values have been corrected by dividing the results by a concentration coefficient, given as the ratio of the sample weight before deactivation to the sample weight after deactivation.

3.4.4 Substrate to water ratio

Once optimal temperature, enzyme, and pH were selected, it has been decided to test a reduced substrate (meat) to water ratio. Water should be removed at the end of the process, therefore, by halving the amount of water it is possible to reduce the water consumption and the energy that will be spent to remove it from the final hydrolysate.

Unfortunately, it is not feasible to obtain a liquid fraction (mollusk waste) with a dilution ratio S:W=1:1, by means of the shredding pump due to a minimum water content needed for the operation. As consequence, mollusks substrate already contained water with a ratio 1:4 or 1:2. Thus, after testing the effect of different pH, the mollusks have not been tested anymore alone.

3.4.5 Effect of endogenous enzymes

The endogenous enzymes deactivation was not provided in the last three tests, where the effects of the endogenous enzymes and of the combination of these with commercial Alcalase were inspected. In particular, the effect of endogenous enzymes was compared with the combined effect of endogenous enzymes and Alcalase. A blank test becher, where the endogenous enzyme was thermically deactivated, was also hydrolyzed for control.

Once the optimal conditions for Alcalase enzyme were defined, and the effect of the endogenous enzyme non-deactivation was determined, it has been chosen to proceed without endogenous enzyme deactivation, in order to optimize the final results, and to reduce the energy consumption. This would be fundamental at pilot scale, where the energy consumption for heating the mixture could be avoided.

3.4.6 Enzyme to substrate ratio

In the last test the effect of halving the substrate enzyme ratio has been investigated, in order to see if half of the initial quantity was still effective in hydrolyzing the mixture. This also would be

important at pilot scale production, mainly for economic reason since the cost of the enzyme is relevant.

3.4.7 Temperature

In the last test, the effect of the temperature on the combination of endogenous enzymes and Alcalase has been inspected, since the previous ones including their presence other than Alcalase have been carried out only at 60°C only on the fish and mix substrates, being the dilution ratio equal to 1.

Therefore, the three substrates have been tested at the three temperatures (28, 40 and 60°C), with Alcalase and without deactivating the endogenous enzymes. The dilution ratio and the E:S ratio have been left respectively at 1:2 and 1:100, in order to have new data in absence of evaporation to implement the LCA methodology for comparing the impacts related to the valorization of each substrate.

3.5 Life Cycle Assessment

The implementation of the LCA methodology with the purpose of delivering the sustainability assessment is reported, step by step, in the following paragraphs.

3.5.1 Goal and Scope Definition

The system under study is a hypothetical pilot scale plant able to treat 1 ton of fish and/or mollusks waste and deliver PHs following the methodology thoroughly described.

Table 19 specifies better the structure of this sustainability assessment. Two system boundaries are defined to assess respectively the environmental impacts related to the treatment of 1 ton of waste (Assessment of valorization), and the production of 1 ton of fish/mollusks-based PH (Assessment of production). For both, three scenarios corresponding to the use of fish, mollusks, and mix substrates were simulated.

***Table 19**: Boundaries and scenarios at the basis of the LCA study. F, fish; M, mollusks' meat; Mix, fish plus mollusks' meat; V, valorization; P, production.*

	Assessment of valorization	**Assessment of production**
Functional unit	1 ton of waste (fish + mollusks' meat)	1 ton of final PH
Scenario 1	F_V	F_P
Scenario 2	M_V	M_P
Scenario 3	Mix_V	Mix_P

3.5.1.1 Description of the system

Equipment chosen to carry out the single stages of the process have been selected on the basis of what is already available, the state-of-the-art on pilot-scale biorefineries, and specifications supplied by technology providers. Basically, it is constituted as follows.

The pre-treatment phase consists of a size reduction unit for fish preparation, a wet separation unit for meat-shell separation of the mollusks, and a homogenization unit. Clearly, when only fish is used as substrate the meat-shell separation stage is not considered, as well as the size reduction stage is eliminated in case the only substrate is mollusks' waste. Only when the mix substrate is used as substrate both the stages are present. It is assumed that an electrical mincer, a shredding pump, and a universal mixer with suitable capacity, in batch functioning were respectively used for these purposes.

The enzymatic hydrolysis is performed in 1000 l thermostatically stirred-batch reactor. Concerning the operating conditions and the results obtained in 4.2, Alcalase is selected as exogenous enzyme to be added to the endogenous ones that are not deactivated, and the temperature of 60°C and the natural pH are used for the reasons discussed above. The deactivation of the enzymes is performed in the same reactor since it is possible to control and modify the temperature.

At this point, hydrolyzed mixture is delivered to a separation decanter suitable for fish processing, and finally the liquid fraction is concentrated in a mechanical vapor recompression evaporator (MVRE), one of the most energy efficient equipment for concentration, in order to achieve a minimum nitrogen content of 4% (w:w), as required by legislation for hydrolyzed animal epithelium biostimulants.

***Table 20**: Phases and stages of the plant model.*

Phase	Stage	Description	Equipment
Pre-treatments	1	Size reduction (fish) Meat-shell separation (mollusks)	Electrical mincer Shredding pump
	2	Homogenization	Mixer
Hydrolysis	3	Enzymatic hydrolysis	Thermostatically stirred-batch reactor
	4	Deactivation of enzymes	
Centrifugation	5	Filtration and solid-liquid separation	Decanter
Concentration	6	Evaporation of excess water	MVRE

3.5.1.2 Main input characterization

Concerning the main input and output characterization, the average values of data outcoming from the lab tests performed at the same operating temperatures are taken for each of the three substrates (Table 21). Information required is the percentage of dry matter, organic content and nitrogen content of substrates, liquid hydrolysates and solid residues, and the mass percentage of the last two (Table 22).

***Table 21**: Operating conditions of the hypothetical pilot-scale plant.*

Enzyme	Alcalase + endogenous
Temperature (°C)	60
pH	natural
S:W	0.5
E:S	0.01
Duration (h)	6

***Table 22**: Input characteristics of substrate, liquid PH and solid residue for the three scenarios.*

Fish				
Parameter	**Unit**	**Substrate**	**Liquid PH**	**Solid residue**
Dry matter	%	28.67	5.44	35.19
Organic content	%DM	85.35	93.69	55.80
Nitrogen content	g TKN/kg DM	74.69	-	43.79
	g TKN/l	-	6.87	-
Mass	%	-	90	10
Mollusks				
Parameter	**Unit**	**Substrate**	**Liquid PH**	**Solid residue**
Dry matter	%	11.60	5.86	53.12
Organic content	%DM	38.03	76.02	26.85
Nitrogen content	g TKN/kg DM	45.87	-	11.33
	g TKN/l	-	5.57	-
Mass	%	-	83	17
Mix				
Parameter	**Unit**	**Substrate**	**Liquid PH**	**Solid residue**
Dry matter	%	11.88	5.72	65.24
Organic content	%DM	55.79	84.38	44.90
Nitrogen content	g TKN/kg DM	55.01	-	16.33
	g TKN/l	-	6.65	-
Mass	%	-	90	10

3.5.1.3 Functional unit

Two different boundaries are defined.

In the first case, i.e., the assessment of valorization, 1 ton of waste treated, intended as fish and/or mollusks' meat, has been used as functional unit; in the second case, i.e., the assessment of production, 1 ton of protein hydrolysate produced is used.

3.5.1.4 System boundaries

The system boundaries are defined on the basis of the "Cradle to gate" approach. In Figure 23, the system boundaries for the most complete case, i.e., with the use of the substrate "mix", are highlighted to identify all the variables that are applied to the system (e.g., energy needed, chemicals consumption, heat consumption, waste transport to disposal, etc...).

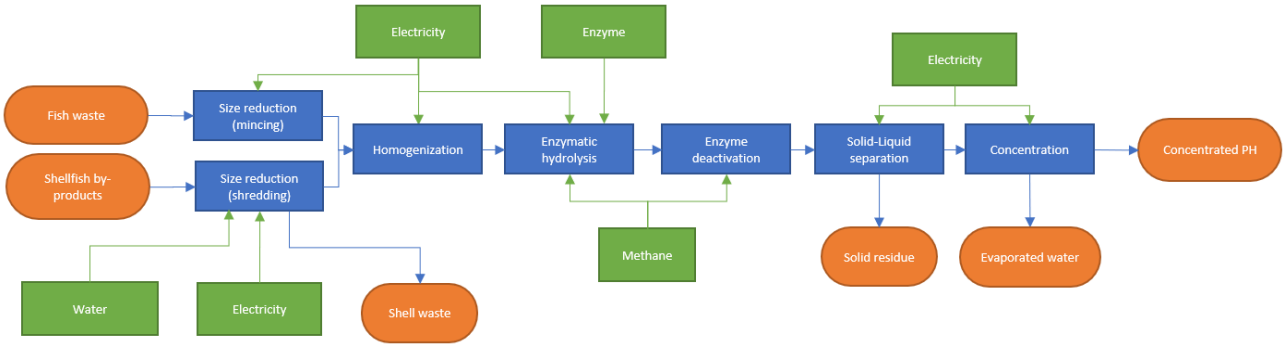


***Figure 23**: Block flow diagram of the model. Start/end products in orange, primary processes in blue, reference supply chain in green.*

3.5.2 Inventory analysis

Once the system boundaries are well defined, with all the inputs and outputs at each stage, mass balances and energy balances are carried out to quantitatively determine the mass and energy flows (Table 23).

The electricity consumption for the phase of pre-treatments is the sum of the contributions given by mincing, shredding, and homogenization; for hydrolysis consists only in electricity for mixing.

In order to assess the energy consumption in kWh, the power value reported in the technical sheet of each equipment selected has been multiplied by the functioning time of the equipment considering the number of batches needed to treat all the quantity, even if this is an overestimation of the real consumption, which depends on the absorbed power. Instead, for what concerns concentration, the value of 300 Wh/kg PH for MVRE reported by Colantoni et al. (2017) has been taken, in absence of reliable data on the energy consumption of this kind of equipment.

The heat request during hydrolysis is considered composed of the following contributions: the heat request to bring the mixture to the selected temperature, the heat necessary to maintain the correct temperature, and the heat for enzyme deactivation.

The heat request to bring the mixture to a certain temperature has been modelled as follows, for both initial heating (subscript 1) and final deactivation (subscript 3).

$$heat\ request_{1,3}\ (kWh) = \frac{M * \left(T_{fin} - T_{in}\right) * h_s}{3600}$$

Where:

- M (kg) is the total weight of the mixture, neglecting the enzyme since it will be added after the correct temperature is reached;
- T_{fin} and T_{in} (°C) are respectively the operating and the initial temperature;
- h_s (kJ/kg°C) is the specific heat of the water, to which the mixture is assimilated;
- 3600 (J/kWh) is a conversion factor.

When the heat for final deactivation is calculated, the M term will comprise the enzyme weight also, and the T_{fin} and T_{in} are respectively the temperature of deactivation (100°C) and the operating temperature.

The heat request for maintaining the correct temperature (subscript 2) has been modelled as follows, simplifying the reactor as a cylinder.

$$heat\ request_2\ (kWh) = \frac{(hl_{walls} + hl_{floor} + hl_{cup}) * d * n}{1000}$$

Where:

- hl_{walls}, hl_{floor}, hl_{cup} (W) are respectively the heat lost for conduction from the walls, floor and cup of the reactor;
- d (h) is the duration of hydrolysis;
- n is the number of batches required, given by the ratio of total volume of the mixture and enzyme to be treated and the volume of one reactor.

The heat losses have been calculated as:

$$\frac{tc_{reactor}}{t} * A * \left(T_{hydrol} - T_{air}\right)$$

Where:

- $tc_{reactor}$ (W/m°C) is the thermal conductivity of the reactor insulated with glass wool;
- t (m) is the thickness of the walls, floor and cup;
- A (m^2) is the area of the walls, floor, cup.
- T_{hydrol}, T_{air} (°C) are respectively the operating temperature and the temperature of the air.

The input quantity of the enzyme is known from the E/S ratio selected as operating condition and the kg of substrate used. The value in kg is known by multiplying the liters by the density of the enzyme.

Relating to the outputs, the solid residue out of pre-treatments consists of inorganic shells separated during shredding. This quantity is calculated as the difference between the total shellfish by-products and the meat, that is calculated from the observed yield corresponding to about 17% of the total.

In the same way, the solid residue output of centrifugation is calculated by multiplying the total weight of the mixture after hydrolysis, assuming that evaporation does not occur, and the average solid yield of all the lab tests conducted on this substrate under the same conditions.

The quantity of the final product, the concentrated PH, is retrieved by the ratio of the liquid PH outcoming of centrifugation stage and the concentration ratio, defined as the ratio of the minimum nitrogen content defined by the D.Lgs. n. 75/2010 and the average nitrogen content (TKN) obtained during the tests at lab scale under the same conditions.

The evaporated water is the difference between the weight of the liquid PH after centrifugation and of the final concentrated PH.

***Table 23**: LCI table for fish substrate.*

Fish						
INPUTS	**u.m.**	**Pre-treatments**	**Hydrolysis**	**Enzymes deactivation**	**Centrifugation**	**Concentration**
Water	kg/ton S	2000.00	-	-	-	-
Substrate (S)	kg/ton S	1000.00	-	-	-	-
Enzyme	kg/ton S	-	11.70	-	-	-
Heat	kWh/ton S	-	171.35	130.51	-	-
Electricity	kWh/ton S	63.67	33.77	-	22.58	140.23
OUTPUTS	**u.m.**	**Pre-treatments**	**Hydrolysis**	**Enzymes deactivation**	**Centrifugation**	**Concentration**
Solid residue	kg/ton S	-	-	-	291.63	-
Concentrated protein hydrolysate	kg/ton S	-	-	-	-	467.42
Evaporated water	kg/ton S	-	-	-	-	2252.65

***Table 24**: LCI table for mollusks substrate.*

Mollusks						
INPUTS	**u.m.**	**Pre-treatments**	**Hydrolysis**	**Enzymes deactivation**	**Centrifugation**	**Concentration**
Water	kg/ton S	2000.00	-	-	-	-
Substrate (S)	kg/ton S	1000.00	-	-	-	-
Enzyme	kg/ton S	-	11.70	-	-	-
Heat	kWh/ton S	-	171.35	130.51	-	-
Electricity	kWh/ton S	70.71	33.77	-	22.58	104.09
OUTPUTS	**u.m.**	**Pre-treatments**	**Hydrolysis**	**Enzymes deactivation**	**Centrifugation**	**Concentration**
Solid residue	kg/ton S	4882	-	-	518.25	-
Concentrated protein hydrolysate	kg/ton S	-	-	-	-	346.98
Evaporated water	kg/ton S	-	-	-	-	2146.47

***Table 25**: LCI table for mix substrate.*

Mix						
INPUTS	**u.m.**	**Pre-treatments**	**Hydrolysis**	**Enzymes deactivation**	**Centrifugation**	**Concentration**
Water	kg/ton S	2000.00	-	-	-	-
Substrate (S)	kg/ton S	1000.00	-	-	-	-
Enzyme	kg/ton S	-	11.70	-	-	-
Heat	kWh/ton S	-	171.35	130.51	-	-
Electricity	kWh/ton S	67.19	33.77	-	22.58	134.74
OUTPUTS	**u.m.**	**Pre-treatments**	**Hydrolysis**	**Enzymes deactivation**	**Centrifugation**	**Concentration**
Solid residue	kg/ton S	2441.18	-	-	310.15	-
Concentrated protein hydrolysate	kg/ton S	-	-	-	-	449.12
Evaporated water	kg/ton S	-	-	-	-	2252.43

3.5.2.1 Implementation in Umberto LCA+ software

At this point, the block flow diagrams have been implemented in Umberto LCA+ software complete with the flows outcoming from the inventory table.

To do a LCA analysis with Umberto LCA+ software, the starting step plans to draw the life cycle model (or SYSTEM MODEL). Processes (sketched as a squared box) represent the core of the model. As a prerequisite for a successful calculation of all material and energy flows of the system, and subsequently for the LCIA results, all processes shall be specified. A process specification can be made by entering materials on the input (green circle) and output (red circle) inside of the process and specifying a coefficient for each entry. These coefficients do not have to be absolute values. Rather they represent the size of flows on the input and the output side in relation to each other. The system model can also be divided into different life cycle phases, in this case called "pre-treatments", "hydrolysis", "centrifugation", and "concentration", each one comprising the respective processes. It will help in the interpretation and discussion of the results.

For a unit process this model stub has one input and an output place, to which elementary exchanges will be assigned, and two connection places: one for the intermediate exchanges on the input side, and one for the intermediate exchange(s) on the output side. These inputs and outputs are called intermediate exchanges since these flows run between the processes, within the Technosphere. They could be flows that are outputs of a technical process, such as a product, a semi-finished product, processed goods, or a component. Elementary Exchanges, on the contrary, are the flows that cross the system boundary and play a central role in impact assessment as they do have characterization factors assigned, for which the contribution to an environmental impact is calculated.

It is possible to define a new intermediate exchange in the project clicking on the button "New Material". The information for the newly created intermediate exchange material that can be edited in the Property Editor window is the material name (and description), the unit type (unit of measure) and material type (good, bad, or neutral). Materials with the material type GOOD are expenditures of raw materials, intermediate products, or auxiliary materials. The products of any process also have the material type GOOD. Direct emissions, as well as wastes causing indirect emissions (like waste being transported to the landfill), are shown with the material type BAD. Materials which should not contribute to the life cycle inventories are marked with the material type NEUTRAL. This operation has been done to create the raw materials like fish and mollusks waste, intermediate products like the hydrolyzing mixture, and wastes like shells and solid residues.

Background data are available through the implementation of ecoinvent 3 database within the Umberto LCA+ software. The ecoinvent database is published and maintained by the Ecoinvent Centre in Switzerland. It is the most renowned database for life cycle inventory (LCI) datasets. It contains approximately 4500-5000 harmonized, reviewed, and validated datasets for use in Life Cycle Assessments (LCA). The ecoinvent activities typically are available as both “Unit” and “System terminated” (“Result”) type. The ecoinvent 3 database has been used to model electricity and heat production, enzyme production and transport, tap water. In addition, also calcium carbonate and nitrogen fertilizer have been accounted for considering the avoided impacts related to the possible production of calcium carbonate from shells waste and of the PH production from fish and/or mollusks waste instead of a generic nitrogen-based fertilizer. All have been taken as intermediate exchanges.

More in detail, electricity to be used in the process (pre-treatments, mixing, centrifugation, concentration) has been modelled with the material "electricity, medium voltage [IT]" (intermediate exchange), which describes the electricity available on the medium voltage level in Italy for year 2014. This is done by showing the transmission of 1 kWh electricity at medium voltage. A similar approach has been used to simulate the electricity at the base of the enzyme production. This has been modelled with the activity market for electricity, medium voltage [NL], which describes the electricity available on the medium voltage level in Netherlands (the country where Alcalase enzyme has been produced) for year 2014.

The heat necessary to reach the operating temperature, to maintain it, and to deactivate the enzymes at the end of the process, has been modelled with the material “heat production, natural gas, at boiler condensing modulating < 100kW [Europe without Switzerland].

To model the contribution of the enzyme, life cycle data for Savinase, the most similar enzyme to Alcalase for type and action, were taken from Nielsen at al. (2007). In particular, a value of 60 MJ/kg of enzyme for energy consumption, and 4 kg CO_2eq/kg for global warming have been considered. To simulate the transport of the enzyme “transport, freight, lorry 3.5-7.5 metric ton, EURO5” has been used, by using as input data a value of 1700 km for the distance from the production site to the pilot-plant.

Avoided impacts are the negative impacts that are calculated because of a waste production that can be considered as a good for downstream processes, and therefore should not be extracted or produced by different processes. In particular, to model the avoided impact related to the production of calcium carbonate (shell waste), “limestone, unprocessed” has been taken from the ecoinvent 3 database and

added as input to the shredding process in terms of kg of $CaCO_3$ equivalent with a negative sign. In the following equation, an example of the calculation performed for the mollusks model is reported.

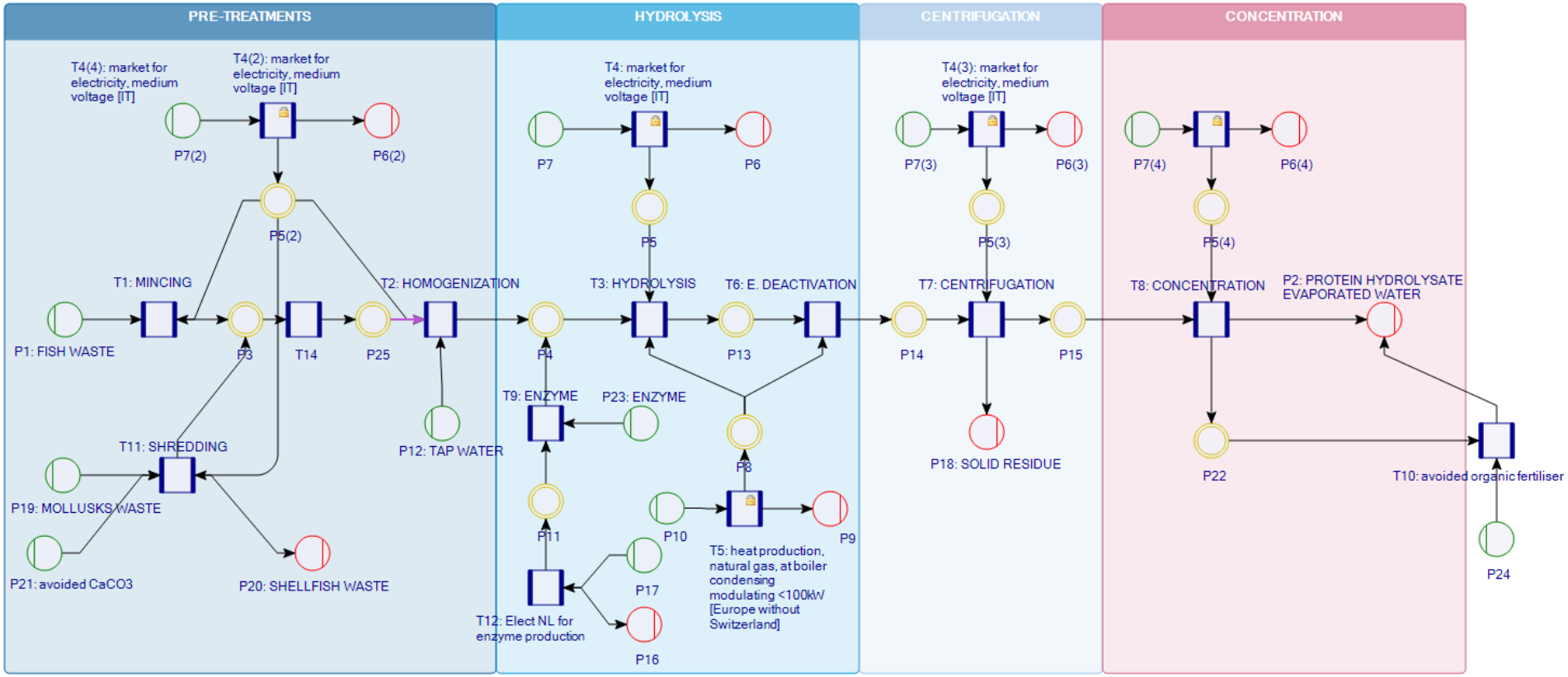
$$4882\ kg\ shell\ waste * 0.93 \frac{kg\ TS}{kg\ shell\ waste} * 0.87 \frac{kg\ CaCO_3}{kg\ TS}$$

The avoided impact due to the production of a nitrogen-based fertilizer instead of a generic one, produced with traditional methods, has been modelled considering that not all the nitrogen contained in the PH is directly available for plants. In fact, the available nitrogen should be calculated as the difference between the mineralized nitrogen and the nitrogen lost in the nitrification process, which could be in turn calculated by knowing the kinetics of mineralization and nitrification related to soil. For simplicity, the material "nitrogen fertilizer, as N" has been taken and the input quantity has been calculated by multiplying the kg of hydrolysate by the minimum nitrogen concentration required (4%, achieved with concentration step) and by the Fraction of Mineralization (FM), defined by Lopes et al. (2021) as the ratio of mineralized nitrogen accumulated to the total nitrogen applied. In particular, the value of the 28.93% for FM has been taken as an average of the ones calculated for four fish waste-based compost at different doses.

After a model has been built up and all processes have been properly specified, the flows can be calculated. As a prerequisite for launching the calculation, it is required to enter at least one flow manually. This start flow can be located anywhere in the model, but it typically is the flow of one unit of product for which the model is calculated. In this case, the substrate material is defined as manual flow, with a specified quantity of 1 ton. Another simulation has been tried by using 1 ton of final PH as manual flow. At this point, the mass and energy flows within the system model are evaluated. They refer to Inventory results.

The life cycle model can be displayed as Sankey diagrams, both for material and energy flows, as well as for weighted "impact flows", i.e., the environmental impact loads cumulated along the stages of the life cycle. Sankey diagrams are flow diagrams, where the width of the arrows represents the flow quantity.

***Table 26**: Example of a system model for mix substrate.*



3.5.3 Life Cycle Impact Assessment

When calculating the product life cycle model, all material expenses (raw materials, energy, components) in the life cycle inventory are considered with their quantities and their characterization factors for impact assessment. For newly defined intermediate exchanges, typically no such characterization factors exist, and the intermediate exchanges will most likely not appear in the life cycle impact assessment as contributing to environmental impacts.

To obtain LCIA results, an impact assessment should be performed. It is the part in LCA where predictions on environmental effects of the production system are made. It is required to choose the methodologies and impact categories, as well as the level of detail, depending on the goal and scope of the study. The link between life cycle inventory outcomes and impact categories are the characterization factors for each elementary flow.

This LCA study uses a midpoint-oriented approach for impact assessment, mainly based on the indicator models described in the Dutch LCIA method ReCiPe 2008, the same used García-Santiago et al. (2021), by reporting the impacts on the physical effect level of the respective category of environmental concern. The categories selected for the impact assessment in this LCA are listed in Table 27

***Table 27**: LCIA categories (method ReCiPe 2008 Midpoint (H) V1.13 no LT)*

Acronym	Name	Unit
ALO	Agricultural land occupation	m^2a
CC	Climate change	kg CO_2-Eq
FD	Fossil fuel depletion	kg oil-Eq
FET	Freshwater ecotoxicity	kg 1.4-DCB-Eq
FE	Freshwater eutrophication	kg P-Eq
HT	Human toxicity	kg 1.4-DCB-Eq
IR	Ionizing radiation	kg U235-Eq
MET	Marine ecotoxicity	kg 1.4-DB-Eq
ME	Marine eutrophication	kg N-Eq
MRD	Mineral resource depletion	kg Fe-Eq
NLT	Natural land occupation	m^2

OD	Ozone depletion	kg CFC^{-11}-Eq
PMF	Particulate matter formation	kg PM_{10}-Eq
POF	Photochemical oxidant formation	kg NMVOC-Eq
TA	Terrestrial acidification	kg SO_2^-Eq
TET	Terrestrial ecotoxicity	kg 1.4-DCB-Eq
ULO	Urban land occupation	m^2a
WD	Water depletion	m^3

Finally, the results are provided in graphical form and several views. They shall also be exported as spreadsheet files allowing for creating any other customized table with selected results and diagrams based upon these.

