This project receives funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 773330 (GAIN)

GAIN Green Aquaculture Intensification

SUMMER SCHOOL

Course: Enhancing Circularity In The Aquaculture Sectors. Presentation: Obtaining of valuable products and chemicals from fish farming by-products

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Obtaining of valuable products and chemicals from fish farming by-products

Summary

1. Definition of the problem and current status of the issue.

2. Production, characterization and application of valuable biocompounds from aquaculture wastes.

    2.1. Production of fish protein hydrolysates (FPH) and fish oils.

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    2.4. Recovery of bioapatites from fish bones.

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1. Definition of the problem and current status of the issue

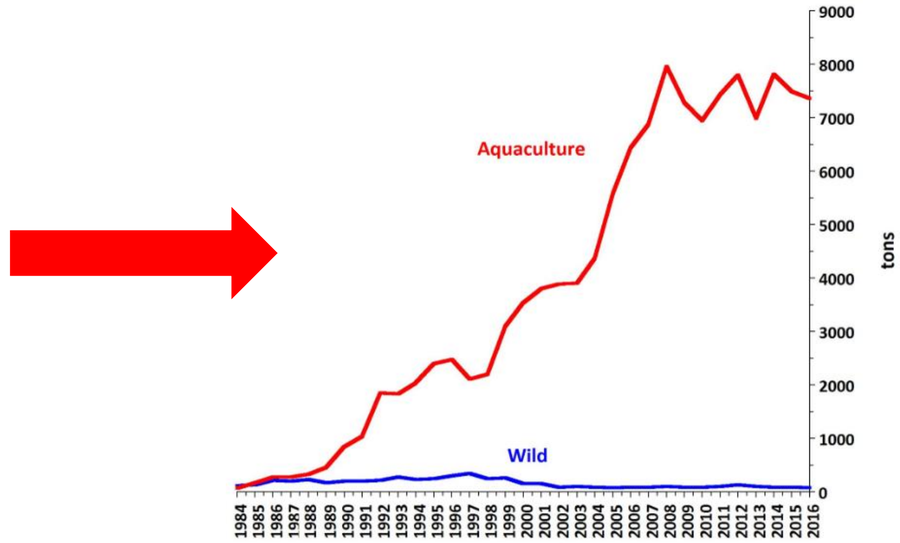
One of the most critical challenges that humanity currently faces is the production of enough food for an expected population of 9.6 billion people by 2050. Global per capita fish consumption has more than doubled in the last fifty years, in line with increasing demand by a growing, wealthier, and more urbanized population.

Up to the 1980s, demand was mainly met by increased wild capture, but aquaculture has rapidly closed the gap, equating production and consumption with fisheries in the last years.

The production of aquaculture fish around the world achieved 80 million tons in 2016, supposing **48%** of the total fish captured, transformed, and marketed.

In some cases, as for example turbot (***Scophthalmus maximus***) production in Europe, only from 1984-1988 at the beginning of farming activities of this species, tons of wild and cultured were equal.

The increase in the period 1984-2016 showed a global **sigmoid profile**: exponential between 1989 and 2007, and with a stationary phase with ripples from 2008 to 2016. The balance between wild-aquaculture production, as percentage, moved from 100%-0% to 5%-95% in 30 years.



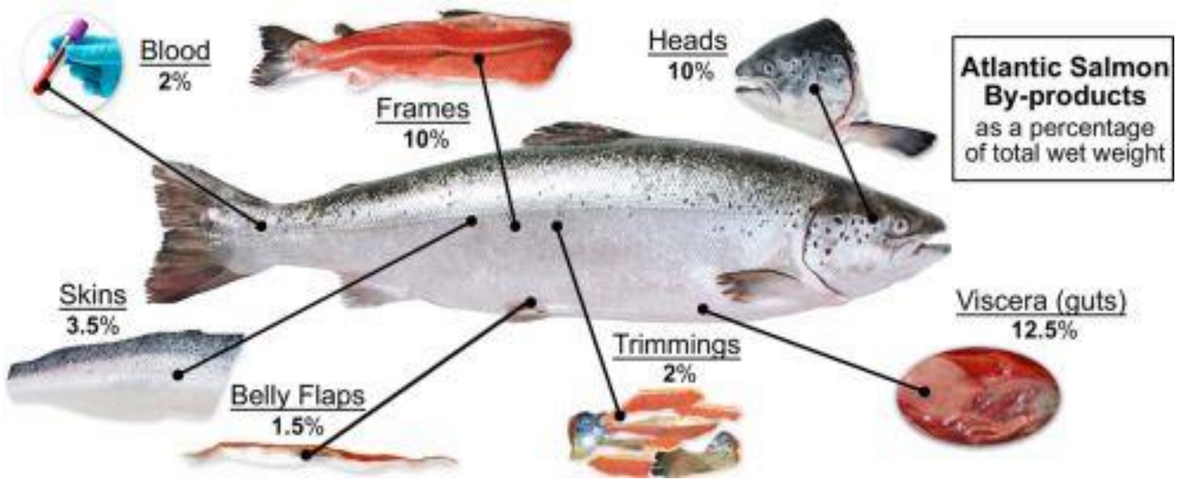
1. Definition of the problem and current status of the issue