3.6 Economic assessment

A simplified economic analysis is conducted. The total cost is reported as annual costs, summarizing the annual operational costs with the capital costs per annum:

$$Annual\ costs\ =\ OPEX\ \left(\frac{€}{y}\right) +\ CAPEX\ \left(\frac{€}{y}\right)\ ,$$

$$CAPEX\ =\ \frac{\sum investment\ costs\ (€)}{economic\ lifetime\ (y)}\ ,$$

$$OPEX\ =\ Annual\ production\ \left(\frac{kg}{y}\right) * Operating\ costs\ \left(\frac{€}{kg}\right) + Maintenance\ and\ other\ fixed\ costs\ \left(\frac{€}{y}\right) + Labor\ cost\ \left(\frac{€}{y}\right)$$

For CAPEX, it has been taken a lifetime period of 20 years, considering that the typical one is between 20-30 years (Pecha et al., 2021). Investment costs (IC) have been obtained by searching for suitable equipment in terms of capacity. In some cases, since in most of the cases these prices were not available, Matches, a tool with educational content, has been used, assisting in the evaluation of process alternatives. In any case, the uncertainties related to the cost of the equipment must be taken into account to justify the results. To these costs the equipment installation (39% IC), instrumentation

and control (26% IC), piping (31% IC), electrical systems (10% IC), engineering and supervision (32% IC), and construction expenses (34% IC) have been added (Pecha et al., 2021).

***Table 28**: Purchase cost for major equipment.*

Life Cycle Stage	Unit Process	€	Source
Size reduction	Mincer	1956	ggmgastro.com
Shell-meat separation	Cage mill	20995	matche.com
Homogenization	Ribbon blender	19380	matche.com
Hydrolysis	Kettle, jacketed, agitated reactor (1000 L)	38760	matche.com
Centrifugation	Decanter for Fish Processing	28770	alibaba.com
Concentration	MVR Film Evaporator	19180	alibaba.com
TOTAL		129041	

For OPEX, it has been assumed an annual processing of 225 tons of waste/y, assuming to process 300 kg/d of waste, corresponding to 900 kg/d of mixture by using the same dilution ratio of LCA, 1 cycles/d of 4 hours in a 1000 l reactor, and 250 working days/y. The operating costs have been obtained by multiplying the data from the LCI by the respective costs, reported in Table 29. The price of the waste has been taken as 0, since it has not any economic value; the price of the enzyme is known from the producer (Novozymes); the price of the water (€/m^3) is taken from the manager of the integrated water cycle in Ancona, considering the cost for other uses, including sewage and wastewater treatment; the cost of electricity (€/kWh) for medium tensions other uses is taken from ARERA; the heat cost has been obtained as follows, starting from the methane cost for consumptions below 26k (m^3/y) always taken from ARERA.

$$Heat\ cost\left(\frac{€}{kWh}\right) = \frac{methane\ cost\left(\frac{€}{Sm^3}\right)}{calorific\ power\left(\frac{kWh}{Sm^3}\right) * boiler\ efficiency}$$

The maintenance and other fixed costs have been calculated as the 10% of the investment costs (Pecha et al., 2021). Finally, the labor costs have been estimated by considering an average salary of 15 €/h and 2 operators.

***Table 29**: Unitary operating costs, valid for both fish, mollusks, and mix.*

Unitary operating costs	u.m.	Value	Source
Water	€/m3	2.85	VIVA Servizi S.p.A.
Waste	€/kg	0	Assumed
Enzyme	€/kg	46.36	Novozymes A/S
Heat	€/kWh	0.08	ARERA annual relation 2021 (Data 2020)
Electricity	€/kWh	0.1632	ARERA annual relation 2021 (Data 2020)

The economic feasibility can be evaluated in terms of return on investment (ROI), expressed as:

$$ROI = \frac{Annual\ net\ profit}{Investment\ costs} * 100\ ,$$

where the profit is the difference between the revenues and the costs (Venslauskas et al., 2021). By inverting the ROI, the payback period (PBP) can be easily calculated (Pecha et al., 2021).

The annual net profit is calculated by assuming the cost of the PH taking into account the products available on the market (2.2) in the worst case, and multiplying this by the kg of hydrolysate produced per ton of waste and the annual productivity as tons of waste/y. The same unitary price for the three cases has been taken. In case of mollusks and mix, the economic value of calcium carbonate to be used as soil conditioner has been accounted also in the same way of PH. In any case, it was decided to attribute zero economic value to the solid residue, as its potential use in the form of biochar or compost is not the subject of this argument.

***Table 30**: Unitary profit in the case of mollusks and mix.*

Unitary profit	u.m.	Value	Source
Calcium carbonate	€/kg	0.72	Average price on internet
Solid residue	€/kg	0	Assumed
Concentrated PH	€/kg	5	Assumed according to PH on market
Evaporated water	€/kg	0	Assumed

CHAPTER 4: RESULTS AND DISCUSSION

In this chapter, the main results of the preliminary functional characterization of seafood waste, of the experimental study core of this thesis work, and of the sustainability assessment, in terms of environmental and economic impacts, are discussed.

4.1 Functional characterization of seafood waste

4.1.1 Chemical-physical composition

The chemical-physical composition of seafood waste has been analyzed in order to assess the potential uses of raw materials and of the respective organic and inorganic fractions in the treatment scheme. For example, to be used as substrates for the extraction of protein hydrolysates, seafood waste must contain a considerable amount of proteins, and, with the aim of applying the principles of circular economy, it is good to propose also a way of valorization for materials that do not have an adequate protein content.

4.1.1.1 Shellfish by-products

The two main components of the shellfish by-product, shell, and residual meat were manually separated from the overall by-products obtaining a meat yield for murex, clam, and mussel of 15.75%, 33% and 17.27% respectively.

As expected, the shell has a low moisture content (<8%) and involves mainly inorganic compounds (>94% of ashes). On the other hand, the residual meat has only 19-28% of dry matter and the organic fraction represents between 74-89%. There is no significant difference in the dry matter content of the three shellfish species (Table 31).

***Table 31**: Dry matter (DM), volatile solids (VS) and ashes in shellfish by-products.*

Shellfish	By-product	DM	VS	Ash
		%	%DM	%DM
clam	organic	24.60 ± 2.77	76.09 ± 8.21	23.91 ± 8.21
murex	organic	28.16 ± 1.93	89.89 ± 4.39	10.11 ± 4.39
mussel	organic	19.11 ± 1.23	74.14 ± 16.11	25.86 ± 16.11

clam	shell	93.00 ± 3.04	3.94 ± .54	96.06 ± .54
murex	shell	92.48 ± 3.91	5.93 ± 1.86	94.07 ± 1.86
mussel	shell	93.65 ± 3.67	3.95 ± 2.05	96.05 ± 2.05
mix	all	65.13 ± 6.37	8.56 ± 1.64	91.44 ± 1.64

Table 32 shows other physicochemical parameters like pH, salinity, chloride, and sulphate concentrations. The latter was not detectable in the shell fraction. On the other hand, the Cl^- concentration in the shell is 70-80% lower than in the residual meat but almost uniform between different shellfish. Chlorides and sulphates content, being very soluble, could be affected by the intensity of the water washings. Similarly, the salinity, which is mainly correlated with the amount of washing carried out in the production line, shows around 90% reduction in the shell fraction.

***Table 32**: pH, salinity, chloride, and sulphate in shellfish by-products.*

Shellfish	**By-product**	**pH**	**Salinity**	**Cl-**	**SO_4^{2-}**
		-	µS	%DM	%DM
clam	organic	5.94 ± 0.42	10’449 ± 3’876	0.60 ± 0.07	1.96 ± 0.58
murex	organic	6.74 ± 0.77	9’851 ± 6’434	0.46 ± 0.00	0.77 ± 0.04
mussel	organic	5.75 ± 0.55	12’436 ± 2’558	0.56 ± 0.03	1.65 ± 0.19
clam	shell	8.48 ±0 .09	733 ± 59	0.10 ± 0.01	Not analyzed
murex	shell	8.71 ± 0.12	692 ± 122	0.12 ± 0.01	Not analyzed
mussel	shell	8.54 ± 0.01	606 ± 145	0.12 ± 0.01	Not analyzed
mix	all	8.10 ± .57	5’572 ± 6’632	0.29 ± .01	Not analyzed

Concerning nutrient content, as shown in Table 33, the organic residue has the highest concentration of Phosphorous and crude protein. In particular, murex meat has, at the same time, a slightly higher level of crude protein content and lower value of fats that should facilitate the protein hydrolyzation process intended to be carried out to valorize such side-streams. On the other hand, all three shells, as expected, contain low nitrogen (0.42 g N/kg of dry matter, on average). The main constituent of mollusk shells, instead, is calcium carbonate (>81.4% of $CaCO_3$ on a dry basis).

***Table 33**: Phosphorous, crude protein, total fat, and carbonate in the shellfish by-products.*

Shellfish	By-product	P	Crude protein	Fats	$CaCO_3$
		mg/kg DM	g/kg DM	g/kg DM	%DM
clam	organic	9'195 ± 1'855	518 ± 76	227 ± 15	Not analyzed
murex	organic	8'354 ± 2'395	643 ± 13	174 ± 82	Not analyzed
mussel	organic	7'051 ± 1'912	497 ± 60	239 ± 51	Not analyzed
clam	shell	368 ± 130	1.18 ± .34	Not analyzed	87.25 ± 3.87
murex	shell	745 ± 229	2.57 ± 1.55	Not analyzed	81.42 ± 2.78
mussel	shell	455 ± 177	4.14 ± .54	Not analyzed	87.40 ± 6.44
mix	all	1'033 ± 305	7.35 ± 5.99	Not analyzed	79.42 ± 3.49

Other nutrients such as Na^{+}, Mg^{2+}, K^{+}, and Ca^{2+} were reported to be present in the mollusk shell as well as in the residual organic fraction (Table 34). Like for most of the analyzed parameter, there is no significant difference between each species.

***Table 34**: Concentrations of cations in shellfish by-products*

Shellfish	By-product	Na^{+}	Mg^{2+}	K^{+}	Ca^{2+}
		mg/kg DM			
clam	organic	16'887 ± 3'035	2'924 ± 538	9'399 ± 3'341	47'780 ± 35'172
murex	organic	11'195 ± 6'336	5'430 ± 1'908	9'715 ± 815	19'763 ± 12'718
mussel	organic	1'699 ± 4'506	3'498 ± 1'598	9'324 ± 419	27'510 ± 17'664
clam	shell	6'406 ± 1'290	323 ± 132	426 ± 179	349'409 ± 15'518
murex	shell	5'812 ± 178	1'002 ± 281	662 ± 55	326'053 ± 11'122
mussel	shell	4'016 ± 113	1'223 ± 83	197 ± 51	349'981 ± 25'787
mix	all	5'997 ± 347	1'816 ± 598	970 ± 62	318'018 ± 13'978

The mixed waste was characterized without separating the shell from the organic fraction. To compare the results with the separately collected sample, a mass balance was carried out to obtain the overall characterization of clam, mussel, and murex by-products before pre-treatment. Similarly,

there is no great difference between the samples confirming homogeneity of the side-stream although the variability could occur daily and seasonally depending on the production activity of Co.Pe.Mo (data not shown).

4.1.1.2 Fish waste

Percentage of dry matter is perfectly in line with values described by Esteban et al. (2007) for fish waste including heads, bones skin, viscera, and some whole fish, while the content of inorganic matter, on average, is lower than the one provided by Esteban et al. (2007) (21.79% DM).

***Table 35**: Dry matter (DM), Volatile Solids (VS), ashes, pH and salinity in combined fish waste.*

Fish waste	DM	VS	Ash	pH	Salinity
	%	%DM	%DM	-	µS
Combined waste	26.16 ± 6.91	86.30 ± 4.45	13.70 ± 4.45	5.96 ± 0.39	10’348 ± 2’570

For what concerns nutrients, crude proteins in percentage of dry matter are in line with literature values that in general go from 31.31% DM for tuna frames (Ahuja et al., 2020) and 57.92% DM for fish waste (Esteban et al., 2007). The content of other nutrients also results in the ranges provided by different authors, Esteban et al. (2007) for fish waste and Pateiro et al. (2020) for fish heads, whereas fish bones and fish frames commonly contain much higher Ca content (143.93-182 g/kg DM) as stated by Ahuja et al. (2020)

***Table 36**: Crude protein and nutrients concentration in combined fish waste.*

Fish waste	Crude protein	N	P	Ca	K	Na	Mg
	%DM	g/kg DM					
Combined waste	43.00 ± 7.57	68.79 ± 12.11	16.69 ± 9.89	39.15 ± 18.39	5.12 ± 1.11	7.72 ± 1.96	2.05 ± 0.42

4.1.2 Contaminants

4.1.2.1 Shellfish by-products

The measured concentration of heavy metals shown in Table 37 is validated by literature values. It is worth mentioning that high variability is detected compared to previously reported results since they are strongly dependent on the quality of the water where mollusk grow. For instance, published values for iron concentration in clamshell varies from 140 to 48,000 mg/kg on a dry basis (Ademolu et al., 2015; Nguyen et al., 2020) and our clamshell contains 1517 mg/kg of iron similarly to Li et al., 2020 (1119 mg Fe/kg). In general, the concentration of heavy metals is comparable to that of the organic fraction of municipal solid waste (Fisgativa et al., 2016), commonly used as raw material in aerobic and/or anaerobic treatments to produce fertilizers.

The concentration of heavy metals in both organic fraction, shell and overall by-products differs from one shellfish to the other, as expected.

***Table 37**: Heavy metals concentration measured in shellfish by-products as mg/kg DM.*

	Clam	**Murex**	**Mussel**	**Clam**	**Murex**	**Mussel**
	organic	**organic**	**organic**	**shell**	**shell**	**shell**
Al	808 ± 307	792 ± 454	723 ± 548	76.28 ± 46.04	712 ± 350	58.22 ± 32.80
As	14.53 ± 3.31	43.51 ± 24.60	28.95 ± 12.51	0.64 ± 0.29	2.78 ± 0.85	0.59 ± 0.30
Cd	0.40 ± 0.12	4.76 ± 5.62	4.04 ± 4.70	<0.01	0.74 ± 0.39	<0.01
Co	1.02 ± 0.24	1.05 ± 0.43	1.37 ± 0.58	0.93 ± 0.30	1.08 ± 0.09	0.98 ± 0.13
Cr	8.32 ± 4.41	8.30 ± 3.68	12.81 ± 17.07	8.14 ± 5.12	15.05 ± 4.06	2.54 ± 2.45
Fe	1’455 ± 668	845 ± 260	610 ± 246	1’517 ± 581	2’005 ± 70	1’247 ± 434
Mn	49.11 ± 12.94	35.17 ± 14.79	19.58 ± +4.05	13.48 ± +6.22	39.13 ± 15.00	8.04 ± 3.07
Hg	0.14 ± 0.04	0.38 ± 0.25	0.44 ± 0.23	0.72 ± 0.60	<0.01	<0.01

Mo	2.20 ± 0.67	1.30 ± 0.49	14.40 ± 19.54	0.91 ± 0.48	0.58 ± 0.09	2.60 ± 0.94
Ni	6.16 ± 1.14	5.99 ± 2.18	42.89 ± 67.16	10.19 ± 2.77	7.45 ± 1.43	8.85 ± 3.71
Pb	1.50 ± 1.07	0.84 ± 0.09	1.01 ± 0.69	<0.01	0.66 ± 0.06	0.72 ± 0.29
Cu	16.34 ± 3.69	117 ± 95	25.43 ± 18.13	6.50 ± 2.03	35.56 ± 25.69	6.41 ± 5.13
Se	2.25 ± 0.54	2.91 ± 1.24	3.67 ± 0.14	<0.2	<0.2	<0.2
Tl	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Zn	74.25 ± 38.52	164 ± 107	157 ± 46	2.88 ± 0.79	21.92 ± 3.91	6.77 ± 4.85

4.1.2.2 Fish waste

Content of heavy metals resulted to be higher with respect to literature values. For instance, Esteban et al. (2007) reported the content of Fe, Zn, Mn and Cu respectively as 100, 62, 6, and 1 mg/kg DM, that are the 66.97%, 28.41%, 57.39%, and 98.12% lower than values obtained on combined fish waste samples from Ittica del Conero (Table 38).

***Table 38**: Concentration of heavy metals on combined fish waste.*

	Combined waste
	mg/kg DM
Al	100.25 ± 52.03
Cd	6.83 ± 4.24
Co	0.08 ± 0.09
Cr	3.28 ± 1.27
Cu	54.16 ± 8.55
Fe	302.80 ± 169.87
Mn	14.08 ± 9.50

Mo	11.62 ± 5.80
Ni	1.44 ± 0.74
Pb	0.28 ± 0.06
V	0.48 ± 0.48
Zn	86.60 ± 32.24

4.1.3 Microbiology

From microbiological analysis, all the shellfish by-products resulted to be pathogen free. Specifically, salmonella resulted absent, while fecal coliforms and E. coli were both below 10 CFU/g.

This analysis has not been performed on fish waste.

4.1.4 Summary of characterization results

Figure 24 shows a summary comparing the results of all the physicochemical analysis done to the shellfish by-products and their fractions. It can be seen that there are no significant differences between the three shellfish species (clam, mussel, and murex) both comparing the shell or the organic fraction as well as the overall by-product. In addition, the mixed side-stream from the three shellfish species is quite a homogenized by-product that ensures replicability and stability of the following valorization chain.

The very low nitrogen content and the high amount of $CaCO_3$ observed in the mineral fraction of the shellfish waste (Figure 24) suggested the possible use of this waste as a soil liming agent, instead of fertilizer. On the other hand, the measured protein content of the organic fraction (Figure 24), instead, confirmed the theoretical possibility to recover protein hydrolysates from the residual meat. It also underlines the need for separating harder shells from the lighter residual meat.

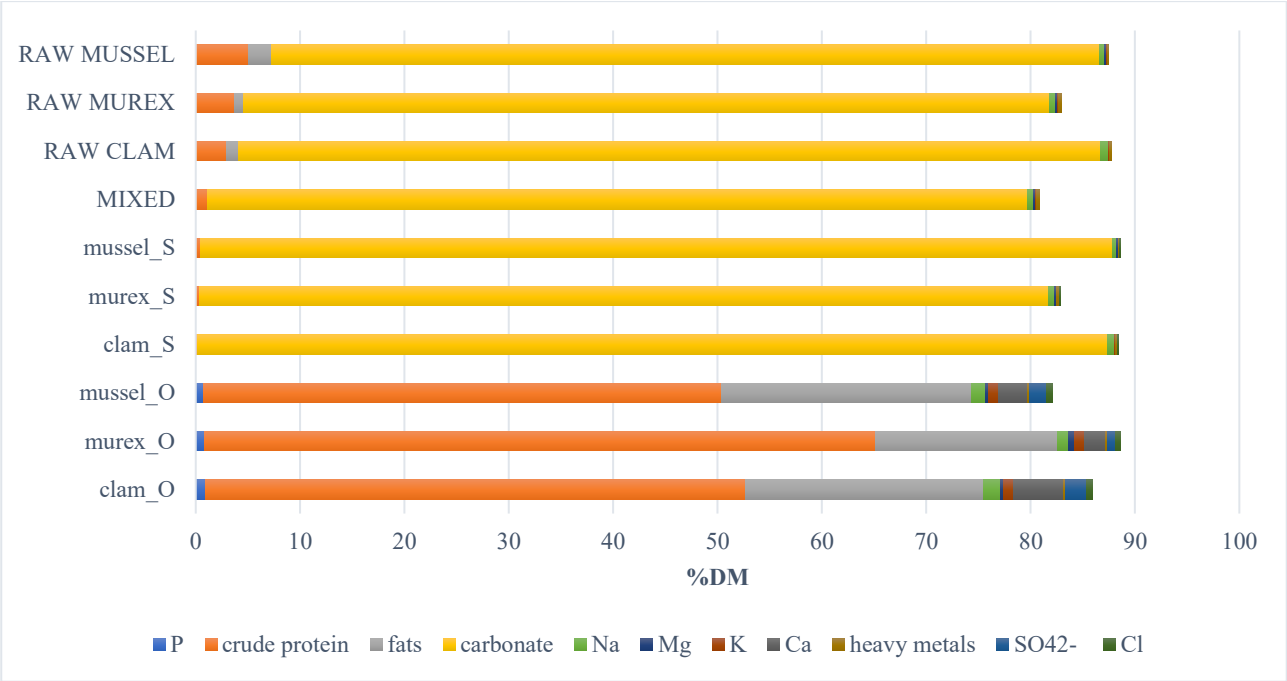


***Figure 24**: A comparison of the composition of different fractions and overall by-products, on a dry basis (S: shell, O: organic fraction)*

The composition of fish waste in terms of humidity and organic (and inorganic) matter is also very similar to the meat fraction of shellfish by-products (Figure 25).

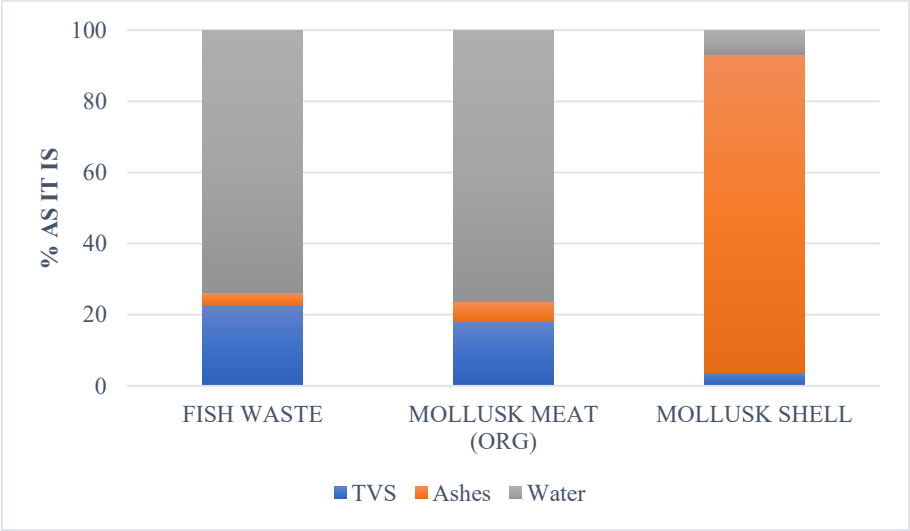


***Figure 25**: Water, ashes and TVS in fish waste, mollusk meat and mollusk shell.*

Mollusks meat resulted to contain the 21.24% more crude proteins as %TVS than fish waste (Figure 26). Furthermore, except for phosphorus, mollusks meat contains a greater content of nutrients (Figure 27).

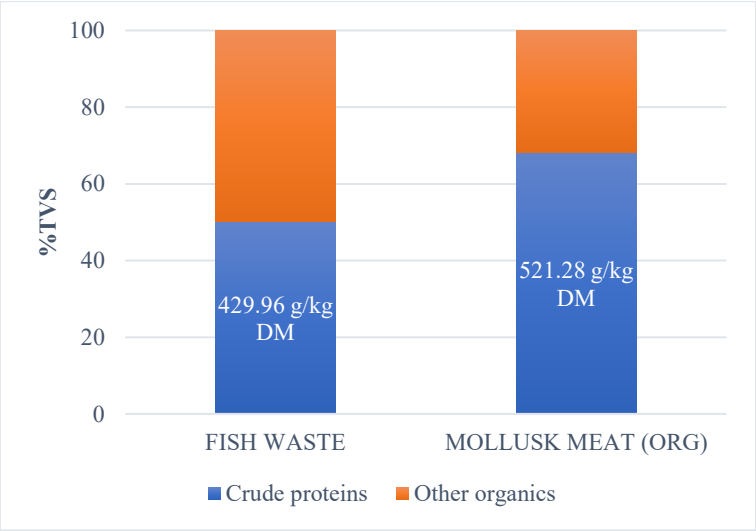


***Figure 26**: Crude proteins and other organics as percentage of TVS, for fish waste and mollusk meat.*

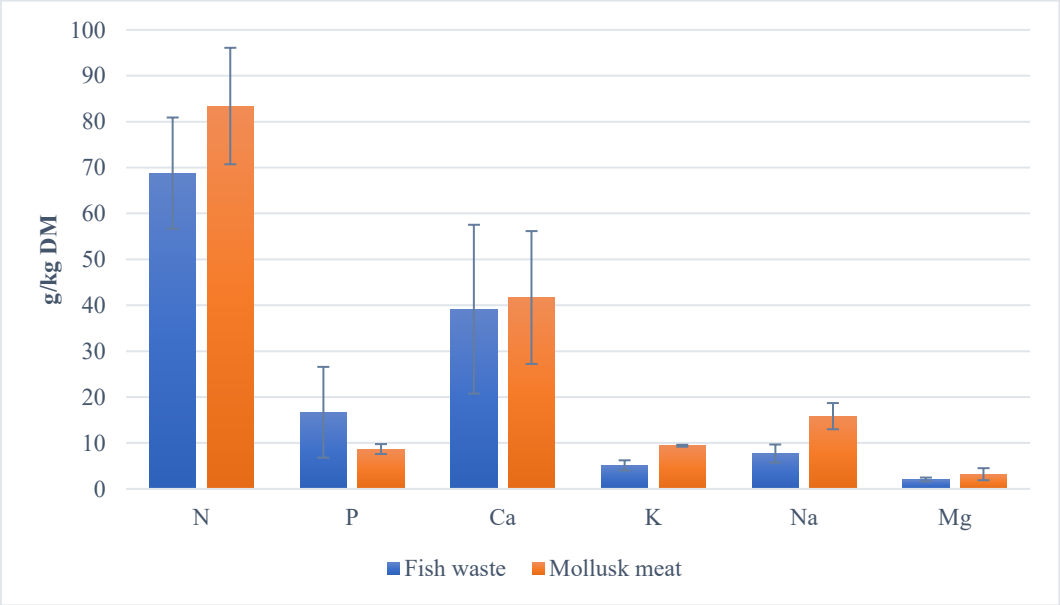


***Figure 27**: Macro and micro nutrients in fish waste, mollusk meat and shells.*

Relating to heavy metals, the most relevant are Al, Fe, and Zn in both fish by-products and mollusk meat. More in detail, in case of fish Al and Fe contents are respectively around the 87% and 75% lower than in mollusks.

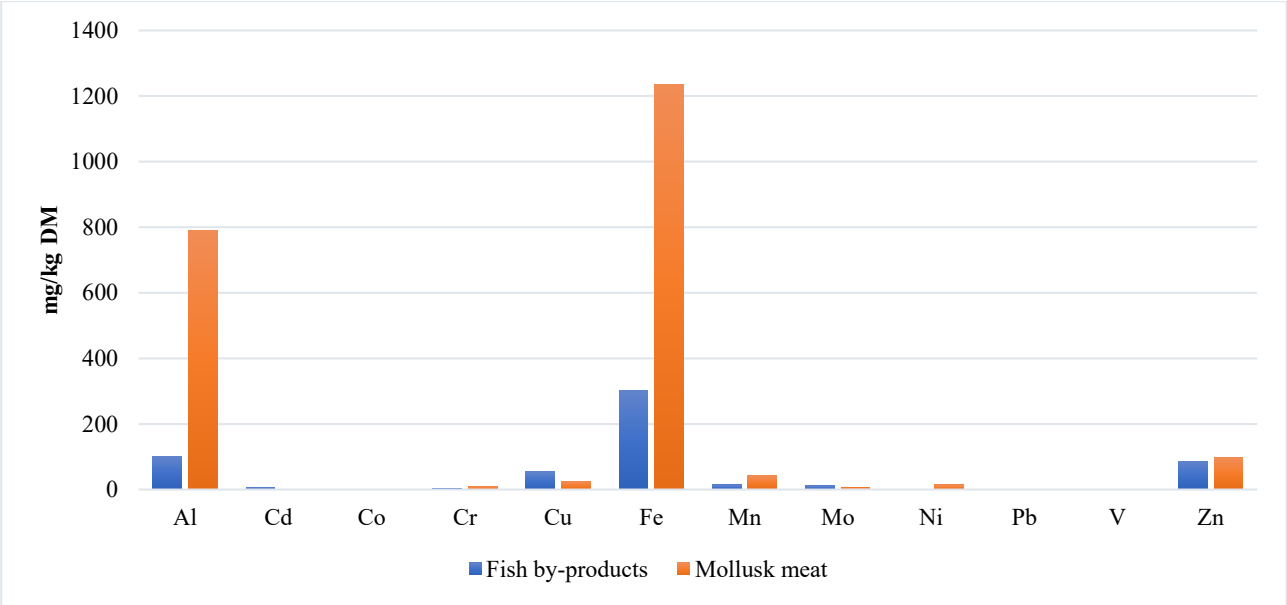


***Figure 28**: Comparison of the heavy metals content between fish waste and mollusk meat.*

4.2 Experimental section

In this chapter the main results of each test are described in chronological order, following the experimental strategy described above.

4.2.1 Correlation between Lowry and TKN methods

Before dealing in depth with the results of the individual tests, it is worth discussing the analytical methods for the determination of proteins on both liquid and solid fractions, since very different results have been obtained using the two methods, probably due to the interfering substances that could overestimate the protein contents, or give inaccurate results (Mæhre et al., 2018).

For what concerns the liquid fraction, Figure 29 shows the correlation existing between the two methods used for protein determination in this study. The best fitting of results is the linear one (R^2=0.7889). The correlation is appropriate; therefore, theoretically, both the methodologies could be used to analyze proteins. Approximately, proteins determined by Lowry method resulted to be the 50% of the crude proteins measured from the TKN method. However, it was chosen to show the results of crude proteins from TKN analysis because, although they are proportional to the proteins determined by the Lowry method, they are more regular. Lowry method, in fact, depends very much on the manual skill of the operator who performs it, and several other substances interfere with the measure. Although less accurate, the Lowry method is faster and allows you to get all the desired

results in a few hours, unlike the TKN method which requires at least 7 hours for 6 samples. This is the reason why the Lowry method has been anyway performed.

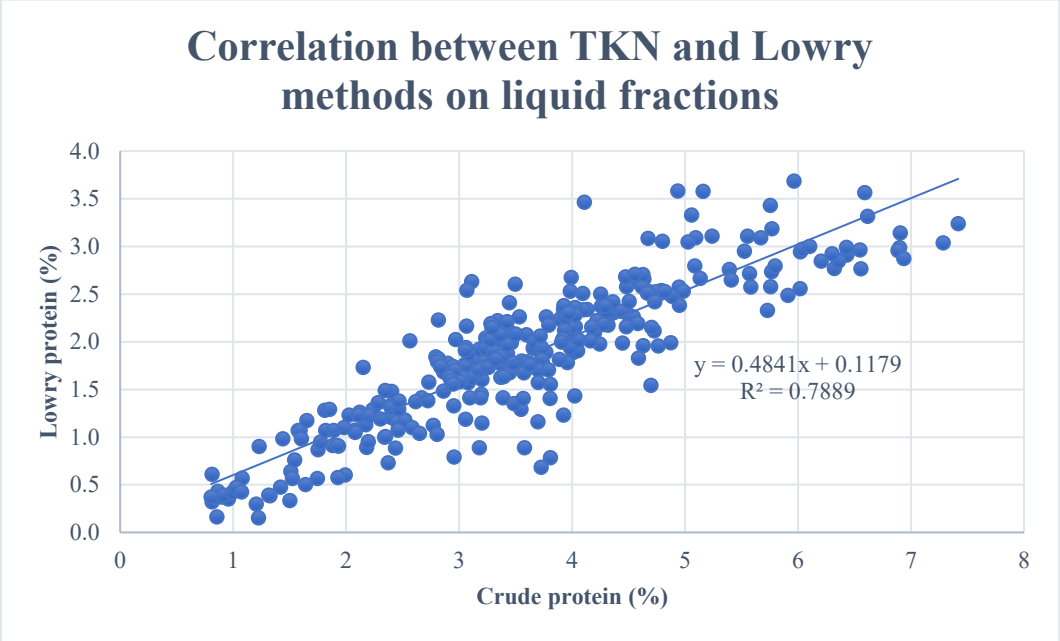


***Figure 29**: Correlation existing between the TKN and Lowry methods for protein determination.*

Figure 30 shows the correlation between the protein obtained with Lowry method (after salt-alkaline extraction) and the ones obtained from the TKN values multiplied by 6.25, to convert the nitrogen values to proteins (crude proteins), for the first four tests. Unfortunately, the correlation was not good enough, therefore the application of the Lowry method for the determination of protein content on the solids fraction has been abandoned. The present results can be affected by the extraction method required prior to analysis (Mæhre et al., 2018).

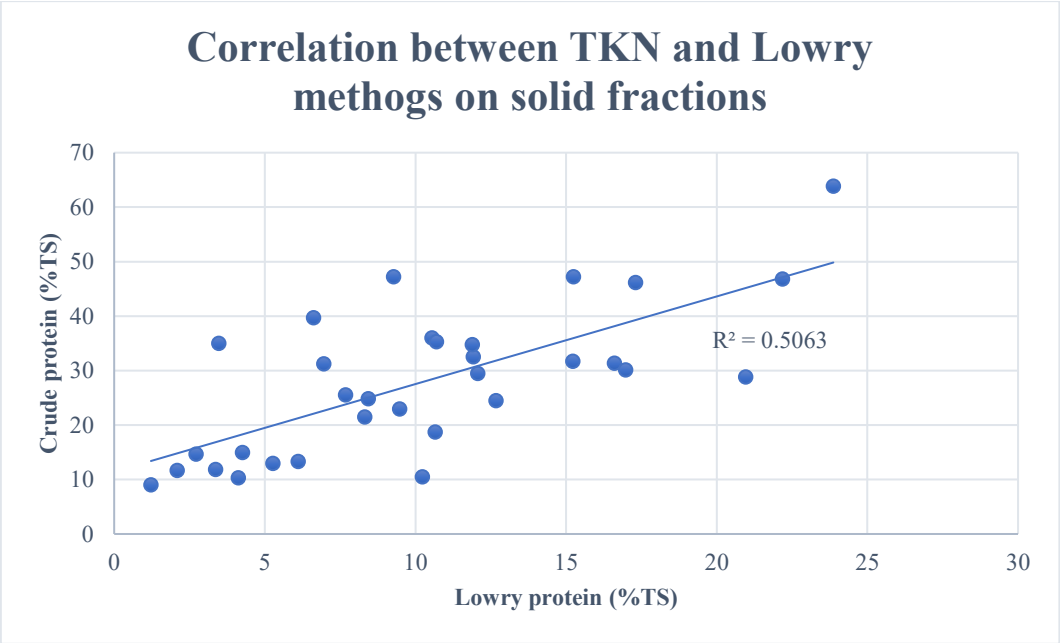


***Figure 30**: Correlation between Lowry and TKN methods for the determination of protein content on solid fractions.*

4.2.2 Substrates characterization

Table 39, Table 40, and Table 41 report the functional characterization of the substrates used for hydrolysis, in terms of humidity, total solids, total volatile solids, nitrogen as TKN, and carbon as percentage of TS.

Fish substrate has been analyzed as it is, without water addition. Total solids (%) are about 25.80 ± 4.65 on average, organic substance (TVS, %TS) is about 86.19 ± 2.94. Nitrogen content is highly variable 7.47 ± 1.76 %TS. This could be due to the presence of different species in fish waste generated in different periods. Instead, carbon content is quite stable and is about 47.10 ± 1.61 %TS.

Mollusks substrate has been taken as it is after shredding, therefore it already contains water. For this reason, humidity values are above 90%. The content of organic matter is much lower than in the fish and on average it is around 45.63 ± 13.82 %TS. This refers to the presence of inorganic material like sand or shells not properly separated after the shredding operation. It can be noted that, to higher level of organic matter correspond a higher content of nitrogen. Both nitrogen and carbon content are quite lower than in the fish. On average, the nitrogen content resulted to be 4.74 ± 1.48 %TS, while the carbon content was 24.94 ± 7.55 %TS.

Mix substrate has been recreated in the lab, according to the right proportion of components selected for the test. Total solids (%) reach 9.82±1.65 for dilution 1:2 and 15.89±1.71 for dilution 1:1. The content of organic matter (60.09 ± 6.42 %TS, on average) is intermediate between the one of mollusks

and fish; the same is for nitrogen and carbon contents (respectively 5.52 ± 1.40 %TS and 32.84 ± 3.51 %TS).

On average, the characterization of the substrates used during hydrolysis resulted to be in line with the preliminary one, presented in § 4.1.

***Table 39**: Fish substrate characterization.*

Fish substrate					
TEST	**Humidity%**	**TS%**	**TVS%**	**N%TS**	**C%TS**
23/09/2021	72.26	27.74	80.33	7.36	43.90
18/11/2021	67.98	32.02	89.06	5.56	48.67
02/12/2021	70.89	29.11	87.43	4.82	47.78
16/12/2021	76.68	23.32	84.16	7.55	45.99
18/01/2022	82.01	17.99	87.37	10.21	47.74
31/01/2022	75.72	24.28	89.29	8.70	48.79
14/02/2022	68.95	31.05	85.31	6.96	46.62
24/05/2022	71.33	28.67	85.35	5.86	46.64
Average ± SD	74.20 ± 4.65	25.80 ± 4.65	86.19 ± 2.94	7.47 ± 1.76	47.10 ± 1.61

***Table 40**: Mollusks substrate characterization.*

Mollusks substrate (M:W=1:2)					
TEST	**Humidity%**	**TS%**	**TVS%**	**N%TS**	**C%TS**
13/10/2021	95.96	4.04	64.87	6.59	35.45
02/12/2021	88.00	12.00	33.58	3.92	18.35
24/05/2022	88.40	11.60	38.03	3.25	20.78
Mollusks substrate (M:W=1:4)					
TEST	**Humidity%**	**TS%**	**TVS%**	**N%TS**	**C%TS**
18/11/2021	93.01	6.99	46.05	5.20	25.17
Average ± SD			45.63 ± 13.82	4.74 ± 1.48	24.94 ± 7.55

***Table 41**: Mix substrate characterization.*

Mix substrate (M:W:F=1:4:1)					
TEST	**Humidity%**	**TS%**	**TVS%**	**N%TS**	**C%TS**
18/11/2021	89.85	10.15	72.00	5.07	39.35
02/12/2021	90.71	9.29	65.24	5.02	35.65
18/01/2022	92.05	7.95	53.28	7.39	29.11
Mix substrate (M:W:F=1:2:1)					
TEST	**Humidity%**	**TS%**	**TVS%**	**N%TS**	**C%TS**
18/01/2022	85.96	14.04	57.06	7.66	31.18
08/02/2022	83.76	16.24	58.29	4.27	31.85
17/02/2022	82.59	17.41	58.98	4.69	32.23
Average ± SD			60.09 ± 6.42	5.52 ± 1.40	32.84 ± 3.51

4.2.3 Preliminary considerations

Figure 31 reports a typical trend of crude protein concentration (%) measured on liquid samples during the test in presence of endogenous enzymes and Alcalase. Time “0” corresponds to the point just before the addition of Alcalase, and time “24” stands for the time of enzymes deactivation. It is clear that most of the hydrolysis occurs within the 4 hours, then the rate starts to decrease, and after 6 hours the concentration became constant. In case of exogenous enzyme only, it has been observed that the constant value has been achieved around 4 hours of test (data not shown). This is the reason why it has been chosen to show the results in terms of rate of hydrolysis and nitrogen recovery at 4 or 6 hours.

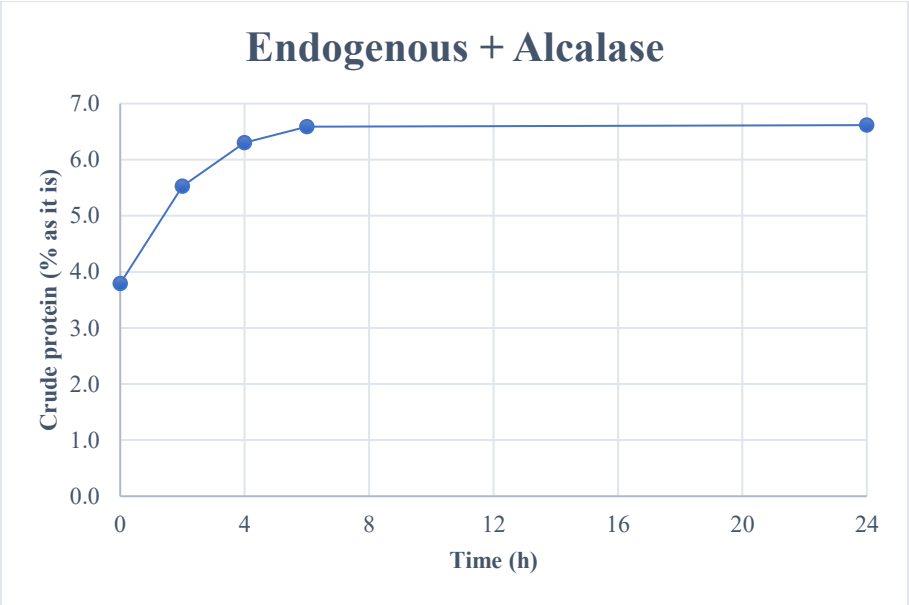


***Figure 31**: Typical crude protein concentration trend.*

In order to distinguish the results obtained during the first two tests, hence in presence of evaporation, from the ones obtained in the following tests during which the evaporation was avoided, the parameters describing the efficiency of the process, i.e., nitrogen recovery and rate of hydrolysis, have been recalled respectively as NR_EVAPO and RATE_EVAPO in the first two tests.

In addition, it is worth mentioning the fact that nitrogen recovery can reach values above 100% in these cases, as the effect of evaporation has resulted in very high nitrogen concentrations, especially towards the end of the test.

4.2.4 Effect of enzymes

The determination of the best exogenous enzyme for hydrolysis was the goal of the test n. 1.

It is clear from Figure 32, Figure 33, and Figure 34 that Alcalase provides the highest nitrogen recovery in all the three substrates, at all the temperatures. This is probably due to the different aims of the two enzymes. In fact, it is known that Alcalase is more efficient in obtaining peptides, whereas Protana aims to improve their quality.

In particular, it can be observed that, at temperatures different from 60°C, for which Protana induces an improvement in nitrogen recovery between 13% and 28% depending on the substrate, there seems to be no benefit from its use compared to blank. These benefits are instead more relevant in the case of Alcalase, and the percentages of improvement compared to blank are all the higher as the temperature is high (Table 42).

Furthermore, by taking a look at the rate of hydrolysis at 60°C (Figure 35), it can be seen that hydrolysis is even faster with Alcalase, so the high values of hydrolyzed proteins in the liquid fraction are reached sooner. It can also be noted that, although nitrogen recovery at the same temperature is higher for mollusks, fish is the substrate that has the highest rate of hydrolysis with Alcalase.

Therefore, it can be concluded that the results outcoming from the first test suggest the use of Alcalase enzyme at 60°C for the following ones, being the enzyme that provides the highest nitrogen recovery, at the highest rate of hydrolysis.

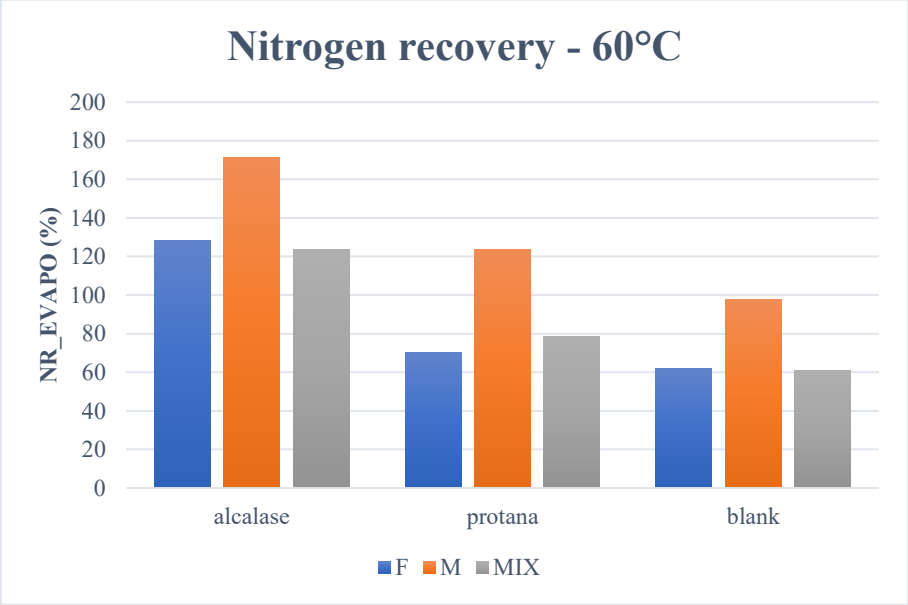


***Figure 32**: Nitrogen recovery at 4 hours (test n. 1 at 60°C).*

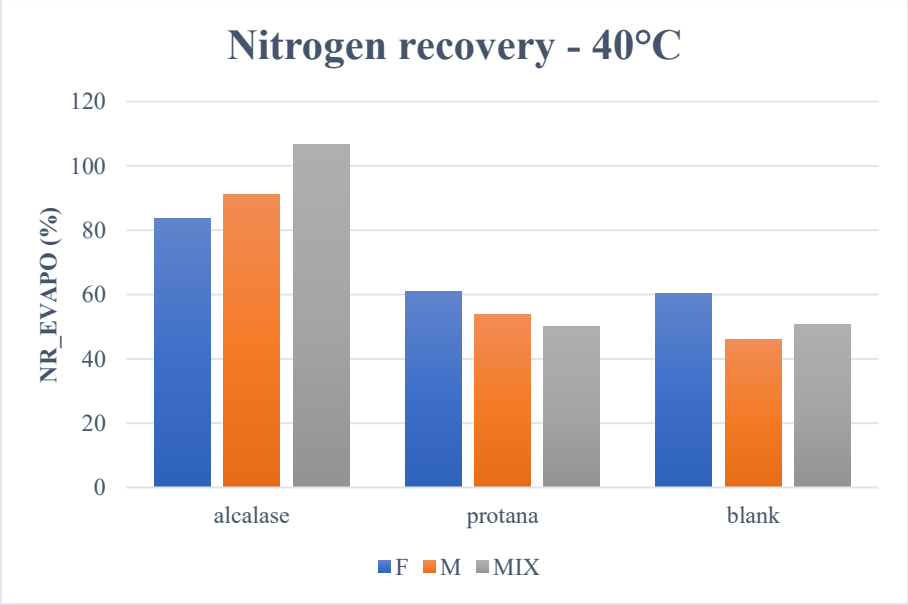


***Figure 33**: Nitrogen recovery at 4 hours (test n. 1 at 40°C).*

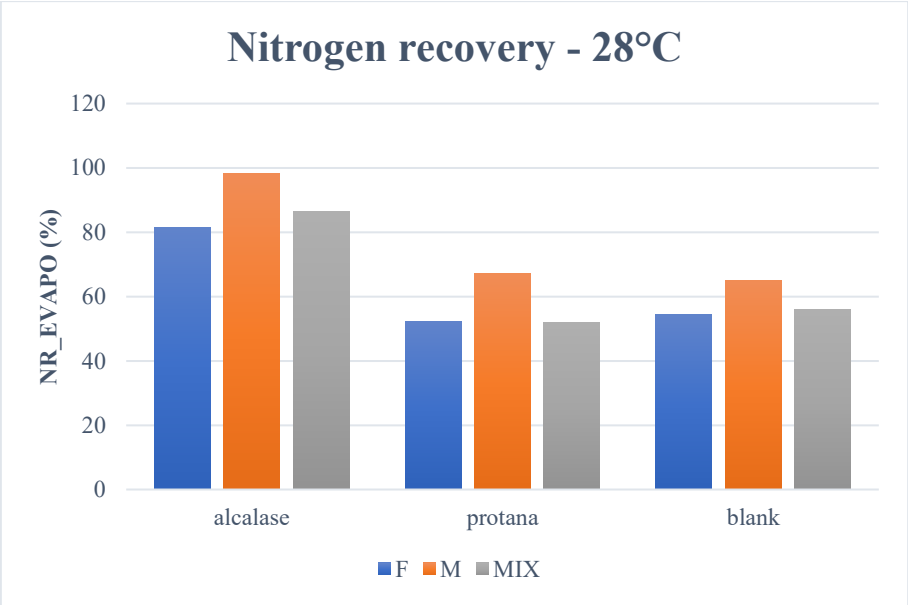


***Figure 34**: Nitrogen recovery at 4 hours (test n. 1 at 28°C).*

***Table 42**: Improvement of nitrogen recovery (%) by using Alcalase or Protana with respect to the blank.*

Enzyme	60°C			40°C			28°C		
-	F	M	MIX	F	M	MIX	F	M	MIX
Alcalase	106	76	102	39	97	111	50	51	55
Protana	13	26	28	1	17	-1	-4	3	-7

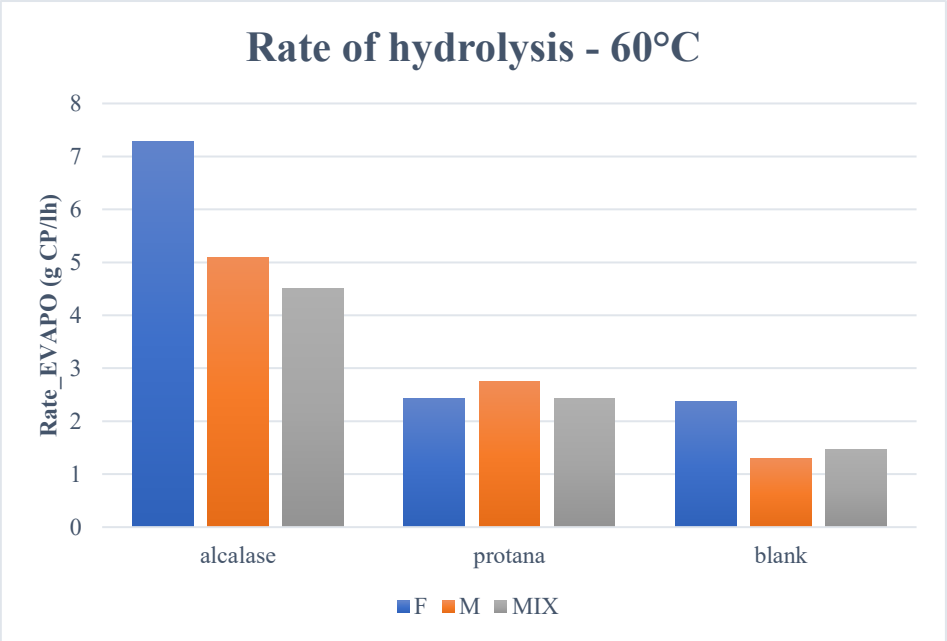


***Figure 35**: Rate of hydrolysis at 4 hours (test n. 1 at 60°C).*

4.2.5 Effect of pH control

As shown in Figure 36, that reports the rate of hydrolysis at 4 hours, with Alcalase, at 60°C, and three different pH, there are not significant differences between the rate of the process at different pH levels for the same substrate, although a slightly higher rate is shown at pH 7.5 for fish and mollusks. However, the rates of the mix substrate at all the pH are quite higher than those of fish and mollusks, meaning that the mix is faster in achieving the final values of crude protein.

By looking at the nitrogen recovery at 4 hours (Figure 37) it seems not to be affected by the pH for fish and mix who achieve almost the same values, while the mollusks show always a slightly better result at pH 7.5.

Therefore, for this test it can be concluded that, by correcting the pH to the stabilized values on the basis of literature review (§ 2.4.3.3), there does not seem to exist significant advantages. Therefore, in subsequent tests, it was preferred to continue with the natural pH, with the advantage of avoiding additional chemicals.

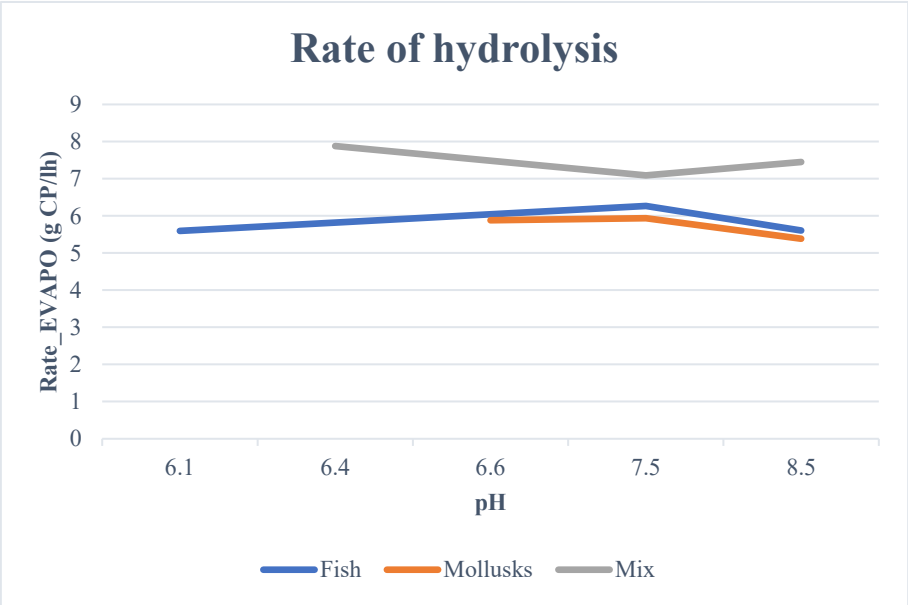


***Figure 36**: Rate of hydrolysis at 4 hours (test n. 2).*

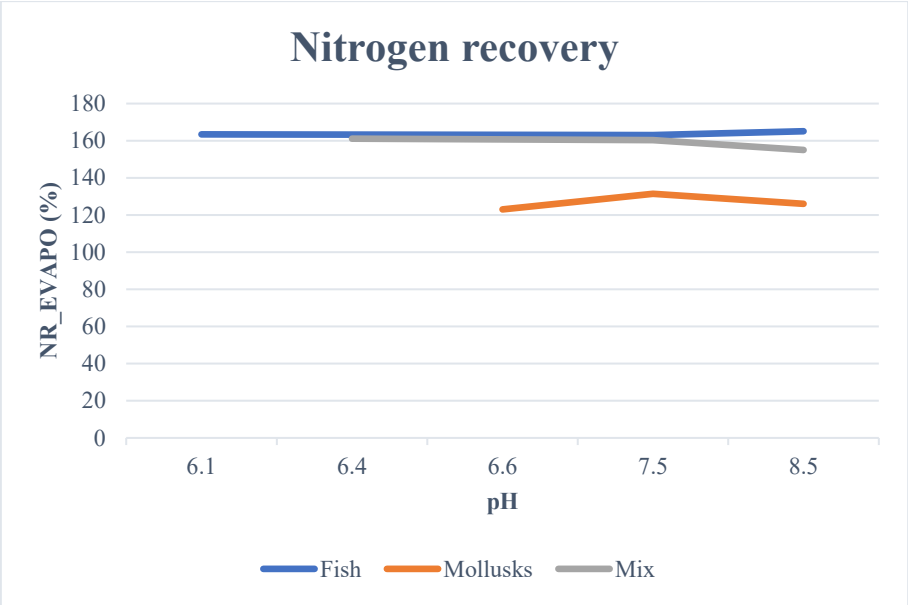


***Figure 37**: Nitrogen recovery at 4 hours (test n. 2).*

4.2.6 Effect of evaporation

Evaporated weight values were obtained as the difference between the total weight of the hydrolyzing mixture, comprising the enzyme and the NaOH when added, and the sum of the weights of samples (post-deactivation) and hydrolyzed mixture, before solid-liquid separation. Hence, the percentage of evaporation has been obtained as the ratio of the evaporated weight to the total weight of hydrolyzing mixture.