Although in many countries fish species are conventionally marketed as a whole fish, fillet presentations are growing year after year around the world.

The **procedure of filleting** (headless, gutted, etc.) generates a huge amount of by-products. In many cases, this residual biomass accounts for about 40-60% of the total fish weight and must be managed to avoid environmental health problems in fish farming plants or in fish processing food companies.

For **Atlantic salmon**, the most abundant species farmed in Europe, the different by-products obtained in the filleting process are: heads (10%), frames and bones (22.5%), trimmings (2%), viscera (12.5%), skins (3.5%) and blood (2%).

Similar percentages are also found in the industrial filleting of other species as rainbow trout, seabream and seabass.



Aquaculture wastes contain a remarkable proportion of valuable components such as high quality proteins and oils, even minerals, that must be recovered to make fish farming production truly sustainable and efficient.
These residues are currently used for the production of **fish meal/fish oil** or **silage/bio-silage**.

1. Definition of the problem and current status of the issue

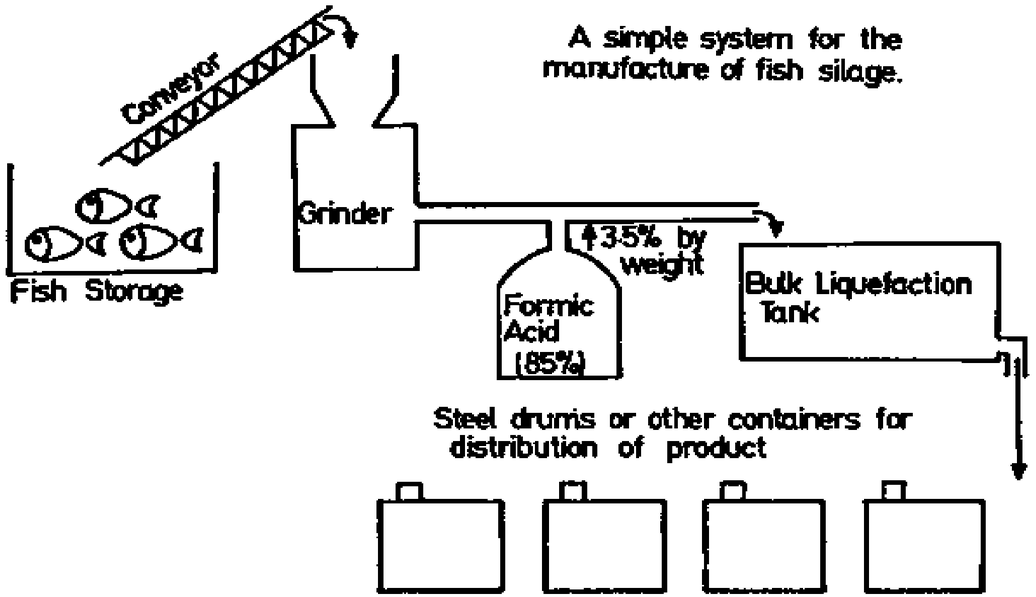
Production of chemical fish silage or biological silage (bio-silage)

Silage is a liquid or dried product resulting mainly from chemical hydrolysis, prepared by combining inorganic acids – as formic or phosphoric acids– to lower pH value of 4 in order to inhibit microbial growth and avoid spoilage.

When viscera are present in the fish substrate, endogenous enzymes included in the pancreas or in the pyrolic caeca can be used as biocatalyst for the production of silage.

Bio-silage is a liquid or dried fermented product obtained when **lactic acid bacteria** are employed as reagent for the fermentation, hydrolysis and stabilization of fish substrate.

In all cases, these products can be applied as liquids, or can be dried by freeze-drying, spray-drying or fluidized bed drying.

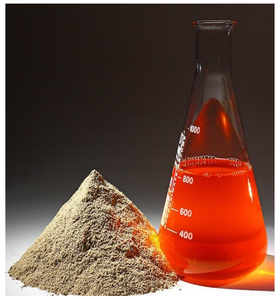


1. Definition of the problem and current status of the issue

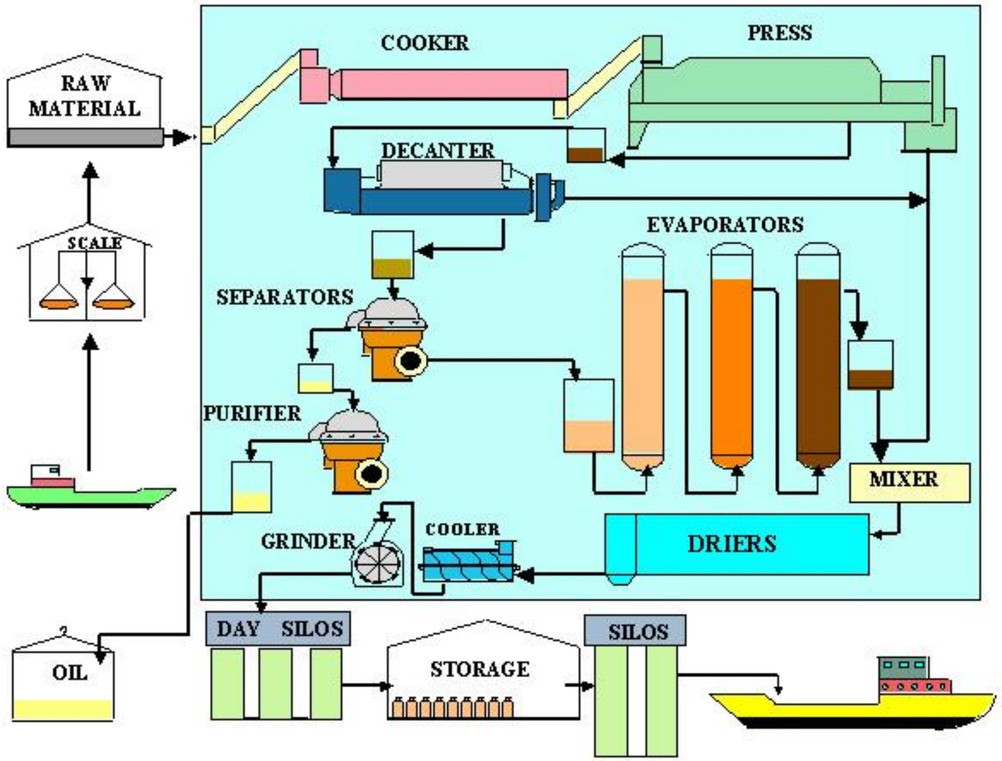
Production of fish meal and fish oil

Fish meal is a material mainly proteic (between 50-75%, w/w) obtained from the crushing, thermal and drying processing of different fish substrates or fish residues. It also contains a variable level of inorganic fraction (ash) depending on the amount of bones present in the wastes and the types of separation process employed.

Fish oil is also recovered concomitantly with the production of meals by cooking, pressing and centrifugation of fish wastes. Its quality in terms of omega-3/omega-6 ratio is a function on the fish species processed.



Fish meals and fish oils are basic ingredients in aquaculture feed formulations, and their commercial value are directly proportional to the concentration of protein and the quaility of oils.



1. Definition of the problem and current status of the issue

When comparing both bioproductions, fish meal/oil and silage, following aspects can be commented:

- **Fish silage** production is **simpler** than **fish meal** since the equipment necessary is **cheaper** and also the **energy-demand and time-consuming** is **lowe**r than fish meal plants.

- In general, **fish meal production** is **more efficient and viable by increasing the volume of waste** to be treated and the size of the processing plant.