Figure 38 reports the evaporation percentages calculated at the different temperatures and pH, outcoming from tests n. 1, 2, and 3. Results of the different substrates are averaged since evaporation does not depend on the type of raw material. There is not a relevant difference between the evaporation at 28 and 40°C. Instead, as expected, evaporation at 60°C and natural pH resulted to be respectively the 90% and 78% higher than that at 40 and 28°C. In addition, it can be observed that evaporation was partially contrasted by the addition of NaOH. In fact, the evaporation was respectively the 4% and 22% lower at pH 7.5 and 8.5. Evaporation percentage in closed vessels was around the 70% lower with respect to case in open vessels at the same temperature. However, evaporation could not be completely avoided as the closure of the vessels was not perfectly airtight, moreover these were opened during the test for sampling.

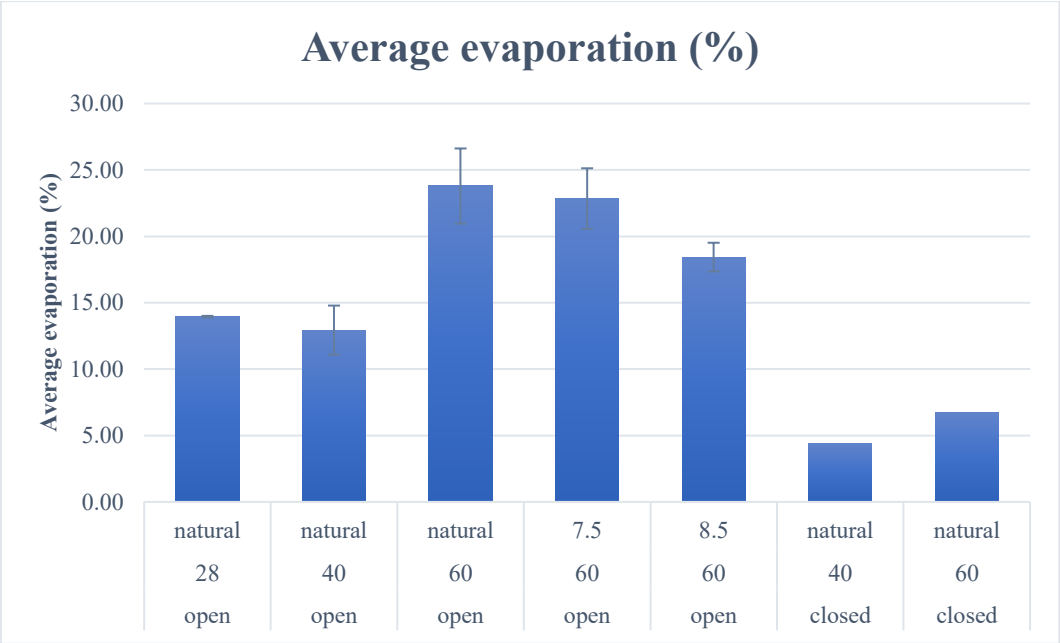


***Figure 38**: Average evaporation for tests n. 1 and 2.*

Figure 39 represents the evaporation trend that has been defined as cumulative evaporated weight during the test n. 3, showing what happens during hydrolysis: all the trends are clearly linear and proportional to the operating temperature. Evaporation rates are reported in Table 43. Closing vessels, the evaporation rate is around the 89.22% and 83.49% lower than with open vessel respectively at 60°C and 40°C.

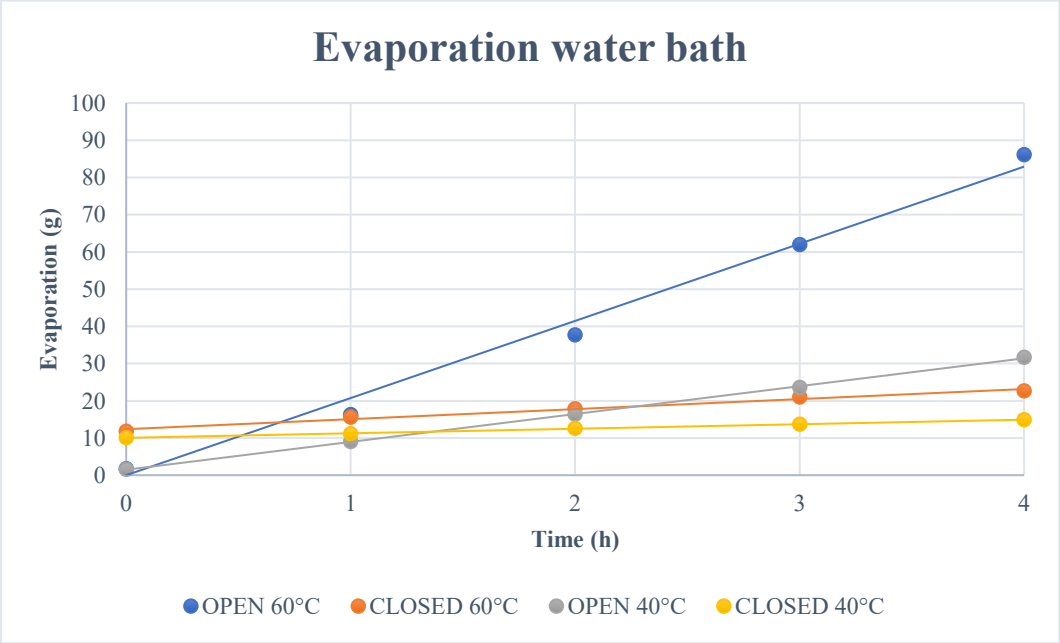


***Figure 39**: Cumulative evaporation during hydrolysis (test n. 3).*

***Table 43**: Evaporation rate and and effectiveness of approximation.*

Vessel	Temperature (°C)	Evaporation rate (g/h)	R^2
Closed	60	2.696	0.9840
Open	60	20.731	0.9964
Closed	40	1.218	0.9975
Open	40	7.486	0.9996

4.2.7 Effect of dilution ratio

The determination of the effect arising from the reduction of dilution ratio has been investigated in test n. 4.

Figure 40 shows the results of crude protein obtained by testing the fish and mix substrates at two different dilution ratios. It is clear that protein values obtained with a S:W ratio of 1:1 are much higher than the ones obtained with the ratio of 1:2, since they are more concentrated.

To compare the effect of the dilution ratio, it is necessary to consider the rate of hydrolysis (Figure 41), or the nitrogen recovery (Figure 42), in which the results are normalized with respect to the nitrogen content of the substrate. In terms of rate of hydrolysis, the dilution ratio 1:1 performed better than the 1:2, therefore the final values of crude protein are reached faster. Instead, if nitrogen recovery is used as parameter describing the efficiency of the process, both the tests conducted with a dilution ratio 1:2 have a better nitrogen recovery than those with a dilution ratio 1:1. For fish substrate this difference is not so relevant, while it is evident for the mix. This result could be expected from the state-of-the-art. In fact, although evaluating the efficiency in different terms from us, Vázquez et al. (2020) found that with substrate to water ratios 1:2 and 1:3 the degrees of hydrolysis was higher than with 1:1.

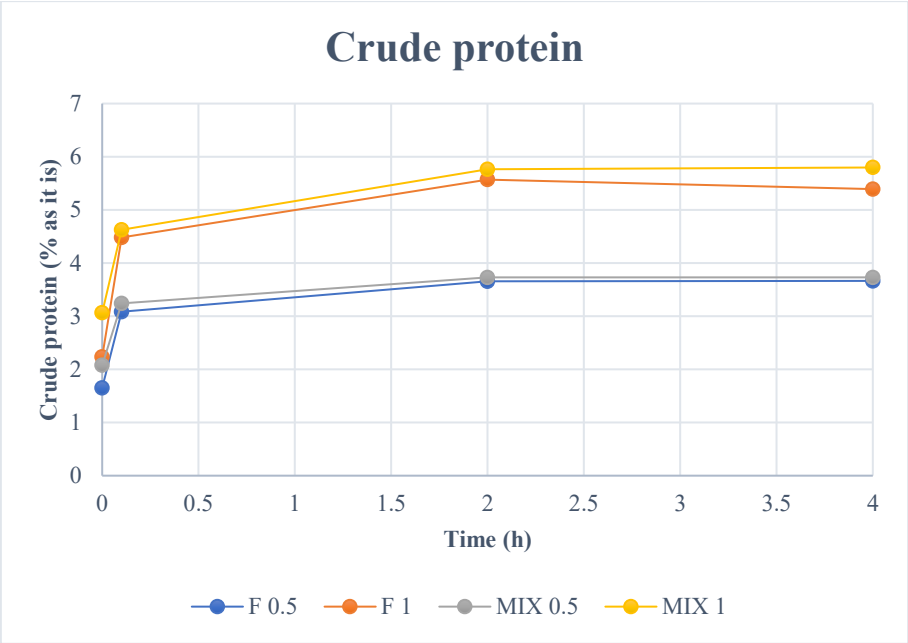


***Figure 40**: Crude protein for test n. 4.*

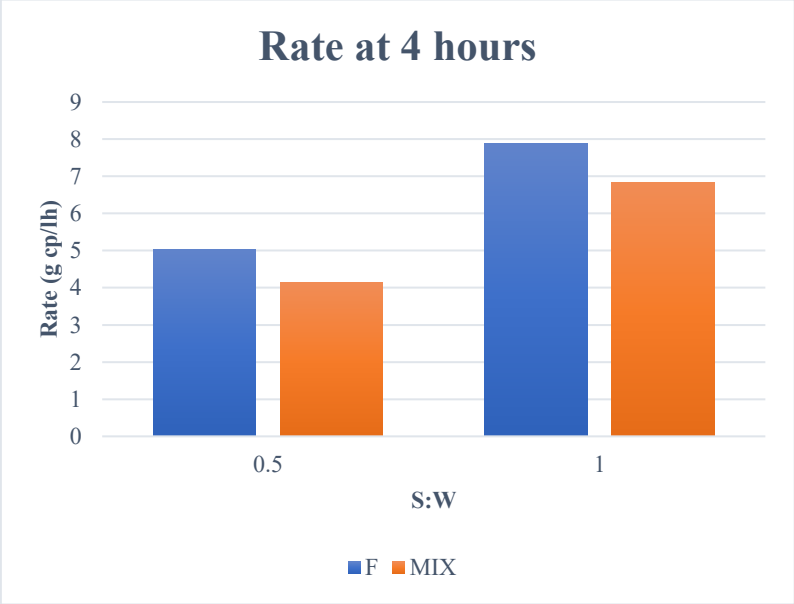


***Figure 41**: Rates of hydrolysis at 4 hours (test n. 4).*

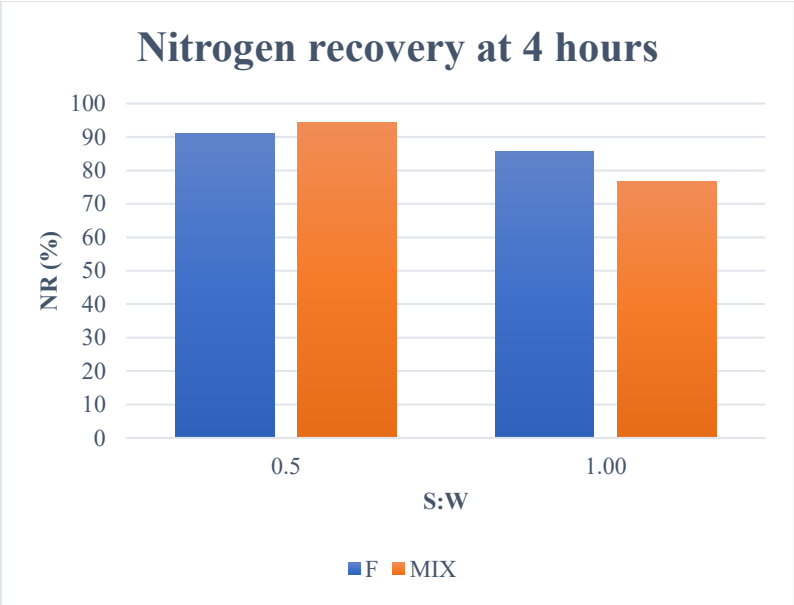


***Figure 42**: Nitrogen recovery (test n. 4).*

4.2.8 Effect of endogenous enzymes

This test lasted for 6 hours since the effect of endogenous enzymes was under exam. Figure 43 shows that the rate of hydrolysis in presence of endogenous enzymes only is very low and almost no difference with the blank exists, while with the addition of Alcalase to the endogenous enzymes it increased of 264% and 172% respectively for fish and mix substrates.

In general, results of the rates are confirmed also for nitrogen recovery (Figure 44). More in detail, the nitrogen recovery is, for fish and mix respectively, the 70% and 62% higher in case of Alcalase addition with respect to the case with endogenous enzymes only.

Finally, to have an idea of the improvement arising from the absent deactivation of endogenous enzymes, the nitrogen recovery obtained with the combination of endogenous enzymes and Alcalase at 4 and 6 hours (test n. 4) and the nitrogen recovery at 4 hours outcoming from the previous test n. 4 under the same conditions (60°C, natural pH, S:W 1:1, E:S 1:100) are roughly compared in Figure 45. Basically, comparing both at 4 hours there are not relevant differences, being the NR in test n. 5 only the 2% and 13% higher than with Alcalase only, respectively for fish and mix. Instead, comparing the final values (at 6 hours in presence of endogenous enzymes, and 4 hours in case of Alcalase only), the same percentages reach respectively the 8% and 18%. Therefore, from this comparison between different tests it can be concluded that the presence of endogenous enzymes can be considered beneficial from the point of view of NR.

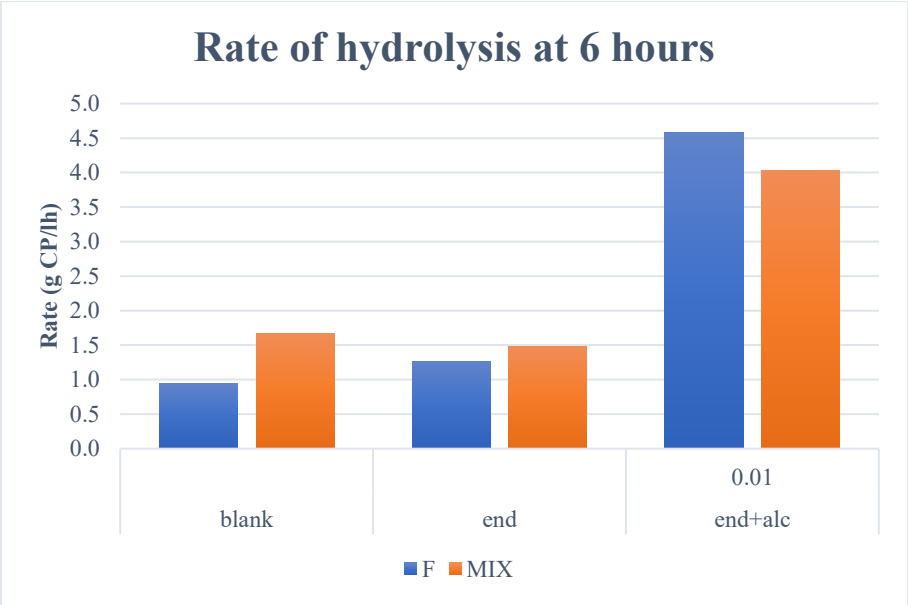


***Figure 43**: Rate at 6 hours of hydrolysis (test n. 5).*

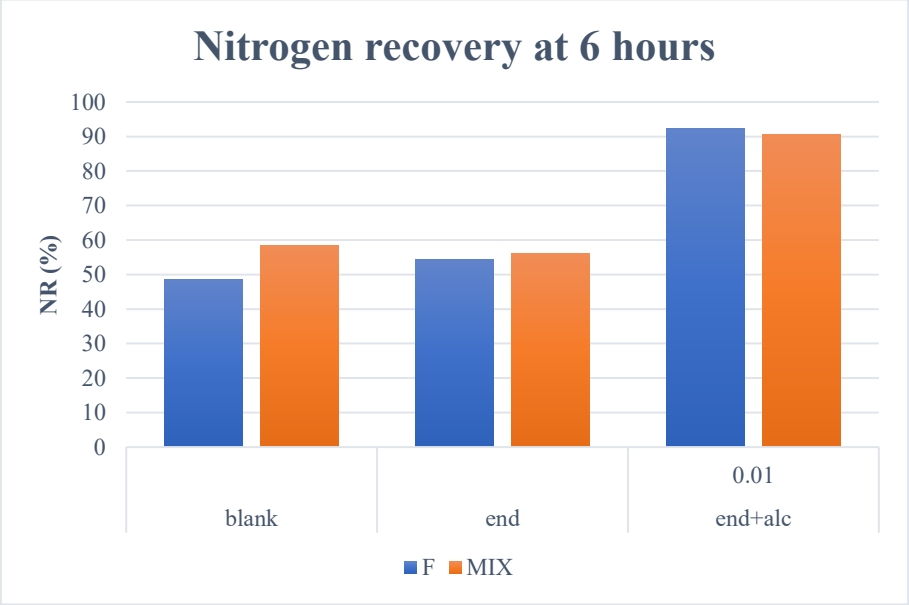


***Figure 44**: Nitrogen recovery at 6 hours (test n. 5).*

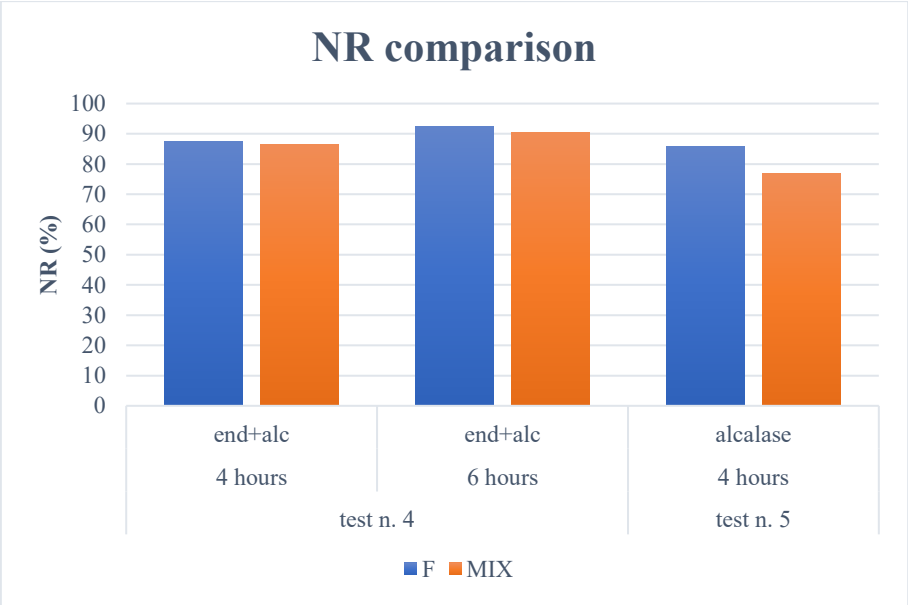


***Figure 45**: Comparison of the nitrogen recovery at 4 and 6 hours in test n. 4 with endogenous enzymes and Alcalase, and at 4 hours in test n. 5 with Alcalase only.*

4.2.9 Effect of E:S ratio

Figure 46 shows the rate of hydrolysis which have been measured during test n. 6. It can be observed that the fish is faster than mix in achieving the highest value of crude protein and the highest rate has been obtained with the E:S ratio equal to 1:100 for both the substrates.

In terms of nitrogen recovery, the values obtained for the same substrate with E:S equal to 1:100 and 1:200 are comparable, although several authors showed that the increase in the enzyme concentration corresponds to an increase in the DH (Quaglia & Orban, 1987; Benjakul & Morrissey, 1997; Klompong at al., 2007; Vázquez et al., 2020) and nitrogen recovery (Benjakul & Morrissey, 1997). This would be an excellent result as it would mean reducing to half the amount of enzyme to be dosed, with a considerable saving on an economic and environmental level. However, it is necessary to test the same products in pot tests to check if the two hydrolysates are comparable also for quality, providing almost the same biostimulant effect.

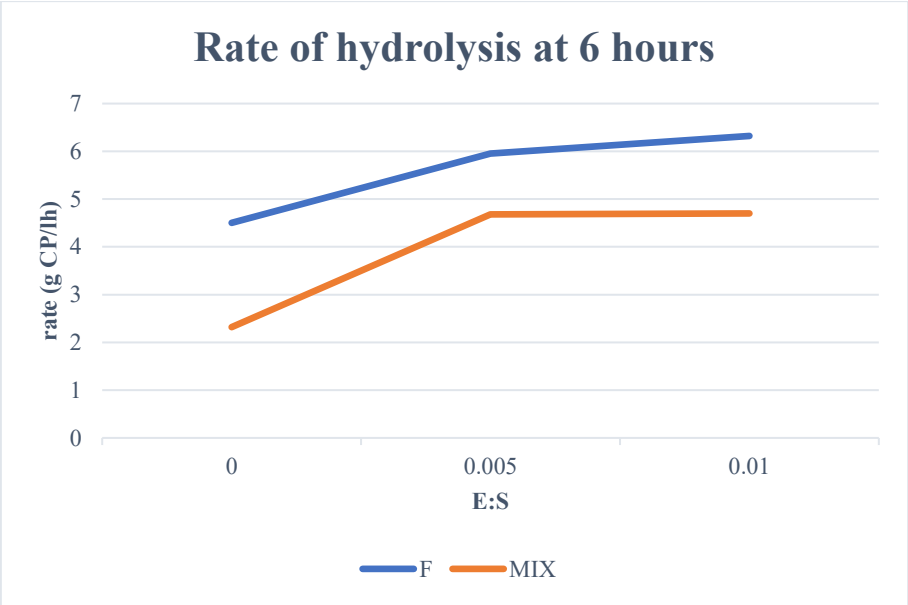


***Figure 46**: Rate of hydrolysis at 6 hours (test n. 6).*

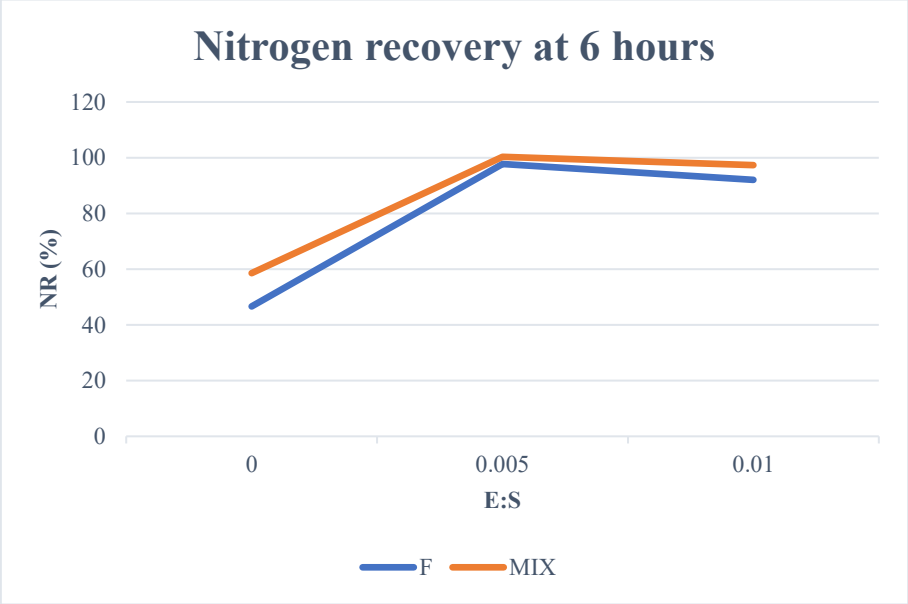


***Figure 47**: Nitrogen recovery at 6 hours (test n. 6).*

4.2.10 Effect of the temperature

The most effective temperature for protein hydrolyzation is 60°C also in the case of endogenous enzymes and Alcalase combination.

In fact, from Figure 48 it is clear that the rate of hydrolysis at 60°C is much higher than that at the other two temperatures. In particular, in case of fish and mix, the rate at 28°C and 40°C are almost the same, with an improvement at 60°C with respect to 40°C respectively of 25% and 57%. Instead,

for mollusks, it can be observed that the improvement with the temperature is much more evident, and equal to 121% between the rates at 28°C and 40°C, and equal to 74% between the rates at 40°C and 60°C. In addition, also in this case, the mix resulted to be the fastest among the three in achieving its highest value at the end of the 6 hours, and mollusks the slowest.

Concerning the nitrogen recovery, there are not relevant differences between the values obtained at 28°C and 40°C, while the values at 60°C are significantly higher, being the differences with the second high value of 11%, 13% and 22% respectively for fish, mollusks, and mix. In this case, the lowest values of nitrogen recovery have been obtained with fish at 40°C and 60°C, and the highest with mix.

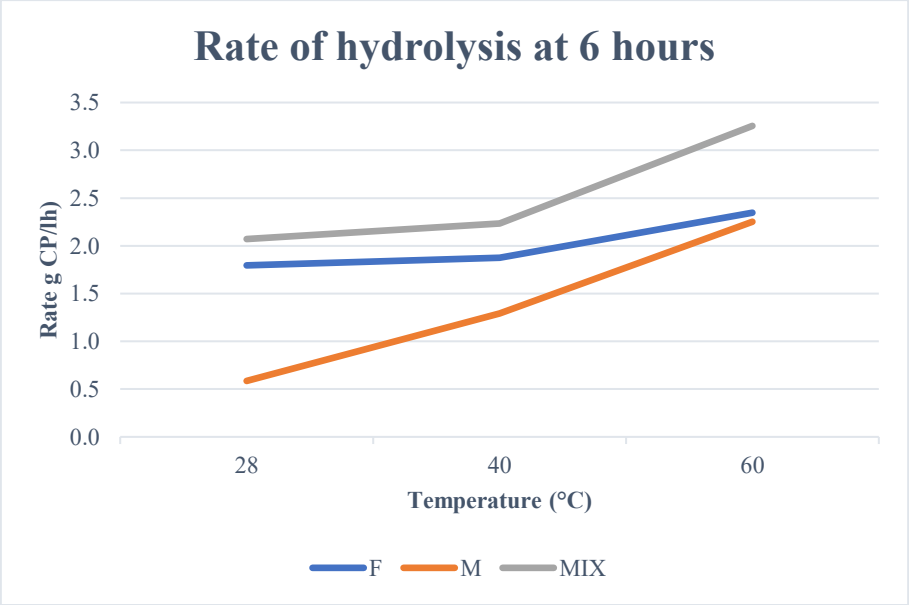


***Figure 48**: Rate of hydrolysis at 6 hours (test n. 7).*

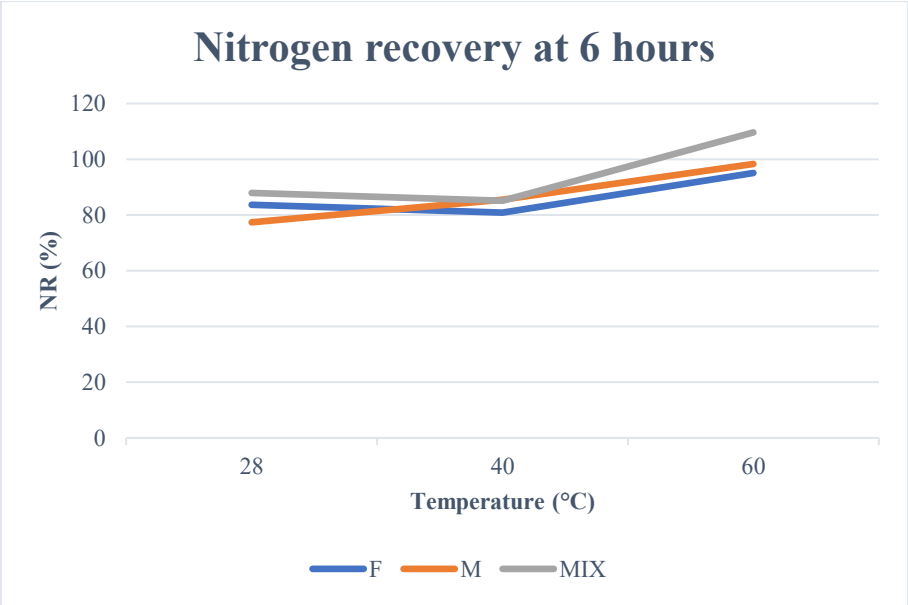


***Figure 49**: Nitrogen recovery at 6 hours for (test n. 7).*

4.2.11 Comparison of the three substrates

It is not possible to directly compare the behavior of the three substrates in tests conducted under different operating conditions, since the results do not show particular behaviors.