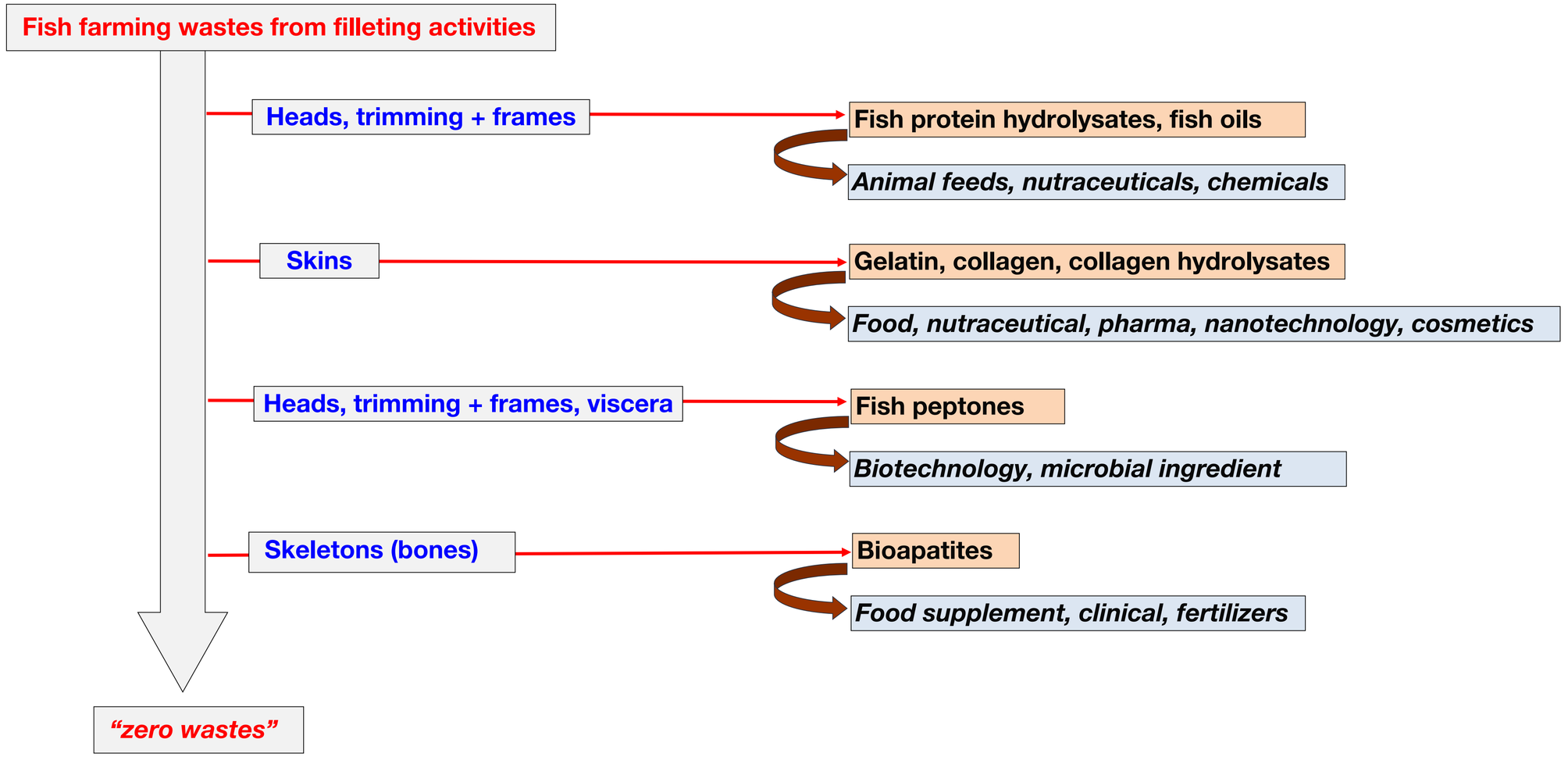
- **Fish silage units are more flexible** and more adequate to be implemented in locations where the volume or seasonal availability of fish by-products is not enough to justify operation in a traditional fish meal plant.

- **Silage** contains more than 60% of water, forcing an intense concentration and/or exhaustive drying if this material wants to be marketed far from its place of production.

- **Fish meal production** is a well established and economically very profitable business, but other valorization processes, in line with circular economy principles, have been explored in the **GAIN project.**

2. Production, characterization and application of valuable biocompounds...

Based on the concept of **marine biorefinery** in GAIN, we have developed and optimized a set of sustainable processes (chemical and physical) and bioprocesses (enzymatic and microbial) for the valorization of aquaculture by-products.



2.1. Production of fish protein hydrolysates (FPH) and fish oils

A **fish protein hydrolysate (FPH)** is defined as the material rich in proteins, peptides and free amino acids obtained from the total or partial hydrolysis of whole fish or fish by-products (skins, heads, viscera, etc.) using chemicals (alkalis, acid) and/or enzyme as proteolytic agent.

Types of FPH

Depending on the proteolytic reagent used we can identified different types of hydrolysis (also known as proteolysis when protein is the substrate to hydrolyse) to produce FPH:

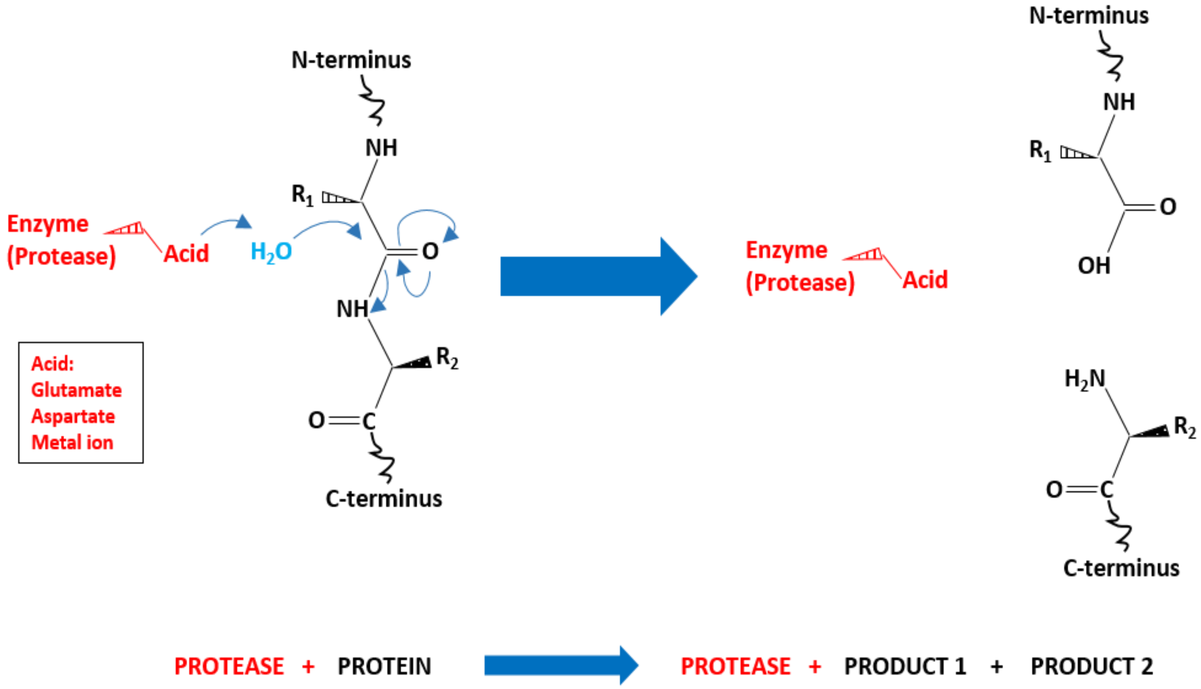
- **Alkaline hydrolysis** when fish waste substrates are treatment with alkalis reagent (NaOH, KOH, etc.).
- **Acid hydrolysis** when acid solution (H_2SO_4, HCOOH, etc.) is applied to the waste substrate. If fish viscera is the substrate, it is also known as fish silage.
- **Enzyme hydrolysis** when a exogenous enzyme is applied for the catalytic process on fish. In viscera, another option is the use of endogenous enzymes from pancreas or pyloric caecum.

2.1. Production of fish protein hydrolysates (FPH) and fish oils

The enzymes that break the peptide bonds of fish protein leading to peptides, of different sizes, and even free amino acids are called **protease** (also known as peptidase or proteinase).