However, the following information can be extracted from the analysis of results: mollusks have always a lower protein content and rate than the other two substrates; although fish has the highest protein content, it does not always have the highest nitrogen rate and recovery; the behavior of the substrate mix is not in the middle between that of fish and shellfish as might be expected.

In particular, it can be observed that the rate and the nitrogen recovery corresponding to the mix are in many tests quite in line with those of fish (test n. 4, 5, 6), while in some tests the rate of the mix is even higher (Figure 35, Figure 48) than those of the fish. This behavior suggests a sort of synergistic effect between the fish and the mollusks that make up the mix.

4.2.12 Liquid yields

It can be observed that the trends of liquid yields (%) during hydrolysis are approximated as logarithmic (data not shown).

In particular, the effect of Alcalase addition to the endogenous enzymes on the liquid yields is reported in Figure 50, for the mix substrate, but the same can be observed for fish. In this case, it can be observed that is the Alcalase addition that gives the logarithmic trend to the liquid yield. In fact, in presence of endogenous enzymes only, the liquid yields during the tests remain almost constant.

In addition, it can be noted that the concentration of exogenous enzymes that is added to the hydrolyzing mixture does not influence the liquid yield. In fact, both the trends for E:S equal to 1:100 and 1:200 are comparable.

Hence, it can be concluded that the addition of the exogenous enzyme allows to reach a final yield of more than 90%. In absence of exogenous enzymes, the final yield remains close to 65-75%.

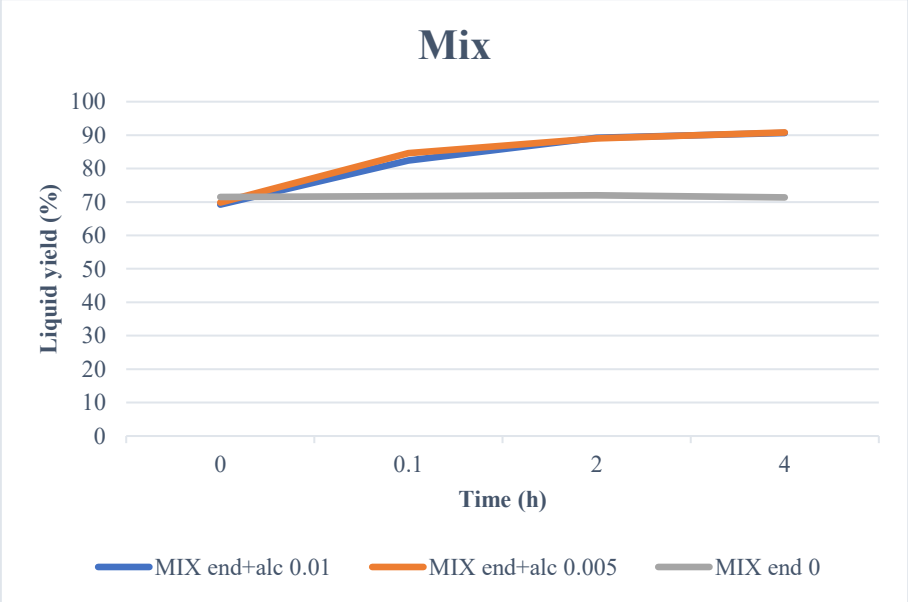


***Figure 50**: Influence of Alcalase addition on liquid yields.*

4.2.13 Characteristics of the final PH in the optimal conditions

Table 44 summarizes the main characteristics of the final hydrolysates at specific operating conditions. In particular, Table 44 reports the operating conditions that will be selected as reference data to compare the LCA of the three scenarios based on the used substrate (left), and the operating conditions which minimize the use of water and Alcalase (right). In this last case, the mollusk substrate is not present, because as already discusses, it was not possible to prepare it with a dilution ratio 1:1.

In particular, with the use of both a S:W ratio equal to 1, and an E:S ratio equal to 0.005, it is possible to improve both the TKN, therefore the crude proteins present in the PH, and the organic carbon content. The final content of the above conditions is due to the higher concentration of the mixture, in turn due to half of the dilution. It must be specified that this does not means that the efficiency of hydrolysis is higher.

***Table 44**: Characteristics of the final hydrolysates to be used for LCA study (left) and the optimal ones from the point of view of resources consumption (right).*

Operating conditions				
Enzyme	end+alc		end+alc	
Set T (°C)	60		60	
Set pH	natural		natural	
S:W (w:w)	0.5		1	
E:S (v:w)	0.01		0.005	
Substrate	**TKN %**	**C org (%)**	**TKN %**	**C org (%)**
F	0.69	2.79	1.10	8.02
M	0.56	2.44	-	-
MIX	0.66	2.64	1.01	4.42

4.2.14 Comparison with law requirements

According to the Italian legislation (D.Lgs. n. 75/2010), this product could not be directly included in any of the existing categories as reported in the regulatory framework in the state-of-the-art section. First, solid products are excluded from the comparison because with our means it is not possible to obtain a solid product, since a spray- or freeze-drying step would be required. Among the liquid products, nitrogenous organic fertilizers must contain a too high content of nitrogen (total or organic) and organic carbon. Therefore, due to the so low nitrogen content and the nature of the raw material, it can be considered more similar to the hydrolysed animal epithelium (solid or fluid), used as biostimulants, even if, generally, this kind of products are produced through chemical or thermal hydrolysis. However, the minimum content of organic carbon was not reached in any case. One possible solution could be considering some external additions. In any case, the nitrogen content of our PHs is evaluated in terms of TKN, therefore it contains both organic nitrogen and ammonium and ammonia nitrogen. Considering that these values contain ammonia nitrogen, the real organic nitrogen may be significantly lower than the minimum required for biostimulants (4%), therefore an efficient concentration step would be necessary to meet the minimum requirement; in addition, it would be necessary to determine the content free amino acids, which must be at least the 10% to understand if

it has biostimulant capacity. Instead, considering the few indications present in new European regulation, our product is closer to the minimum requirements (Table 45).

***Table 45**: Comparison with law requirements.*

Type designation	N (%)	Soluble N org	C org	Other	Note
-	%	%	%	-	-
Hydrolysed animal epithelium (solid or fluid), as biostimulant	4 (organic)	1 of N org	15	C/N ≤ 6	Glicine/(Proline+ Hydroxyproline) = 1.1 Degree of hydrolysis dry matter> 330 Free amino acids > 10%
Liquid organic fertilizer, PFC 1(A)(II)	2 (total)	-	5	-	-
Fish PH	1.10 (TKN)	-	8.02	-	-
Mix PH	1.01 (TKN)	-	4.42	-	-

4.3 Life Cycle Assessment

In this section, the main results of the LCA study are discussed. Basically, the Assessment of valorization represents the assessment of the environmental impact from the point of view of valorization of the three substrates, compared in terms of environmental impact resulting from the treatment of 1 ton of waste, whereas the Assessment of production represents the assessment of the environmental impact from the point of view of the PH production, therefore the comparison is with regard to environmental impact generated by the production of 1 ton of respectively fish-, mollusks-, mix- based PH.

4.3.1 Assessment of valorization

4.3.1.1 Fish

In order to determine which is the most relevant category among all, the normalized impacts have been calculated by dividing the single values outcoming from the LCIA analysis by the impact generated by one person in one year on each category (Sleeswijk et al., 2008). In this way, it is possible to compare the absolute contributions of each category, which was characterized by a different unit of measure. From this analysis, it results that assuming to sum all the potential impacts analyzed without any weighing step, the fossil fuel depletion (FD), contributes for almost the 50% to the total impact in this kind of process; the second most important category is the climate change (CC) for around the 13-14%, followed by photochemical oxidant formation (POF), ionizing radiation (IR), freshwater eutrophication (FE), terrestrial acidification (TA) all between the 9-5%, and so on (Figure 51).

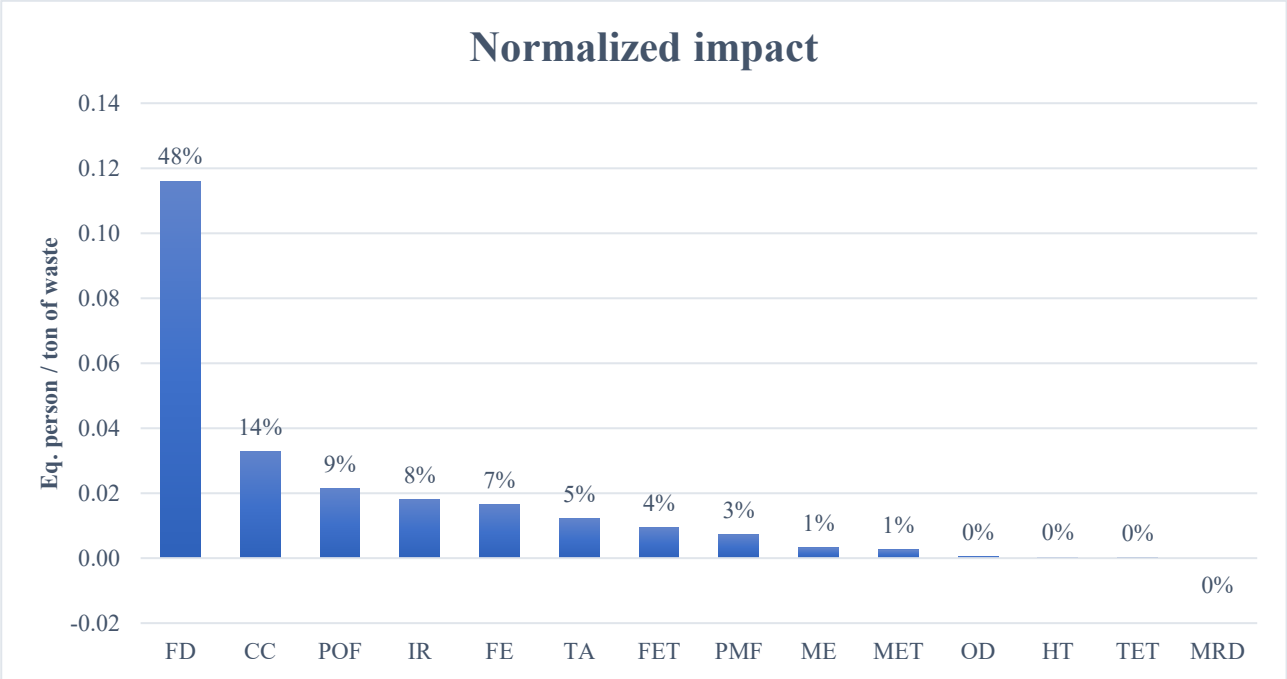


***Figure 51**: Normalized impact on each impact category, for fish.*

However, this view of the results, does not show which are the main contributors to each category and which are the most impacting phases of the process.

To discuss the results in these terms, it is worth specifying that the impacts called hereafter as "positive", are the ones which provide a negative effect on the environment, whereas the impacts called as "negative", are actually the ones which have a positive effect on the environment.

To understand the contribution of each phase the calculations have been reworked so as to make the positive impacts the 100%, and the negative impacts have been rendered as a percentage of the positive impact, to understand in which category the negative impact was more relevant, compared to the positive. In this case, the negative impact consists only of the avoided N fertilizer. This is not considered in none of the operating phases, since it is the result of the process as a whole.

From this point of view, the avoidance of mineral nitrogen-based fertilizer allows to recover between the 30% and 45% of the positive impacts for particulate matter formation (PMF), terrestrial acidification (TA), human toxicity (HT), marine ecotoxicity (MET), marine eutrophication (ME). In case of mineral resource depletion (MRD), the negative impact is even greater than the positive one; i.e. over 100%. With regard to the positive impacts, the most relevant impacts are related to the hydrolysis process, followed by concentration, pre-treatments, and centrifugation, for all the impact categories, with small variations in the relative contributions (Figure 52).

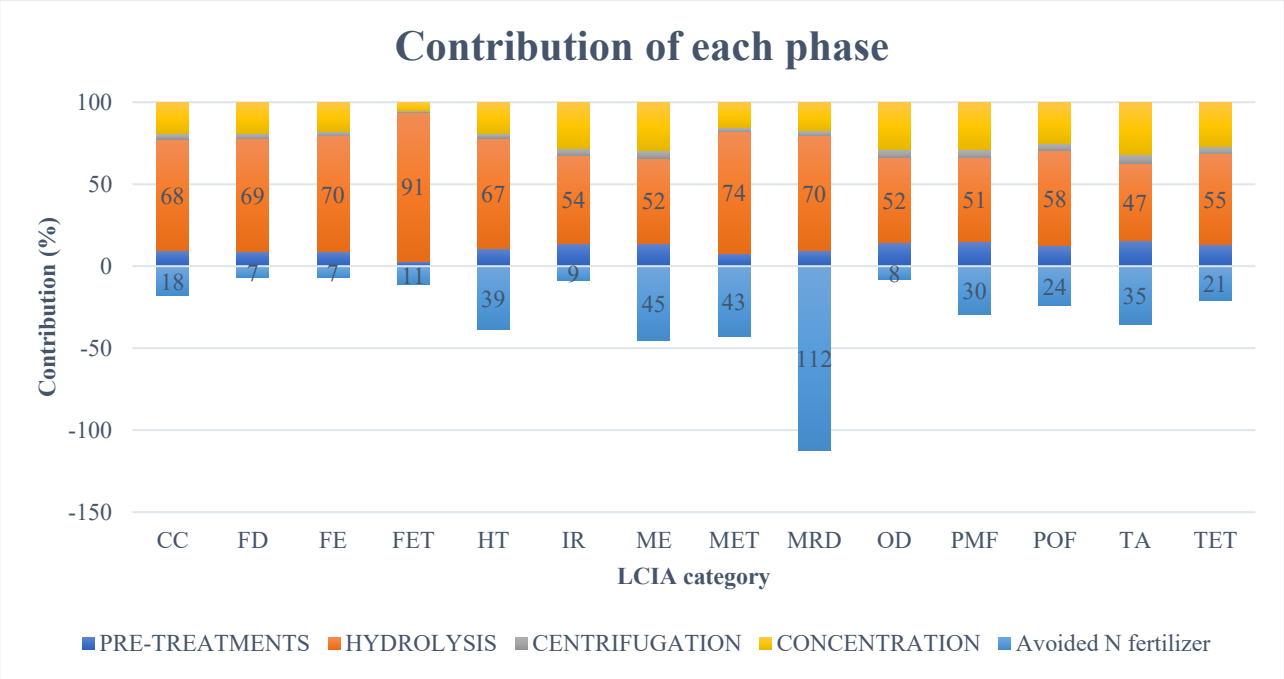


***Figure 52**: Contribution of each LC phase to each impact category, for fish.*

Table 46 reports the details of potential impact of valorizing fish waste in protein hydrolysate, in equivalent person/1000 tons of waste. In particular, avoided production of mineral N fertilizer permits to offset the 79% of impact from mincing and homogenization (pre-treatments) in the fossil fuel depletion category. Similarly for the climate change, such saving is equivalent to the 94% of the kg CO_2-eq derived from the concentration step.

***Table 46**: Summary table of the normalized (Equivalent person/1000 tons of waste) and absolute impacts (PT, pre-treatments; H, hydrolysis; CE, centrifugation; CO, concentration; AF, avoided N-fertilizer).*

	Absolute impact		**Normalized impact**					
LCIA category	**u.m.**	**Total**	**PT**	**H**	**CE**	**CO**	**AF**	**Total**
FD	kg oil-Eq/ton of waste	114.04	11.30	85.92	3.84	23.83	-8.90	115.98
CC	kg CO_2-Eq/ton of waste	261.20	3.70	27.25	1.24	7.71	-7.21	32.69
POF	kg NMVOC-Eq/ton of waste	0.44	3.49	16.47	1.15	7.15	-6.86	21.40
IR	kg U235-Eq/ton of waste	8.65	2.76	10.60	0.89	5.55	-1.79	18.02
FE	kg P-Eq/ton of waste	0.01	1.58	12.45	0.50	3.13	-1.23	16.44
TA	kg SO_2-Eq/ton of waste	0.50	2.89	8.90	0.97	6.02	-6.65	12.12
FET	kg 1,4-DCB-Eq/ton of waste	0.24	0.32	9.56	0.09	0.55	-1.16	9.36
PMF	kg PM_{10}-Eq/ton of waste	0.18	1.52	5.23	0.48	2.97	-3.03	7.18
ME	kg N-Eq/ton of waste	0.01	0.81	3.01	0.27	1.70	-2.62	3.17
MET	kg 1,4-DB-Eq/ton of waste	0.11	0.35	3.28	0.11	0.68	-1.90	2.52
OD	kg CFC^{-11}-Eq/ton of waste	0.00	0.08	0.27	0.02	0.15	-0.04	0.48
HT	kg 1,4-DCB-Eq/ton of waste	8.87	0.05	0.31	0.01	0.09	-0.18	0.28
TET	kg 1,4-DCB-Eq/ton of waste	0.02	0.00	0.00	0.00	0.00	0.00	0.00
MRD	kg Fe-Eq/ton of waste	-0.65	0.00	0.04	0.00	0.01	-0.06	-0.01

Figure 53 describes the same concepts but from another perspective, that is the contribution to all the impact categories of each resource involved. Electricity impacts for the 13-27% in all the categories, except for freshwater ecotoxicity (FET). For FET the impact is for the 81% due to thermal energy. Electricity used for concentration has been divided from the electricity used for the other process because its contribution was significant (15-32%, except for FET), due to the use of the MVRE. Under the assumption of using another kind of equipment, this contribution could be eliminated with the results of an increase in the thermal energy contribution. The enzyme production gives a significant contribution in almost all the categories: it results to be always between 16-37% of positive impact for all the categories, except for FE for which it reaches the 63%, and for FET for which it is only the 6%. The contribution of the enzyme transport to the total positive impact is mostly below

the 10%, except for HT (12%), MET and TET (14%), MRD (16%), therefore the categories over which the transport impacts more.

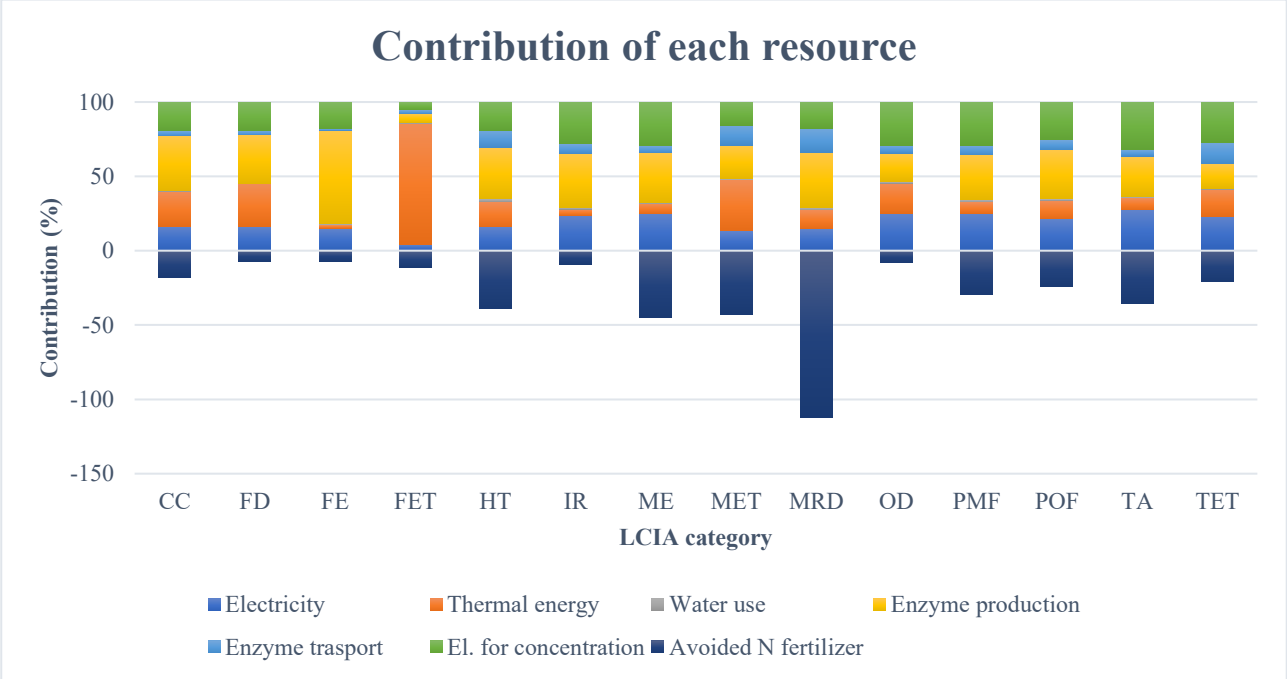


***Figure 53**: Contribution of the resources involved on the different impact categories for fish.*

***Table 47**: Summary table of the normalized impacts (Equivalent person/kg waste) (EL, electricity; EC, electricity for concentration; EP, enzyme production; ET, enzyme transport; WU, water use; AF, avoided N-fertilizer).*

LCIA category	EL	EC	EP	ET	TE	WU	AF	Total Normalized
FD	20.39	23.83	40.65	3.80	35.73	0.48	-8.90	115.98
CC	6.60	7.71	14.68	1.34	9.37	0.19	-7.21	32.69
POF	6.12	7.15	9.37	1.90	3.47	0.24	-6.86	21.40
IR	4.75	5.55	7.16	1.30	0.80	0.24	-1.79	18.02
FE	2.68	3.13	11.09	0.21	0.39	0.16	-1.23	16.44
TA	5.15	6.02	5.11	0.81	1.53	0.16	-6.65	12.12
FET	0.47	0.55	0.62	0.27	8.54	0.07	-1.16	9.36
PMF	2.55	2.97	3.06	0.66	0.80	0.17	-3.03	7.18
ME	1.45	1.70	1.93	0.28	0.39	0.04	-2.62	3.17
MET	0.59	0.68	0.99	0.60	1.52	0.04	-1.90	2.52

OD	0.13	0.15	0.10	0.03	0.11	0.01	-0.04	0.48
HT	0.07	0.09	0.16	0.05	0.08	0.01	-0.18	0.28
TET	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MRD	0.01	0.01	0.02	0.01	0.01	0.00	-0.06	-0.01

Having a more detailed look on the most important categories, the first two for importance, individuated in Figure 51 are very similar in terms of contributions to each phase. Basically, the hydrolysis is the most important process for impact, and the most relevant contributions at this stage are related to the enzyme production and the use of thermal energy. As example, Figure 54 reports the details of fossil fuel depletion.

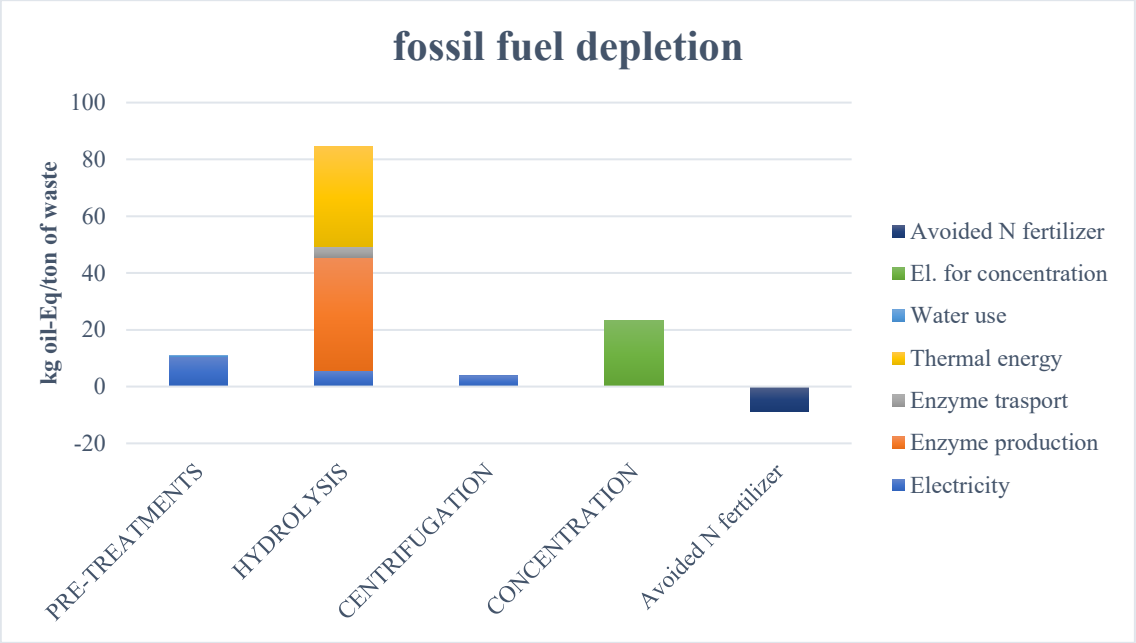


***Figure 54**: Details of the single contributions to each phase, for FD impact category, for fish.*

4.3.1.2 Mollusks

The same type of discussion can be done for mollusks. The calculation of normalized impact, in this case, provides a bit more relevant negative contribution of PMF. The first two categories for global impact remain the FD and CC.

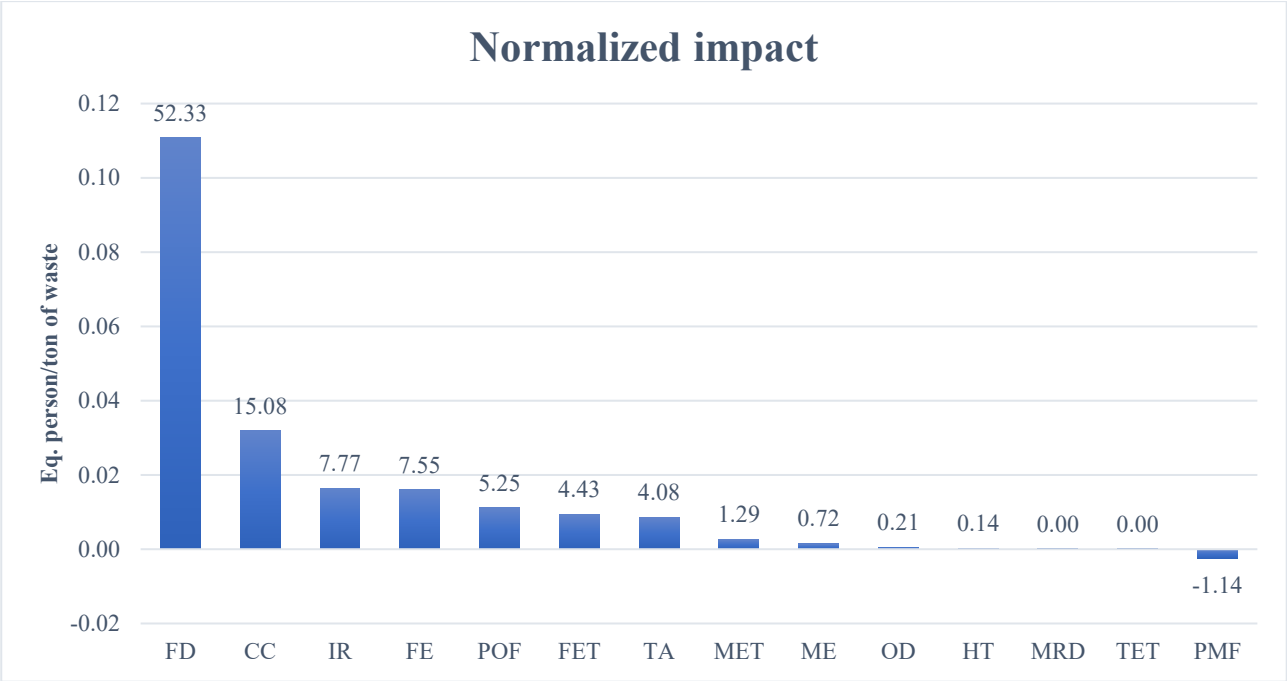


***Figure 55**: Normalized impact on each impact category, for mollusks.*

The main difference with the fish case is that the pre-treatment step negatively contributes to several categories, i.e., marine eutrophication (ME), particulate matter formation (PMF), photochemical oxidant formation (POF), terrestrial acidification (TA), terrestrial ecotoxicity (TET). This is due to the production of shells waste during the wet separation of organic-inorganic fractions of mollusks. In fact, shells can be considered a source of $CaCO_3$, therefore the avoided extraction of limestone, that mainly impact on ME, PMF, POF, TA, and TET, is in this way accounted.

Hence, thanks to the $CaCO_3$ recovery occurring during the pre-treatment steps, the use of mollusks substrate provides a more important negative impact on the environment. In particular, this contribution alone resulted to be the 92% of the total positive impact for MRD; considering also the contribution of the avoided N fertilizer, this resulted to be the 130% of the positive impact for PMF; for other two categories (ME and POF), both the negative contributions give a negative impact above the 50% of the positive one (Figure 56).

For what concerns the other contributions in Figure 57, the same considerations of the fish substrate can be done.

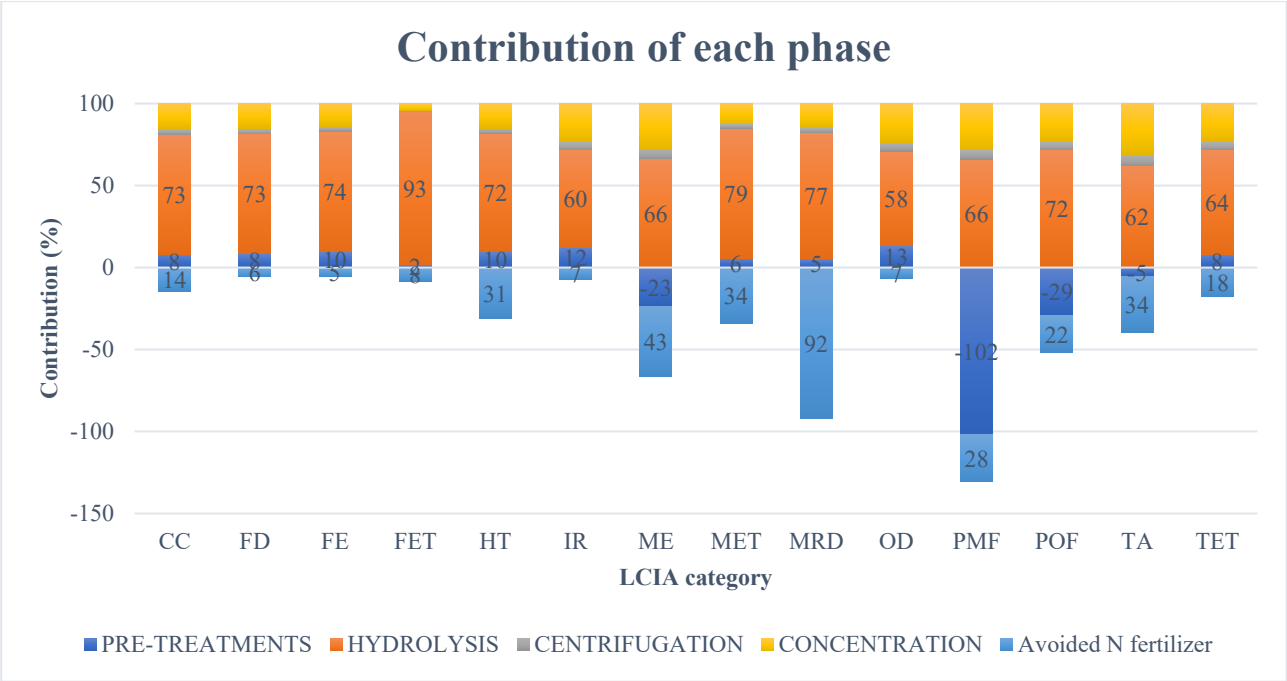


***Figure 56**: Contribution of each LC phase to each impact category, for mollusks.*

***Table 48**: Summary table of the normalized (Equivalent person/1000 tons of waste) and absolute impacts (PT, pre-treatments; H, hydrolysis; CE, centrifugation; CO, concentration; AF, avoided N-fertilizer).*

	Absolute impact		**Normalized impact**					
LCIA category	**u.m.**	**Total**	**PT**	**H**	**CE**	**CO**	**AF**	**Total**
FD	kg oil-Eq/ton of waste	108.89	9.91	85.92	3.84	17.69	-6.61	110.75
CC	kg CO_2-Eq/ton of waste	254.93	3.04	27.25	1.24	5.73	-5.36	31.90
IR	kg U235-Eq/ton of waste	7.89	2.16	10.60	0.89	4.12	-1.33	16.45
FE	kg P-Eq/ton of waste	0.01	1.62	12.45	0.50	2.33	-0.91	15.98
POF	kg NMVOC-Eq/ton of waste	0.23	-6.72	16.47	1.15	5.31	-5.09	11.11
FET	kg 1,4-DCB-Eq/ton of waste	0.24	0.18	9.56	0.09	0.41	-0.86	9.38
TA	kg SO_2-Eq/ton of waste	0.35	-0.76	8.90	0.97	4.47	-4.94	8.64
MET	kg 1,4-DB-Eq/ton of waste	0.12	0.24	3.28	0.11	0.51	-1.41	2.73
ME	kg N-Eq/ton of waste	0.01	-1.07	3.01	0.27	1.26	-1.94	1.53
OD	kg CFC^{-11}-Eq/ton of waste	0.00	0.06	0.27	0.02	0.11	-0.03	0.44
HT	kg 1,4-DCB-Eq/ton of waste	9.34	0.04	0.31	0.01	0.06	-0.13	0.30

MRD	kg Fe-Eq/ton of waste	0.38	0.00	0.04	0.00	0.01	-0.04	0.00
TET	kg 1,4-DCB-Eq/ton of waste	0.02	0.00	0.00	0.00	0.00	0.00	0.00
PMF	kg PM_{10}-Eq/ton of waste	-0.06	-8.08	5.23	0.48	2.21	-2.25	-2.41

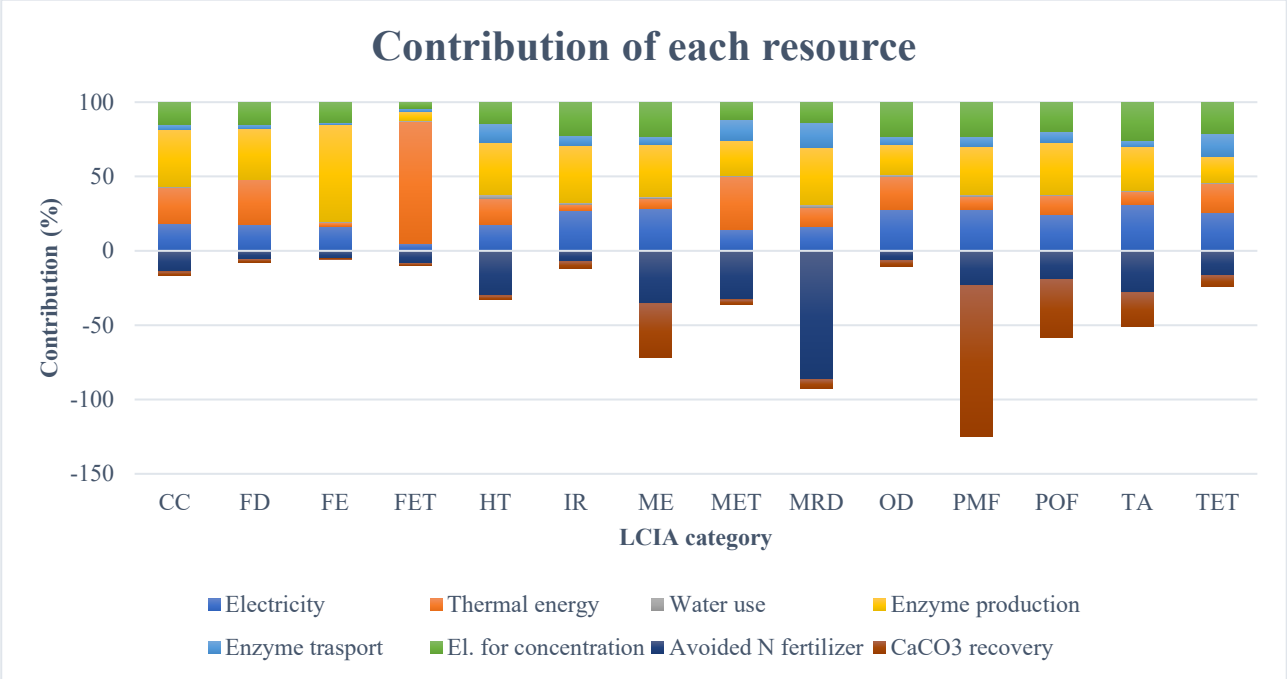


***Figure 57**: Contribution of the resources involved on the different impact categories for mollusks.*

***Table 49**: Summary table of the normalized impacts (Equivalent person/kg waste) (EL, electricity; EC, electricity for concentration; EP, enzyme production; ET, enzyme transport; WU, water use; AF, avoided N-fertilizer).*

LCIA category	EL	EC	EP	ET	TE	WU	AF	Total Normalized
FD	21.59	17.69	40.65	3.80	35.73	0.48	-6.61	113.33
CC	6.99	5.73	14.68	1.34	9.37	0.19	-5.36	32.95
IR	5.03	4.12	7.16	1.30	0.80	0.24	-1.33	17.33
FE	2.84	2.33	11.09	0.21	0.39	0.16	-0.91	16.10
POF	6.48	5.31	9.37	1.90	3.47	0.24	-5.09	21.68
FET	0.50	0.41	0.62	0.27	8.54	0.07	-0.86	9.54
TA	5.45	4.47	5.11	0.81	1.53	0.16	-4.94	12.59

MET	0.62	0.51	0.99	0.60	1.52	0.04	-1.41	2.87
ME	1.54	1.26	1.93	0.28	0.39	0.04	-1.94	3.50
OD	0.14	0.11	0.10	0.03	0.11	0.01	-0.03	0.46
HT	0.08	0.06	0.16	0.05	0.08	0.01	-0.13	0.31
MRD	0.01	0.01	0.02	0.01	0.01	0.00	-0.04	0.01
TET	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PMF	2.70	2.21	3.06	0.66	0.80	0.17	-2.25	7.35

The detail view of the single contributions to each phase for the most important impact category confirms the results obtained for the fish, with the difference that a small negative contribution to the FD category is given by the $CaCO_3$ recovery during pre-treatments (Figure 58).

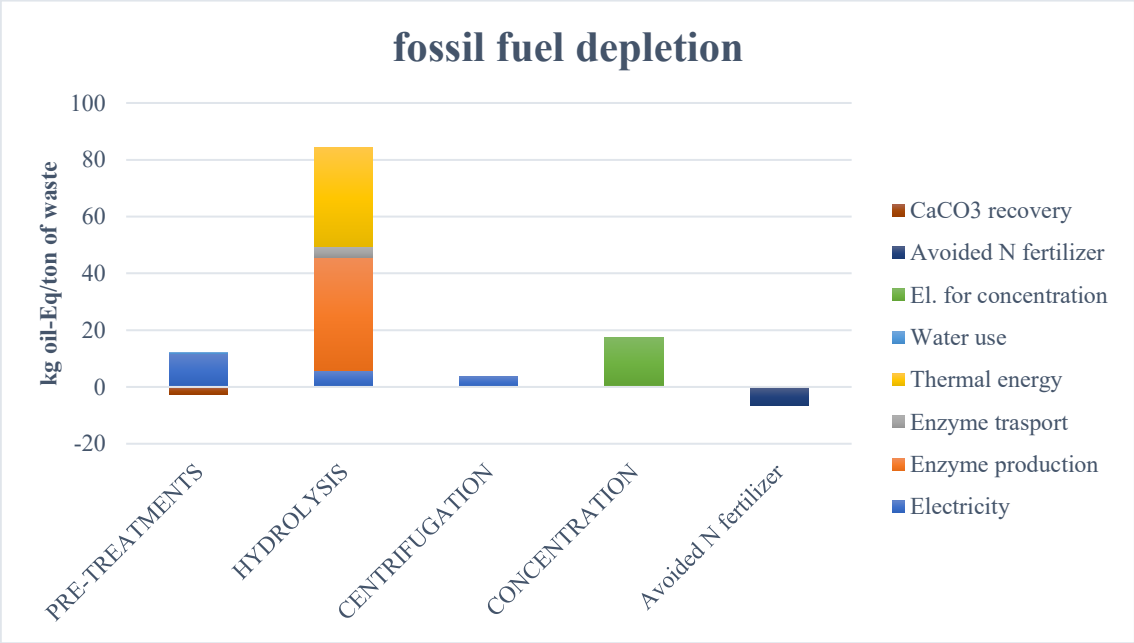


***Figure 58**: Details of the single contributions to each phase, for FD impact category, for mollusks.*

4.3.1.3 Mix

The most important impact category is confirmed to be the fossil fuel depletion, followed by the climate change, also for the mix substrate.

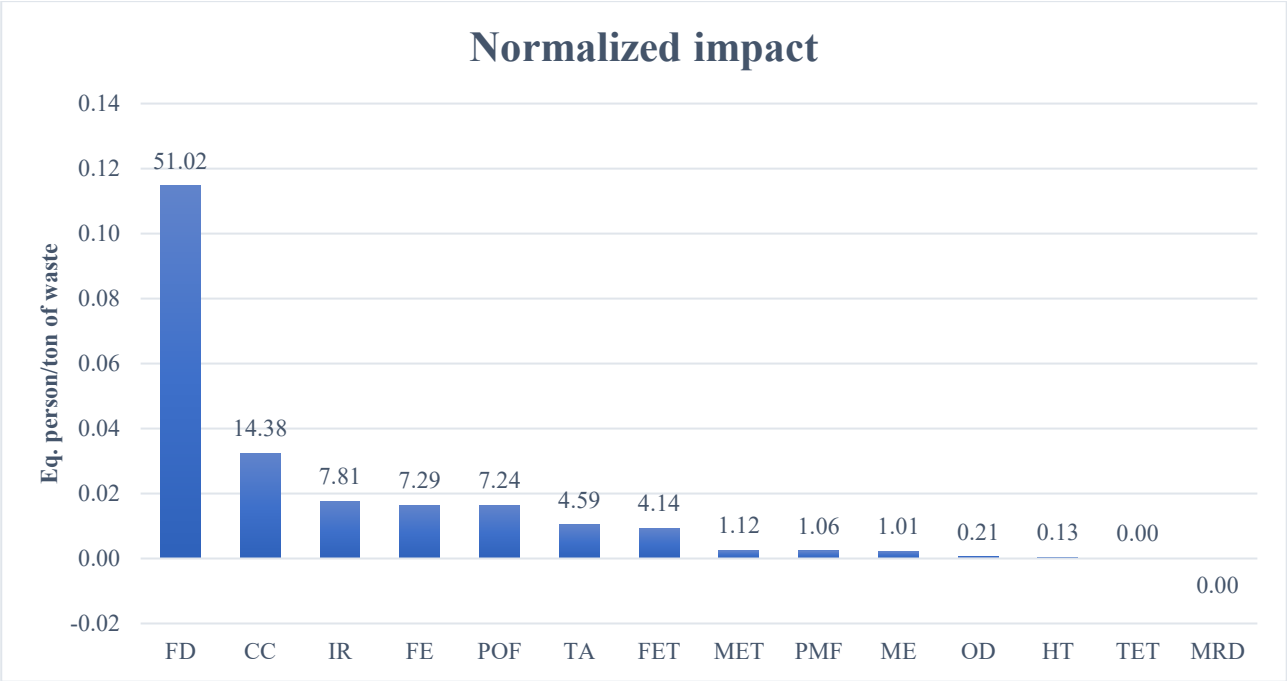


***Figure 59**: Normalized impact on each impact category, for mix.*

In case of mix substrate, a negative contribution due to the $CaCO_3$ recovery also occurs during pre-treatments, but with a less relevant quantity with respect to mollusks' case, since the quantity of mollusks used for the mix preparation is halved (Figure 60). The same can be observed in Figure 61.

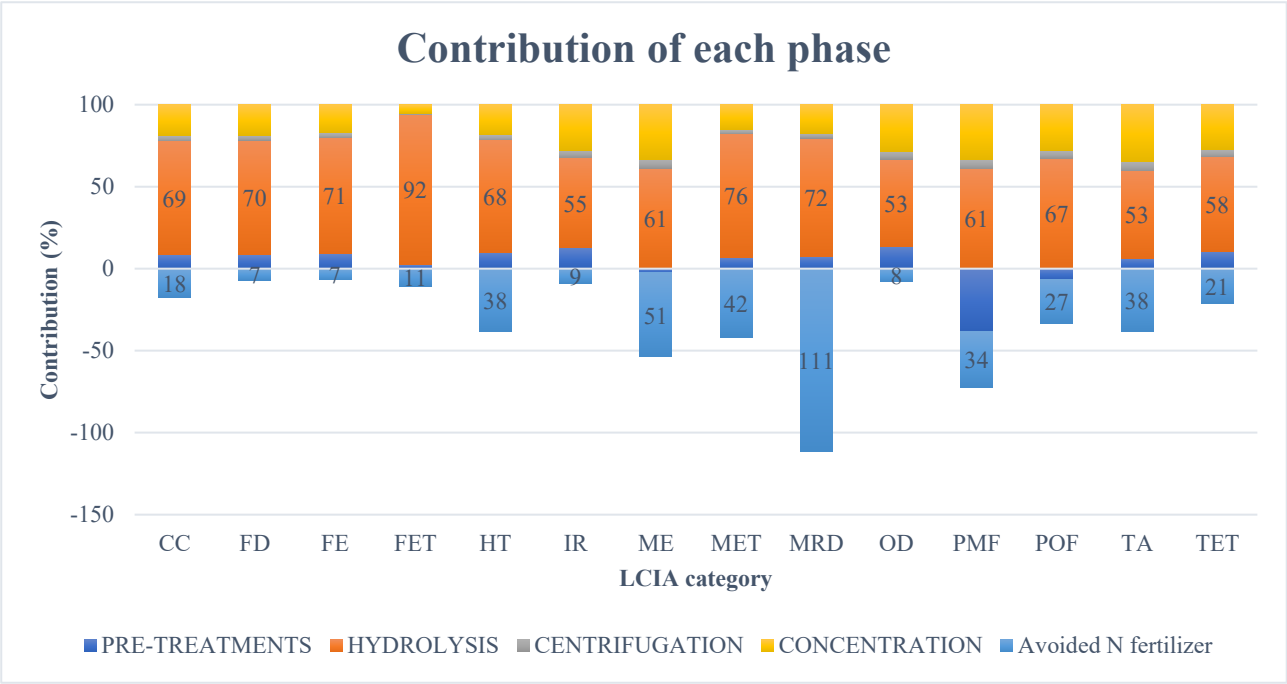


***Figure 60**: Contribution of each LC phase to each impact category, for mix.*

***Table 50**: Summary table of the normalized (Equivalent person/1000 tons of waste) and absolute impacts (PT, pre-treatments; H, hydrolysis; CE, centrifugation; CO, concentration; AF, avoided N-fertilizer).*

	Absolute impact		**Normalized impact**					
LCIA category	**u.m.**	**Total**	**PT**	**H**	**CE**	**CO**	**AF**	**Total**
FD	kg oil-Eq/ton of waste	112.78	10.60	85.92	3.84	22.90	-8.55	114.70
CC	kg CO_2-Eq/ton of waste	258.42	3.37	27.25	1.24	7.41	-6.93	32.34
IR	kg U235-Eq/ton of waste	8.43	2.46	10.60	0.89	5.33	-1.72	17.57
FE	kg P-Eq/ton of waste	0.01	1.60	12.45	0.50	3.01	-1.18	16.38
POF	kg NMVOC-Eq/ton of waste	0.33	-1.62	16.47	1.15	6.87	-6.59	16.28
TA	kg SO_2-Eq/ton of waste	0.42	1.06	8.90	0.97	5.78	-6.39	10.32
FET	kg 1,4-DCB-Eq/ton of waste	0.23	0.25	9.56	0.09	0.53	-1.11	9.31
MET	kg 1,4-DB-Eq/ton of waste	0.11	0.29	3.28	0.11	0.66	-1.82	2.52
PMF	kg PM_{10}-Eq/ton of waste	0.06	-3.28	5.23	0.48	2.86	-2.91	2.38
ME	kg N-Eq/ton of waste	0.01	-0.13	3.01	0.27	1.63	-2.52	2.27
OD	kg CFC^{-11}-Eq/ton of waste	0.00	0.07	0.27	0.02	0.15	-0.04	0.47
HT	kg 1,4-DCB-Eq/ton of waste	8.84	0.05	0.31	0.01	0.08	-0.17	0.28
TET	kg 1,4-DCB-Eq/ton of waste	0.02	0.00	0.00	0.00	0.00	0.00	0.00
MRD	kg Fe-Eq/ton of waste	-0.59	0.00	0.04	0.00	0.01	-0.05	-0.01

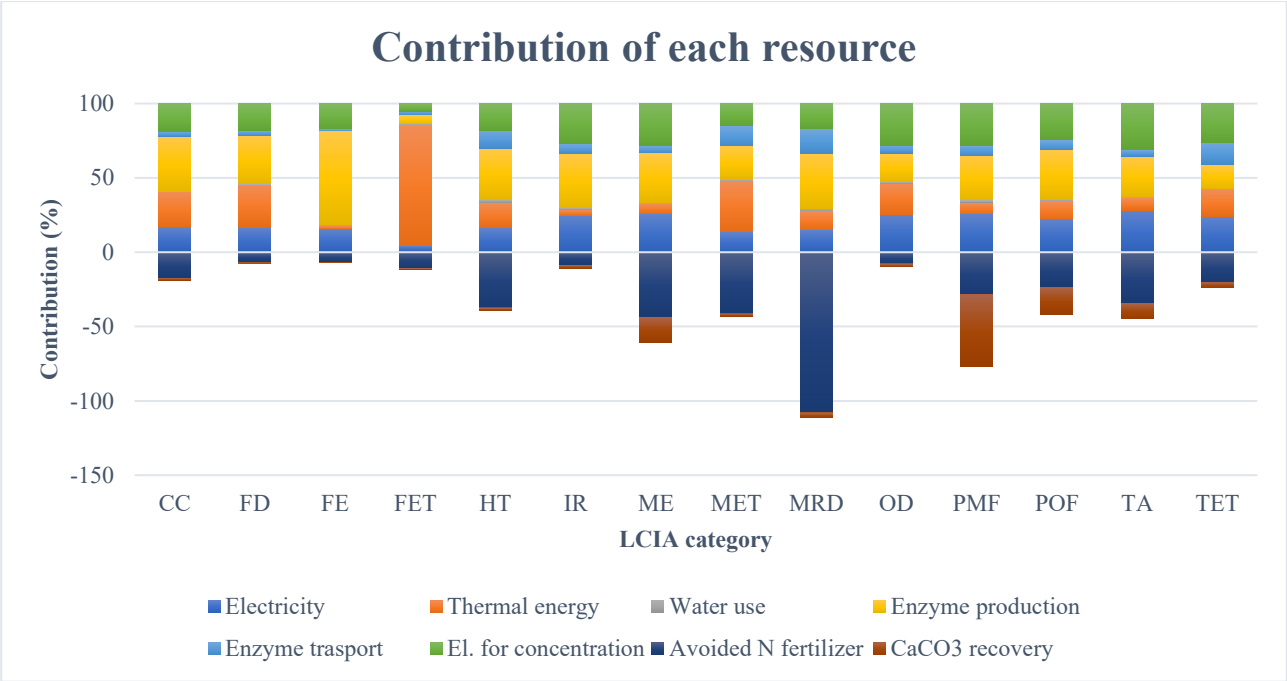


***Figure 61**: Contribution of the resources involved on the different impact categories for mollusks.*

***Table 51**: Summary table of the normalized impacts (Equivalent person/kg waste) (EL, electricity; EC, electricity for concentration; EP, enzyme production; ET, enzyme transport; WU, water use; AF, avoided N-fertilizer).*

LCIA category	EL	EC	EP	ET	TE	WU	AF	Total Normalized
FD	20.99	22.90	40.65	3.80	35.73	0.48	-8.55	116.00
CC	6.80	7.41	14.68	1.34	9.37	0.19	-6.93	32.86
IR	4.89	5.33	7.16	1.30	0.80	0.24	-1.72	18.01
FE	2.76	3.01	11.09	0.21	0.39	0.16	-1.18	16.44
POF	6.30	6.87	9.37	1.90	3.47	0.24	-6.59	21.57
TA	5.30	5.78	5.11	0.81	1.53	0.16	-6.39	12.30
FET	0.48	0.53	0.62	0.27	8.54	0.07	-1.11	9.40
MET	0.60	0.66	0.99	0.60	1.52	0.04	-1.82	2.59
PMF	2.62	2.86	3.06	0.66	0.80	0.17	-2.91	7.26
ME	1.50	1.63	1.93	0.28	0.39	0.04	-2.52	3.25
OD	0.13	0.15	0.10	0.03	0.11	0.01	-0.04	0.48
HT	0.08	0.08	0.16	0.05	0.08	0.01	-0.17	0.29

TET	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MRD	0.01	0.01	0.02	0.01	0.01	0.00	-0.05	0.00

4.3.1.4 Comparison of the three substrates

Comparing the normalized impacts due to the three substrates it emerges that, for most of the impact categories, the most impacting, for the same weight of treated substrate, is fish, followed by the mix and then mollusks (Figure 60). This can have different explanations depending on which is the most impacting contribution on each category.

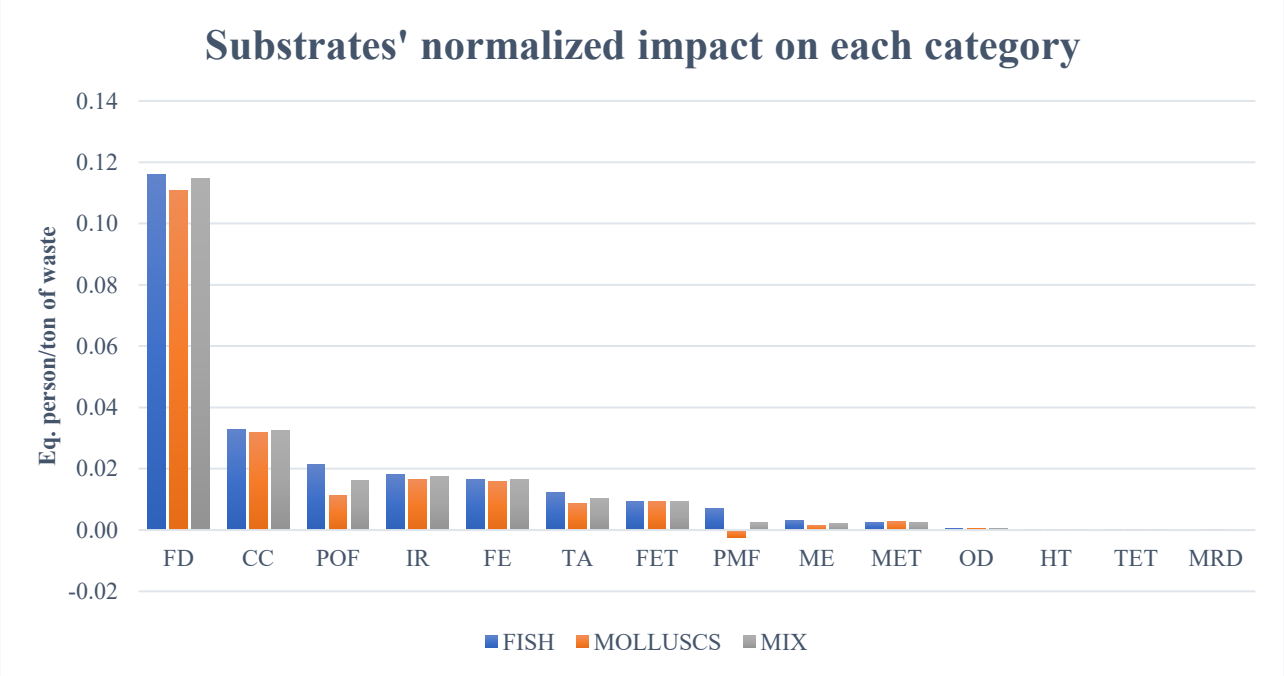


***Figure 62**: Comparison of the normalized impacts due to the three substrates, Assessment of valorization.*

***Table 52**: Comparison of the normalized impacts due to the three substrates.*

LCIA category	Fish	Mollusks	Mix
-	Equivalent person/1000 tons of waste		
FD	115.98	110.75	114.70
CC	32.69	31.90	32.34
POF	21.40	11.11	16.28
IR	18.02	16.45	17.57

FE	16.44	15.98	16.38
TA	12.12	8.64	10.32
FET	9.36	9.38	9.31
PMF	7.18	-2.41	2.38
ME	3.17	1.53	2.27
MET	2.52	2.73	2.52
OD	0.48	0.44	0.47
HT	0.28	0.30	0.28
TET	0.00	0.00	0.00
MRD	-0.01	0.00	-0.01

Figure 63 shows the impact of each substrate with respect to the one for which the impact is the maximum in the category. It is worth mentioning that the contribution of thermal energy, water and enzyme addition is equivalent to all the three analyzed cases since the hydrolysis was conducted at the same conditions.

For most of the categories where the fish is the most impacting substrate, the difference with the other substrates is not relevant (< 10%). The slight difference in potential fossil fuel depletion between mollusk and fish valorization (2.4%) should be caused by the lower liquid yield achieved with mollusk hydrolysis. It results in lower amount of liquid to be concentrated that means lower amount of electricity to be used for concentration and lower amount of avoided N fertilizer (Figure 64). On the other hand, mollusk valorization benefits of $CaCO_3$ recovery. These results are also valid in the case of CC category, for which the contribution of electricity production is also relevant. Anyway, it must be specified that the LCA study does not take into account the amount of solid residues output of the centrifugation step, since it has been assumed that it is not a waste to be disposed and the possibility to recover biochar and/or compost from solid residue is not considered at now.

On the other hand, the impact categories for which valorization of fish substrate are greater than other substrates are TA, POF, PMF, ME. These differences can be explained with the higher value of negative impacts (avoided N fertilizer, $CaCO_3$ recovery) arising from the treatment of mollusks and, in lower percentage, of mix. In these categories, the lower impact of mollusks substrate is certainly due to the fact that the only negative impact in the case of fish is the avoided N fertilizer, while in the

case of the mix there is the contribution of $CaCO_3$ recovery, which is doubled in the case of mollusks. In particular, for PMF the absolute impact for mollusks is negative. Figure 65 reports a detail for PMF that shows how great is the negative contribution due to the $CaCO_3$ recovery in the case of mollusks, this is halved for mix, and null for fish, in comparison to the negative contribution given by the avoided N fertilizer, which is a slightly lower for mollusks, since the quantity of PH produced is lower as effect of a higher solid content.

The situation is reversed for MRD, for which the most relevant contribution is the avoided N fertilizer (Table 47 for fish, Table 49 for mollusks, Table 51 for mix), and only in the case of mollusks the impact is positive, due to the lower PH production (Figure 66).

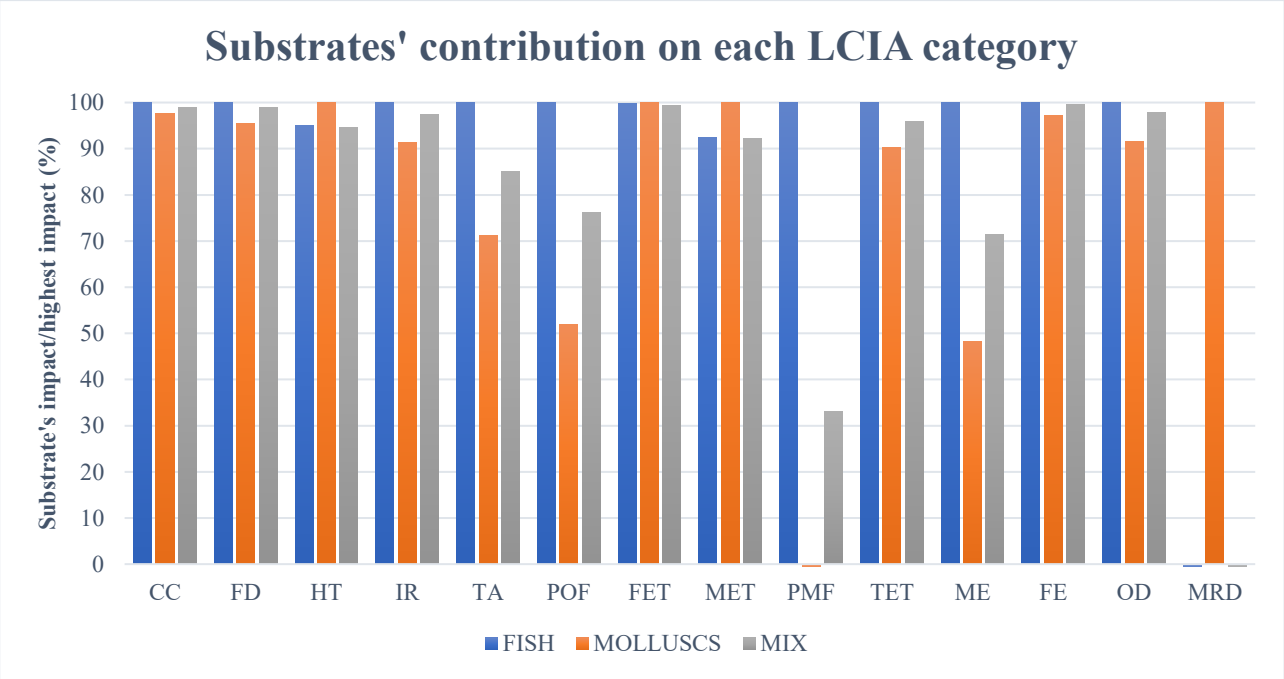


***Figure 63**: Substrates' contribution on each LCIA category.*

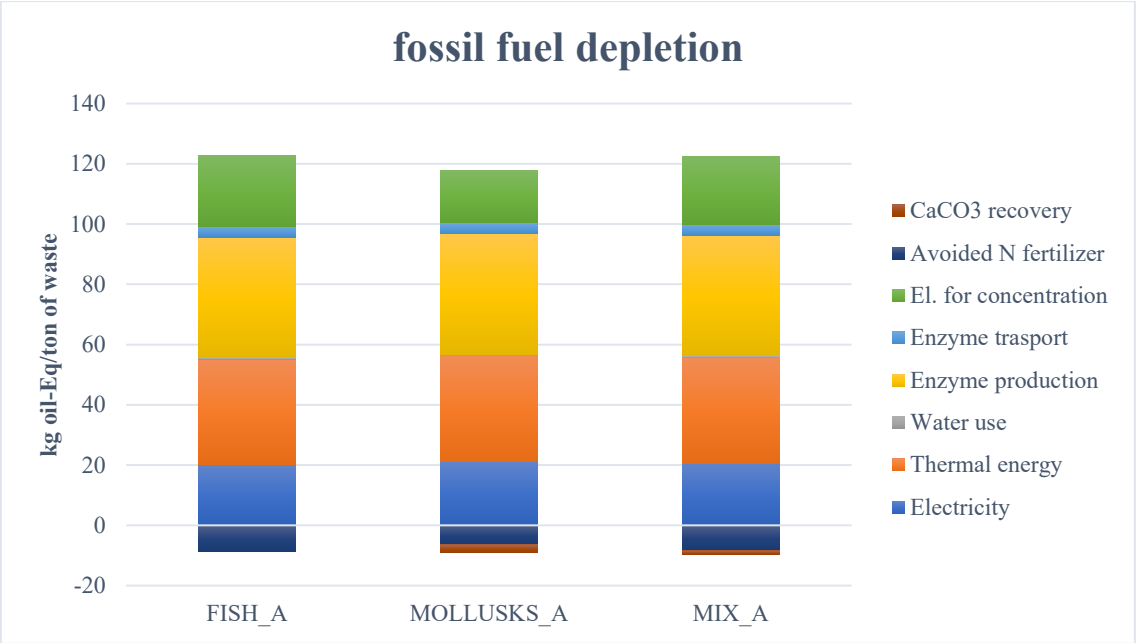


***Figure 64**: Detail of the contributions to the FD category, comparing the three substrates.*

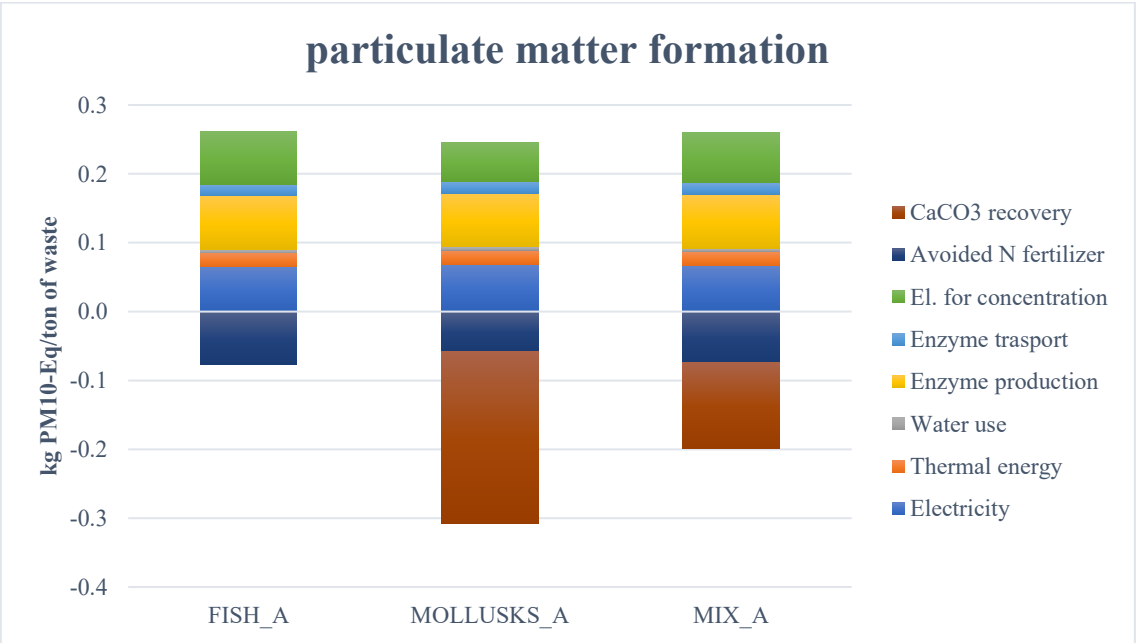


***Figure 65**: Detail of the contributions to the PMF category, comparing the three substrates.*

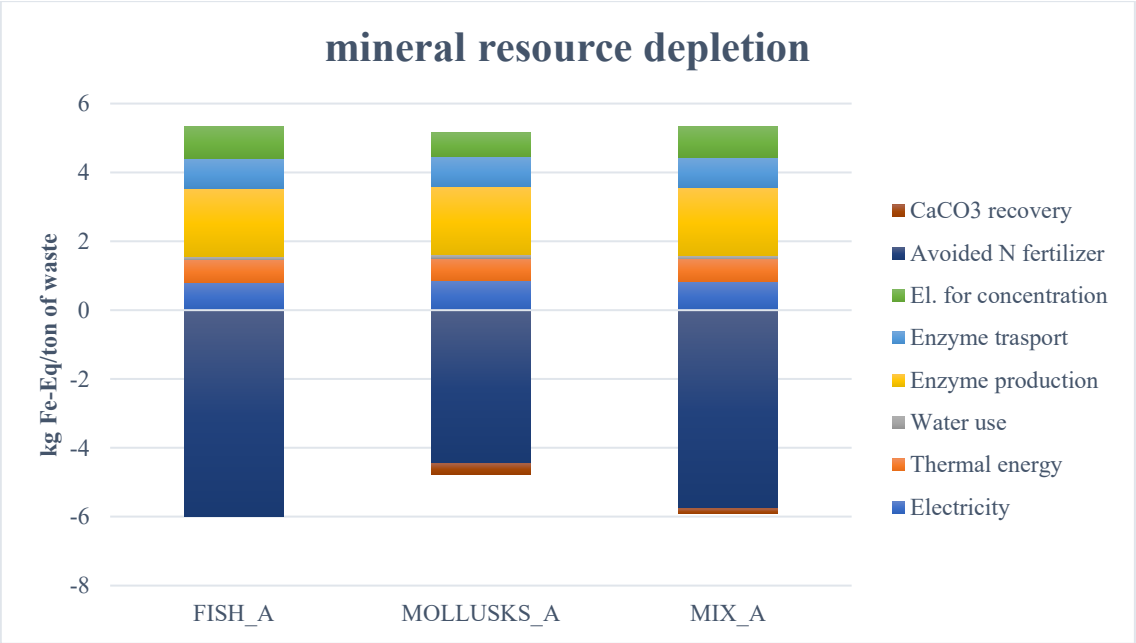


***Figure 66**: Detail of the contributions to the MRD category, comparing the three substrates.*

Finally, it is possible to evaluate if any savings could be obtained by creating and treating the substrate mix, instead of treating fish and mollusks in two separate lines. Results are reported as percentage of saving (negative part) or worsening (positive part) from the point of view of the impact on each category in Figure 67. Basically, there are no particular savings or worsening arising from the treatment of the created mix substrate since they are below 5%, and the ones that reach the 3-4% are related to the categories for which the avoided N fertilizer is the most important contribution. In fact, the yield obtained in the lab by treating the mix substrate is higher than the one which can be calculated as average of the fish a mollusks yields, resulting in a higher amount of PH produced and therefore of avoided N fertilizer.

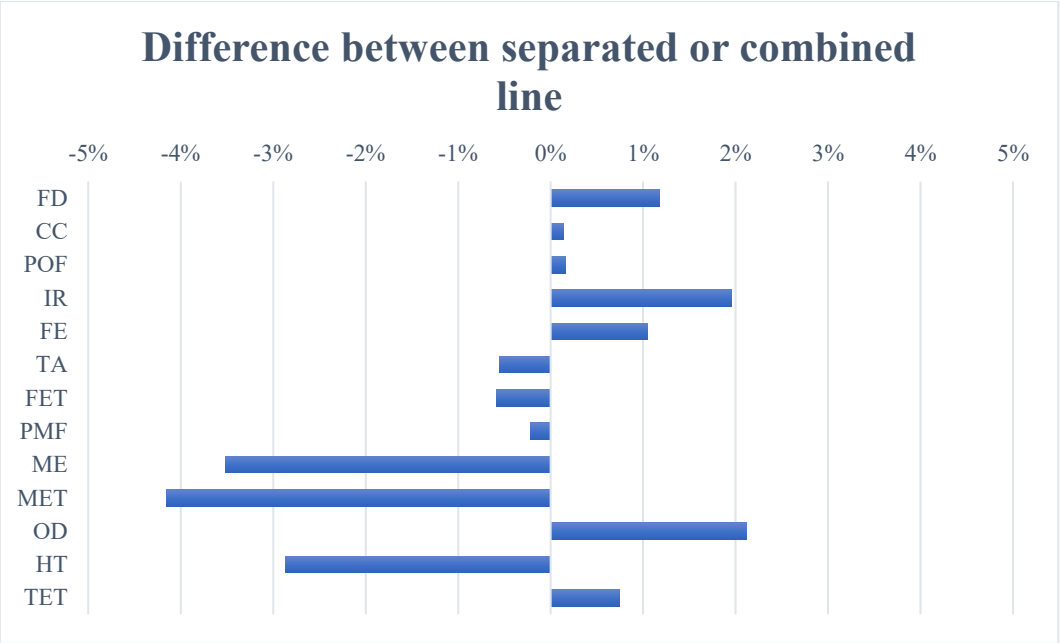


***Figure 67**: Saving (negative values) or worsening (positive value) from the treatment of mix substrate with respect to the treatment of two separated lines.*

4.3.2 Assessment of production

The considerations done for assessment of valorization regarding the comparison of the three substrates are here reversed. In fact, as expected, since the quantity of final product that is possible to obtain using the mollusks is the lowest, comparing the results in terms of quantity of final product, the impacts for mollusks are much more relevant than the others for most of the impact categories. Therefore, PH recovered by mollusk waste resulted to be the lower environmentally friendly solution compared to the use of fish waste or a mix substrate.

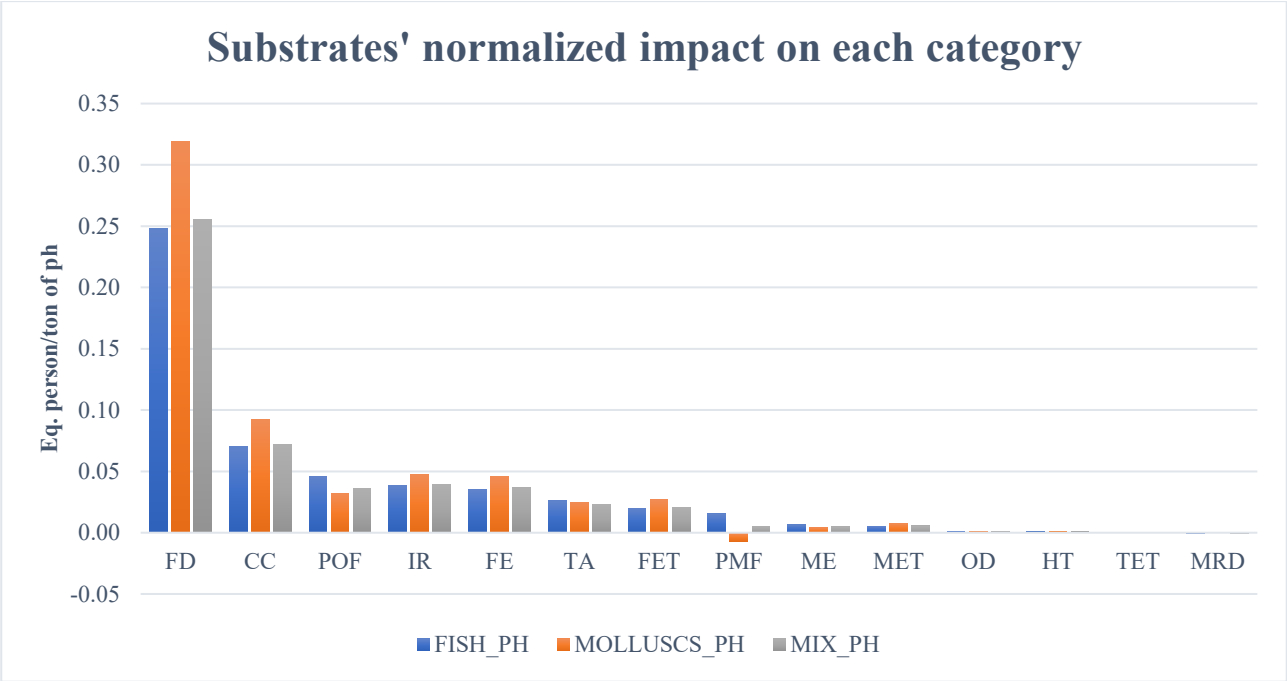


***Figure 68**: Comparison of the normalized impacts due to the three substrates, Assessment of production.*

4.3.3 Summary of the LCA results

The quantified impacts of the three most impacting categories are reported in Table 53.

As already stated, comparing the valorization train of three different substrates, treatment of 1 ton of fish waste results to be the most impacting, followed by the treatment of 1 ton of mix substrate, and then 1 ton of mollusks by-products, in 11 out of 14 impact categories analyzed. Anyway, slight difference (lower than 10%) is assessed mainly related to the energy used for concentration step since the liquid yield was greater for fish. Greater differences are highlighted in POF and PMF categories correlated to the environmentally positive effect of recovering $CaCO_3$ when mollusk by-products are treated. On the other hand, in terms of impact per ton of PH produced, the order of the most impacting substrate is reversed, for the same reasons: the liquid yield of mollusks is lower than the other two substrates, therefore more substrate and resources must be employed to produce the same quantity of final product. However, these differences are not so relevant.

Having a look on the quantities, the first three categories for magnitude of impact have been found in fossil fuel depletion, climate change, and photochemical oxidant formation. In particular, the impact on climate change obtained for ton of PH, i.e., 558.8-734.7 g CO_2-Eq/kg of PH produced (Assessment of production) is in line with those obtained by Colantoni et al. (2017) for PH production from lupine grains, who determined an impact on climate change equal to 586.9 g CO_2-Eq/kg of PH, excluding the agricultural phase necessary to the growth of lupine grains, and 743.2 g CO_2-Eq/kg of PH

considering the whole process. In any case, the calculated impacts are much lower than the ones which the same authors retrieved for PH production from leather wastes through chemical hydrolysis. In particular 1532.2 g CO_2-Eq/kg of PH is emitted in that case. Instead, considering the values obtained by García-Santiago et al. (2020) who retrieved an impact on climate change of 115.23 kg CO_2-eq/100 kg of waste treated, our results (25,49-26,12 kg CO_2-Eq/100 kg of waste) is around 5 times lower. In this case the difference can be due to the use of different equipment (e.g., the spray drier instead of the MVRE, a microfiltration equipment, a solid-drying oven and other auxiliary equipment), and also to the fact that no negative impact, i.e., in favor of the environment, has been taken into account.

***Table 53**: The main results of LCA on the most relevant categories, i.e., fossil fuel depletion (FD), climate change (CC), photochemical oxidant formation (POF).*

LCIA category	**FD**		**CC**	
Boundary	V	P	V	P
	kg oil-Eq/ ton of waste	kg oil-Eq/ ton of PH	kg CO_2-Eq/ ton of waste	kg CO_2-Eq/ ton of PH
FISH total	**114.04**	**243.99**	**261.20**	**558.82**
Pre-treatments	11.11	23.77	29.54	63.21
Hydrolysis	84.49	180.75	217.74	465.83
Centrifugation	3.77	8.07	9.93	21.23
Concentration	23.43	50.13	61.64	131.87
Avoided N fertilizer	-8.75	-18.73	-57.65	-123.33
MOLLUSCS total	**108.89**	**313.83**	**254.93**	**734.71**
Pre-treatments	9.74	28.07	24.30	70.05
Hydrolysis	84.49	243.49	217.74	627.53
Centrifugation	3.77	10.87	9.93	28.61
Concentration	17.39	50.12	45.75	131.87
Avoided N fertilizer	-6.50	-18.73	-42.79	-123.33
MIX total	**112.78**	**251.12**	**258.42**	**575.40**
Pre-treatments	10.42	23.21	26.92	59.94

Hydrolysis	84.49	188.12	217.74	484.81
Centrifugation	3.77	8.40	9.93	22.10
Concentration	22.51	50.13	59.23	131.88
Avoided N fertilizer	-8.41	-18.73	-55.39	-123.33

4.4 Economic assessment

Results of the economic assessment are discussed here.

The highest CAPEX, whose calculation is reported in Table 54, is for mollusks and mix, since an equipment able to carry out meat-shell separation must be provided in those cases. The difference with the case of fish is equal to 57.107 €.

***Table 54**: Comparison of the CAPEX calculated for the three substrates.*

Parameter	**u.m.**	**Fish**	**Mollusks**	**Mix**
Equipment cost	€	108046	129041	129041
Equipment installation	€	42138	50326	50326
Instrumentation and control	€	28092	33551	33551
Piping	€	33494	40003	40003
Electrical system	€	10805	12904	12904
Engineering and supervision	€	34575	41293	41293
Construction expenses	€	36736	43874	43874
Total capital investment	**€**	**293884**	**350991**	**350991**
Lifetime	y	20	20	20
CAPEX	**€/y**	**14694**	**17550**	**17550**

For OPEX, Table 55 shows the calculation procedure and the final result, comparing the three substrates. In general, significant differences between the three substrates are not highlighted. By looking at the annual operating costs alone, the bigger cost is for the fish, followed by the mix and then the mollusks, but the differences are irrelevant (1.068 €/y for mollusks, 73 €/y for mix). Adding

the costs of maintenance and other fixed costs, instead, the result is different, as this cost is linked to the investment costs that are greater in the case of mix and mollusks, with differences with respect to the greatest (mix) of 5.638 €/y with the fish case, and 1001 €/y with the mollusks.

***Table 55**: Comparison of the OPEX calculated for the three substrates.*

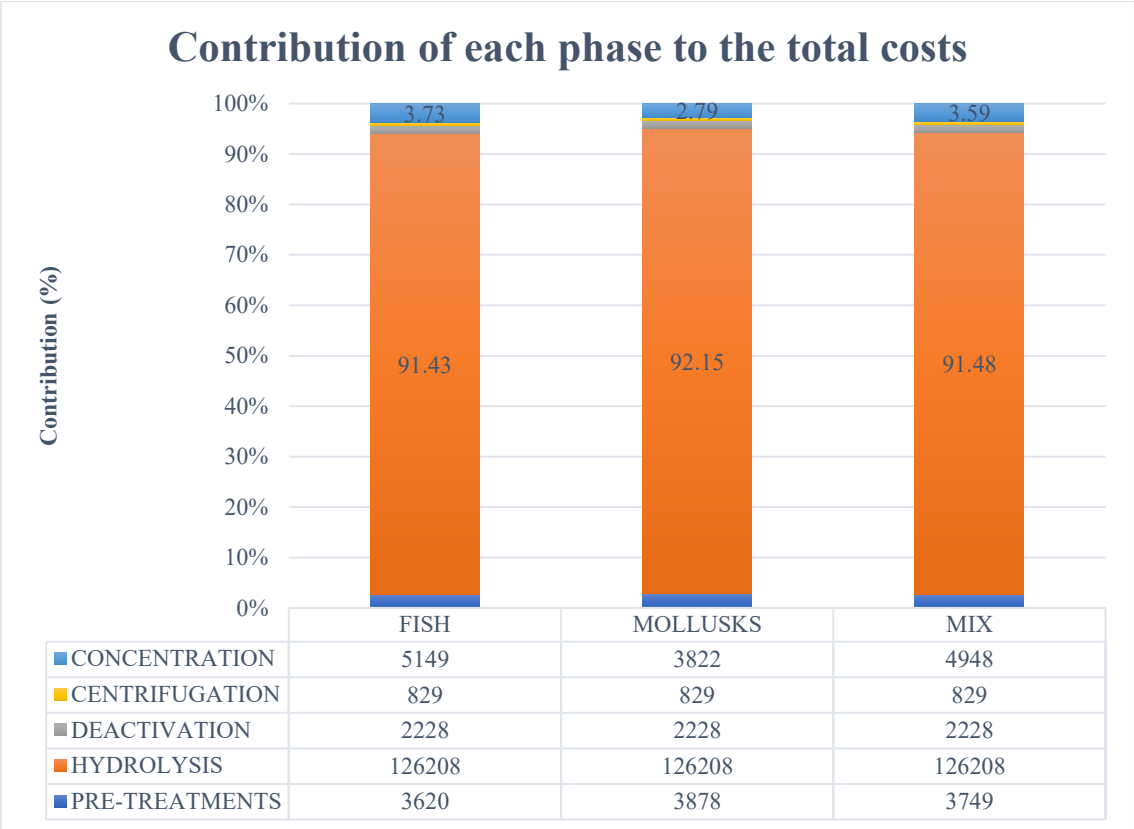
Parameter	u.m.	Fish	Mollusks	Mix
Annual processing	ton/y	225	225	225
Operating costs	€/ton	613	609	613
Annual operating costs	€/y	138034	136966	137961
Maintenance and other fixed costs	€/y	29388	35099	35099
Labor	€/y	60000	60000	60000
OPEX	**€/y**	**228261**	**232898**	**233899**

It is clear from Figure 69 and Figure 70, respectively summarizing the annual operating costs for phase and resources involved, that the most impacting phase for costs is the hydrolysis, accounting from the 91.43-92.15% of the total depending on the substrate used. The 96.70% of the costs during hydrolysis, is due to the enzyme, which represents also the 88.42-89.10% of the total, with a unit cost of 46.36 €/kg.

Anyway, the absolute costs (€/y in the tables reported in the figures) related to this stage, as well as the enzyme deactivation and the centrifugation, are equal for the three substrates, since the results have been obtained analyzing the operating costs for tons of waste treated, intended as meat.

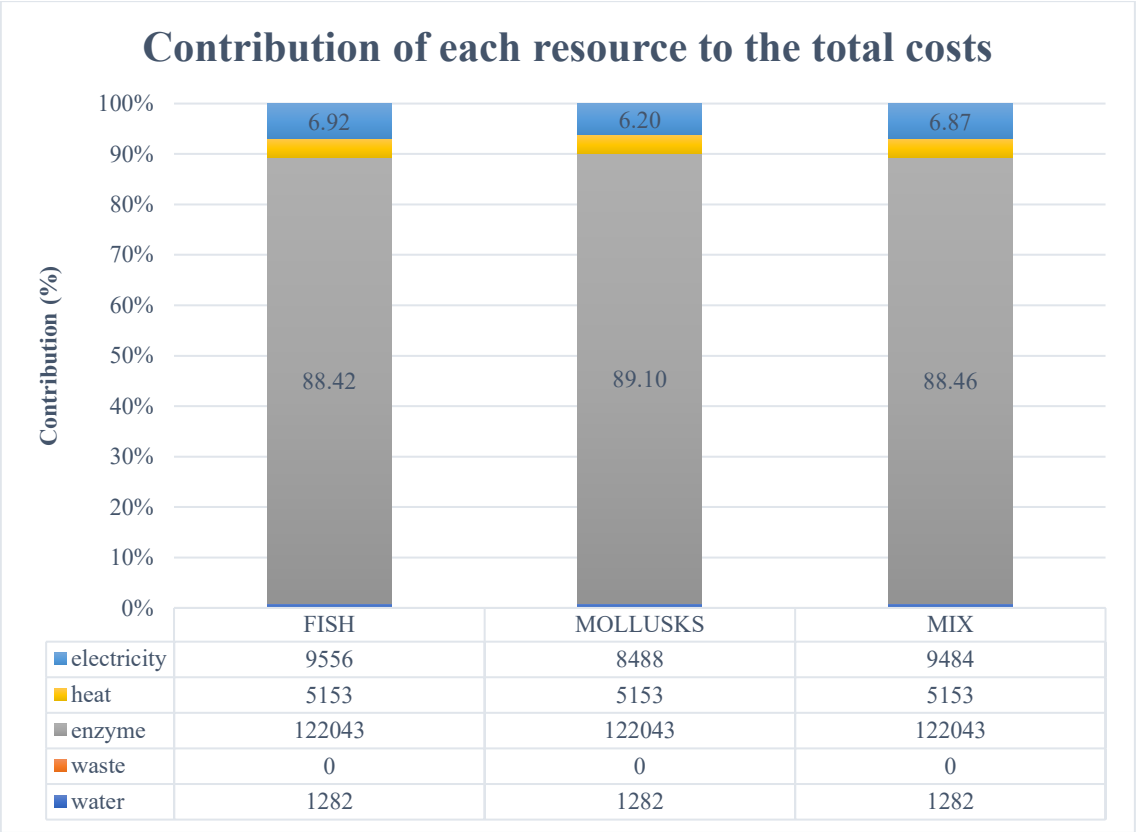
The second cost for importance is that of electricity (6.20-6.92% of the total), mainly due to the concentration step for the 45.03-53.88% (Figure 71). Anyway, the concentration step represents just the 2.79-3.73% of the total costs.

The only differences in terms of absolute costs between the three substrates are linked to the pre-treatments and concentration phases: pre-treatments have a slightly higher cost for mollusks since it comprises the whole quantity of mollusks to be shredded to obtain the ton of mollusks meat to be hydrolyzed; concentration has a slightly lower cost for mollusks, since they have a lower liquid yield.



	FISH	MOLLUSKS	MIX
CONCENTRATION	5149	3822	4948
CENTRIFUGATION	829	829	829
DEACTIVATION	2228	2228	2228
HYDROLYSIS	126208	126208	126208
PRE-TREATMENTS	3620	3878	3749

***Figure 69**: Contribution of each phase to the total costs. Costs in the table are in €/y.*



	FISH	MOLLUSKS	MIX
electricity	9556	8488	9484
heat	5153	5153	5153
enzyme	122043	122043	122043
waste	0	0	0
water	1282	1282	1282

***Figure 70**: Contribution of each resource to the total costs. Costs in the table are in €/y.*

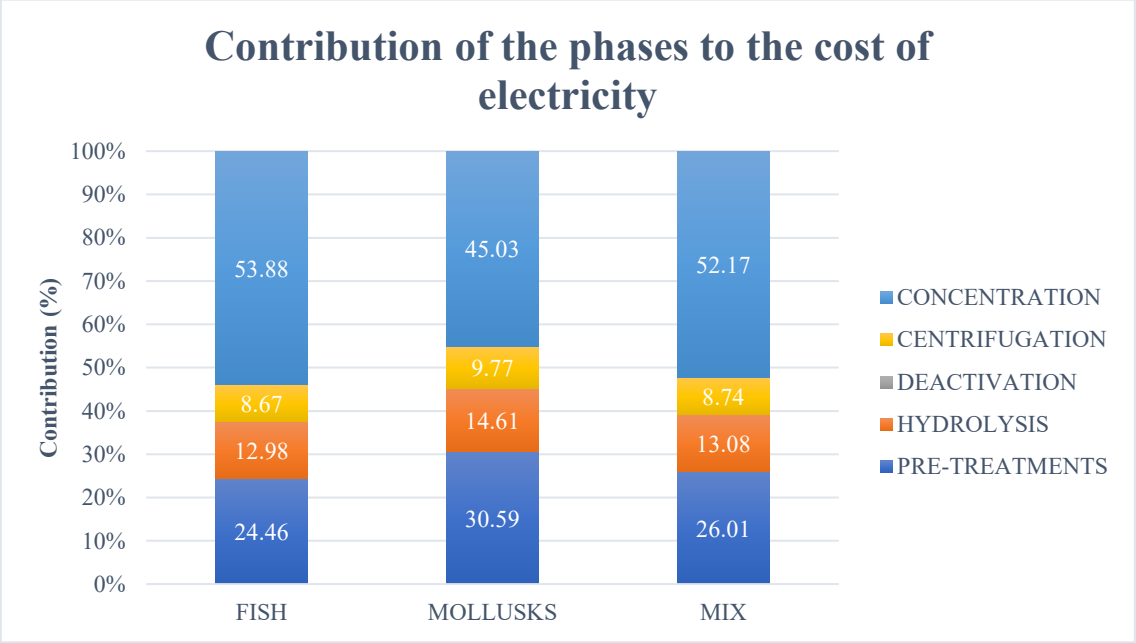


***Figure 71**: Contribution of the phases to the cost of electricity.*

At this point, the parameters describing the economic sustainability of our choices are calculated (Table 56). Annual costs are very similar in the three cases: the highest are for mix, differences are just of 1000 €/y with mollusks, and 8493 €/y with fish. The annual profit which could be obtained

using mollusks is strongly higher than that using fish, and it is in the middle by using the mix. This is due to the fact that the calcium carbonate recovered during mollusks and, in lower amount, mix pre-treatments has an economic value (Figure 72). As consequence, the return of investment, that is very high in any case, for mollusks is significantly higher than that of fish, with the ROI of mix in the middle, and the payback periods resulted to be of about 1 year in the case of fish, a little more than six months for mix, and just four months and half in the case of mollusks. Therefore, treating the mollusks can be considered the most economically proficient choice. This is even more clear by looking at Figure 73, and Table 57. Under the strong assumption of constant operating costs and market value of the products during the 20 years, with mollusks it is possible to obtain a profit that is respectively the 43,36% and the 229,04% higher than those of mix and fish.

***Table 56**: Comparison of economic sustainability.*

Parameter	u.m.	Fish	Mollusks	Mix
Annual costs	€/y	242955	250448	251448
Annual profit	€/y	525850	1181290	900734
Annual net profit	€/y	297589	948391	666836
ROI	%	101.26	270.20	189.99
PB	y	0.9876	0.3701	0.5264

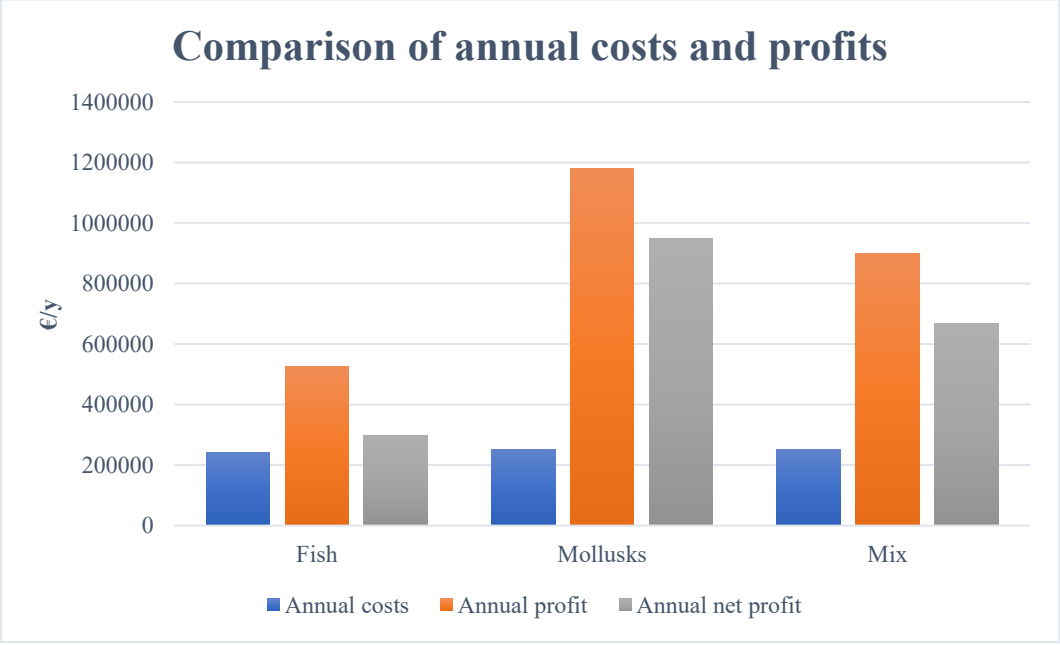


***Figure 72**: Comparison of annual costs and profits by using fish, mollusks, and mix.*

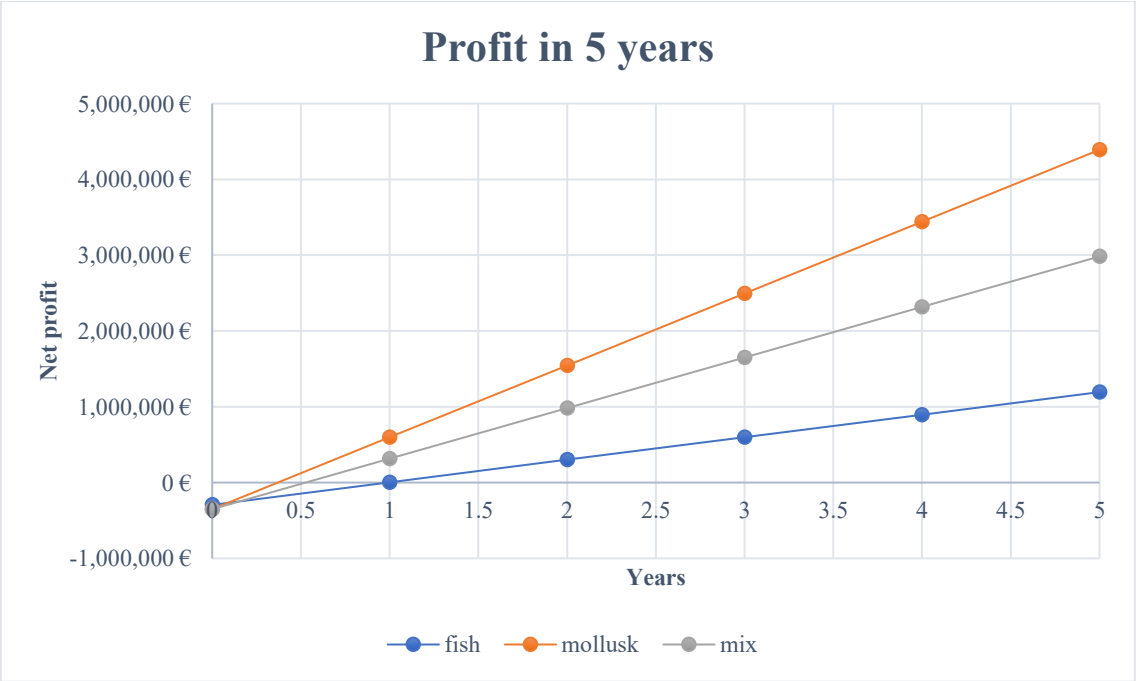


***Figure 73**: Profit in 5 years for the three substrates.*

***Table 57**: Comparison of the profit at 5 years and 20 years for the three substrates.*

Substrate	Profit in 5 years	Profit in 20 years
Fish	1,194,060 €	5,657,894 €
Mollusks	4,390,967 €	18,616,839 €
Mix	2,983,188 €	12,985,723 €

As done in the LCA, the differences in treating the mix substrate instead of the separated fish and mollusks have been accounted. As expected, also from the economic point of view, treating the mix substrate is more profitable than treating fish and mollusks in two separated lines, always due to the higher liquid yield which can be obtained in this case. Basically, the annual costs resulted to be 9494 € higher, but the annual net profit could be 87691 € more (Figure 74).

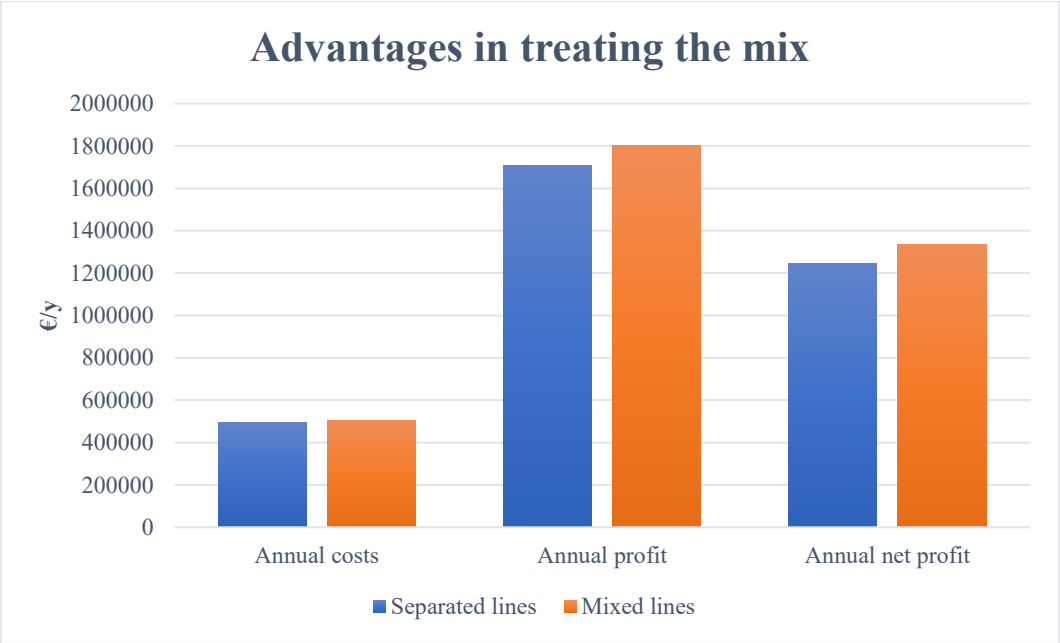


***Figure 74**: Comparison of annual costs and annual profits in treating the mix substrate instead of two separated lines.*

CHAPTER 5: CONCLUSIONS

The production scheme developed in the laboratory, has allowed to obtain the fish and/or mollusks based PHs, object of the Adriatic Sea Case Study within the SEA2LAND project. This scheme could be effectively scaled-up in a pilot plant, by using the best operating conditions highlighted during the experimental study and optimized having in mind the results outcoming from the sustainability assessment, in terms of environmental and economic impacts.

5.1 Experimental study

The single stages of the process have been set-up by taking into account the reference state-of-the-art, but always considering possible system optimizations from an economic, energy, and environmental point of views. For example, it was chosen not to carry out a defatting pre-treatment, although strongly suggested by several authors, among all He et al. (2013) and Kristinsson & Rasco (2000), to see if it was possible to achieve a good performance even in the presence of fats, whose removal is highly impacting due to the use of particular chemicals and high temperatures, which most likely denature proteins (Hoyle & Merritt, 1994) and inactivate endogenous enzymes (Klompong at al., 2007). However, in this case, it must be said that fat caused problems in liquid-solid separation and, especially in the case of fish, it was not possible to achieve a complete three-phase separation, and fat oxidation has caused unpleasant odors.

The results obtained in this experimental study are in line with those found in the literature for nitrogen recovery and hydrolyzed yield. In fact, in most of the cases, a hydrolyzed yield close to 90% has been found thanks to the addition of Alcalase to the endogenous enzymes (Ovissipour et al., 2013), and the same enzyme has allowed to obtain a nitrogen recovery between the 80% and, in many cases, close to 100%. On the other hand, the final values of nitrogen recovery are sometimes quite higher than those reported in from different authors (Table 14). The most likely motivation is related to possible overestimation of the numerator due to the not perfect solid-liquid separation in presence of semi-solids fats, which could bring non-hydrolyzed solid particles with them when the content of the test probe is spilled to collect the separated liquid after centrifugation. In the case of mollusks and mix, instead, the high nitrogen recovery could be due to an underestimation of the denominator, not being sure that the assumed dilution of the substrate is correct. In fact, it is worth to remember that the wet separation of the mollusks has been carried out by assuming a meat yield of 20% , that actually could vary depending on the waste sampled.

Concerning the operating conditions, as expected, Alcalase has been selected as the best exogenous enzyme to be added to the endogenous ones which should not be deactivated in order to improve the process and, at the same time, reduce the thermal energy consumption; in addition, the 60°C resulted to be the best operating temperature for Alcalase, in line with literature evidence. The other operating conditions selected as optimal during the experimental study are not always in line with the ones found in literature, but this can represent an advantage for this study, from the point of view of a possible up-scale of the process. For instance, the natural pH has been selected is the easiest and less expensive solution due to the avoided use of further chemicals for its control; the dilution ratio 1:1, while not always giving better results, has been defined optimal as quite similar results are obtained with half the amount of added water; the enzyme concentration (E:S) 1:200 (ml:g) provides almost the same results of the doubled one in terms of nitrogen recovery, in contrast with literature evidence that showed a reduction of the performance in that case (Figure 8), therefore it allows to save the costs for the enzyme and, indirectly, the energy spent in its production.

Although the operating parameters are satisfying from nitrogen recovery point of view, in terms of nitrogen concentration we are very far from the minimum required by the current legislation, and a very high energy consuming concentration step should be provided in a full-scale plant to achieve that minimum. In addition, the minimum content of organic carbon is not met by our product, therefore possible additions could be necessary.

Unfortunately, at now, it is not possible to know if the biostimulant activity of these products is present and, in case, how much it is effective since this kind of assessment is out of scope for this thesis.

5.2 Sustainability assessment

For what concerns the environmental assessment, it has been found that the production of mollusks and/or fish based PHs impacts on fossil fuel depletion and climate change respectively for the 48-62%, and 13.5-18% with variations depending on the substrate, while the other categories have an incidence below the 10% on the normalized impact.

In any case, most of the impact is related to the hydrolysis phase, in accordance with García-Santiago et al. (2020) and Colantoni et al. (2017), mostly due to the use of enzyme and its production, and thermal energy. The second phase in order of impact on fossil fuel depletion and climate change is the concentration, due to the use of electricity.

From the point of view of environmental assessment of waste valorization (results for 1 ton of waste treated) the impact on climate change and on fossil fuel depletion resulted to be respectively between 254.93-261.20 kg CO_2-Eq, 108.89-114.04 kg oil-Eq, whereas considering the assessment of production, the impacts were respectively 558.82-734.71 kg CO_2-Eq, and 243.99-313.83 kg oil-Eq, with variations depending on the substrate used. The results are in line with the ones obtained by Colantoni et al. (2017) for an enzymatically produced PH from lupine grains, and much lower in comparison with chemically produced PH from leather waste. This, although the pilot plant on which the LCA study was conducted is only hypothetical, and the technologies cannot be directly compared, represents a promising result.

What can be concluded is that most of the environmental impact is related to the enzyme production, the use of thermal energy and of electricity. By summing the use of electricity for all the steps and for concentration (elaborated separately), this results to be the most relevant contribution on both fossil fuels depletion and climate change.

Having this in mind, it is possible to determine where it is possible to act in order to optimize the process from this point of view. In particular, by halving the concentration of the enzyme, it is possible to save 50% of the contribution related to the enzyme. By using another type of apparatus for concentration, it is possible to reduce the contribution of electricity but the one related to the thermal energy would increase, and, if a less efficient concentrator is used, this could result in a more relevant impact.

From the economic assessment, it can be concluded that, the payback period is very short, being from four months and half to about 1 year depending on the substrate, therefore the solution studied seems to be highly efficient from an economic point of view. The operating conditions to carry out the economic assessment have been selected as the most expensive ones (S:W = 1:2; E:S = 1:100). Therefore, the payback period and the long-term profit could be respectively much shorter and higher by selecting the most efficient operating conditions.

Anyway, uncertainties related to the data obtained, especially to the selling price of the machinery, which are very approximate, and the selling price of the protein hydrolysate and calcium carbonate, should be kept in mind. Most probabily, in the reality, the CAPEX would be greater than those calculated here, and the payback period would be longer. In addition, it is recalled that electricity and heat prices refer to the year 2020, as the current energy situation would have greatly increased operating costs, without guarantee that these would be permanent over time.

5.3 Comparison of the three substrates

From technical point of view, the comparison of the behavior of the three substrates showed, in some cases, a sort of synergistic effect between the fish and the mollusks that make up the mix, resulting in a higher rate of hydrolysis by using it.

The differences between the environmental impacts generated by the three substrates are not so relevant. However, the limitation on using mollusks is that, under the assumption to use the available technology for meat-shell separation, it is necessary to use a dilution ratio of at least 1:2, differently for the other two substrates for which half of the water can be added with promising results, as obtained at lab scale. With half of the water, the electricity and thermal energy used will be reduced, therefore also the impacts on fossil fuel depletion and climate change. In addition, it has been studied also the presence of savings or worsening arising from the treatment of the created mix, in comparison with the treatment of both fish and mollusks. Anyway, no particular differences have been highlighted, being them below 5%.

The economic comparison between the three substrates showed, as expected from mass and energy balances, comparable CAPEX and OPEX. What is of main interest is that treating the mollusks can be considered the most economically proficient choice, being the profit much higher than those of the others, due to the possible commercialization of the calcium carbonate obtained during meat-shell separation, in addition to the revenues arising from the protein hydrolysate. As expected, also from the economic point of view, treating the mix substrate is more profitable than treating fish and mollusks in two separated lines, always due to the higher liquid yield which can be obtained in this case.

Therefore, although the lowest protein content, from the point of view of waste valorization, mollusks are the best choice, because with an appropriate concentration of the mollusks-based protein hydrolysate it is possible to obtain a suitable final value of nitrogen content, moreover they are characterized by a slightly lower environmental impact and, at the same time, the economic profit is highly greater than the other two.

However, from the point of view of the protein hydrolysate production, this result is reversed since the mollusks have a lower yield than the other two and therefore more waste and more resources are needed to obtain a comparable amount of protein hydrolysate. From this point of view, the best substrate is the mix, as it has yield values more similar to those of fish, so it is characterized by a slightly higher environmental impact than that of fish, but lower than that of mollusks. Also, from the

economic point of view, the profit that you can get with the mix is closer to what you can get with mollusks than to that of fish.

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