They are hydrolytic enzymes and are globally classified into two groups: **endopeptidase** (breakdown of nonterminal amino acids) or **exopeptidase** (breakdown at the ends of the protein chain releasing amino acids).

Another classification is based on the reactivity of the catalytic amino acid presents on the protease, that is, aspartate protease, glutamate protease, metalloprotease, cysteine protease, serine protease, threonine protease and asparagine protease.



2.1. Production of fish protein hydrolysates (FPH) and fish oils

The commercial name of proteases is established for different reasons such as, its origin, the substrate that especifically catalyses or the optimal range of pH activity. Thus, some examples of these proteases are:

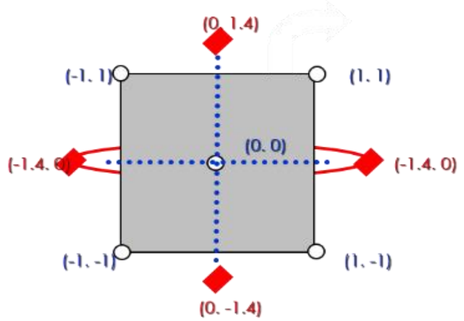
- **Papain** is an endopeptidase (cysteine protease) isolated from papaya and actives in the range of pH (6-8) and T (40-80ºC).
- **Bromelain** is an endopeptidase (cysteine protease) obtained from pineapple and actives in the range of pH (6-8) and T (30-60ºC).
- **Trypsin** is an endopeptidase (serine protease) recovered from digestive system of vertebrates and actives in the range of pH (6.5-9.5) and T (4-65ºC).
- **Pepsin** is an endopeptidase (aspartate protease) isolated from digestive system of vertebrates and actives in the range of pH (1.5-3.0) and T (30-45ºC).
- **Carboxypeptidase A** is an exopeptidase obtained from pancreas with activity in the range of pH (7.0-10.0) and T (45-75ºC).
- **Aminopeptidase N** is an exopeptidase recovered from small intestine with activity in the range of pH (7.0-9.0) and T (30-60ºC).
- **Alcalase** (subtilisin A) is an endopeptidase (serine protease) produced by ***Bacillus licheniformes*** with activity in the range of pH (6.5-9.0) and T (40-70ºC).

1) High proteolytic activity, 2) Excellent capacity and effectiveness for the hydrolysis several fish substrates, 3) Cost-effective.

2.1. Production of fish protein hydrolysates (FPH) and fish oils

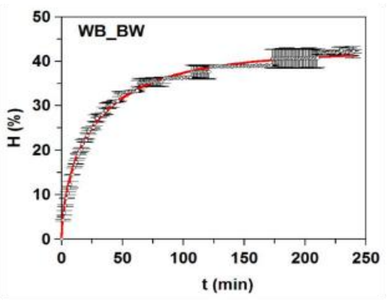
The first stage was to optimize the most relevant variables that affect the proteolytic activity of the protease employed for the digestion of the fish wastes. The **independent variables** to control were pH, temperature (T), solid:liquid ratio (r(S:L)), [protease], agitation and time of hydrolysis. The **dependent variables (responses)** to determine were: degree of hydrolysis, total soluble protein, yield of digestion, bioactivities, etc.

Second order design
(response surface methodology)



- Establish the range of values for each independent variable (pH and T).
- Variables codification to homogeneize statistical weights.
- Statistical assessment of the results:
  - Coefficients significance (t-*Student*)
  - Consistency of equation (F-*Fisher*)
  - Validity of equation (R^2 and R^2_{adj})

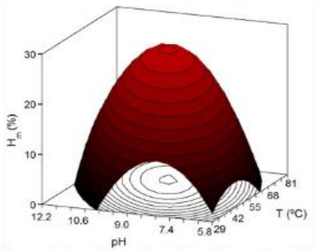
Kinetical approach
(hydrolysis degree *vs* time)



Weibull model

$$H = H_m\left\{1-\exp\left[-\ln 2\left(\frac{t}{\tau}\right)^{\beta}\right]\right\}$$

$$v_m = \frac{\beta H_m \ln 2}{2\tau}$$



$$H_m = b_0 + b_1 pH + b_2 T + b_{12} TpH + b_{11} pH^2 + b_{22} T^2$$

pH-stat thermostated reactor

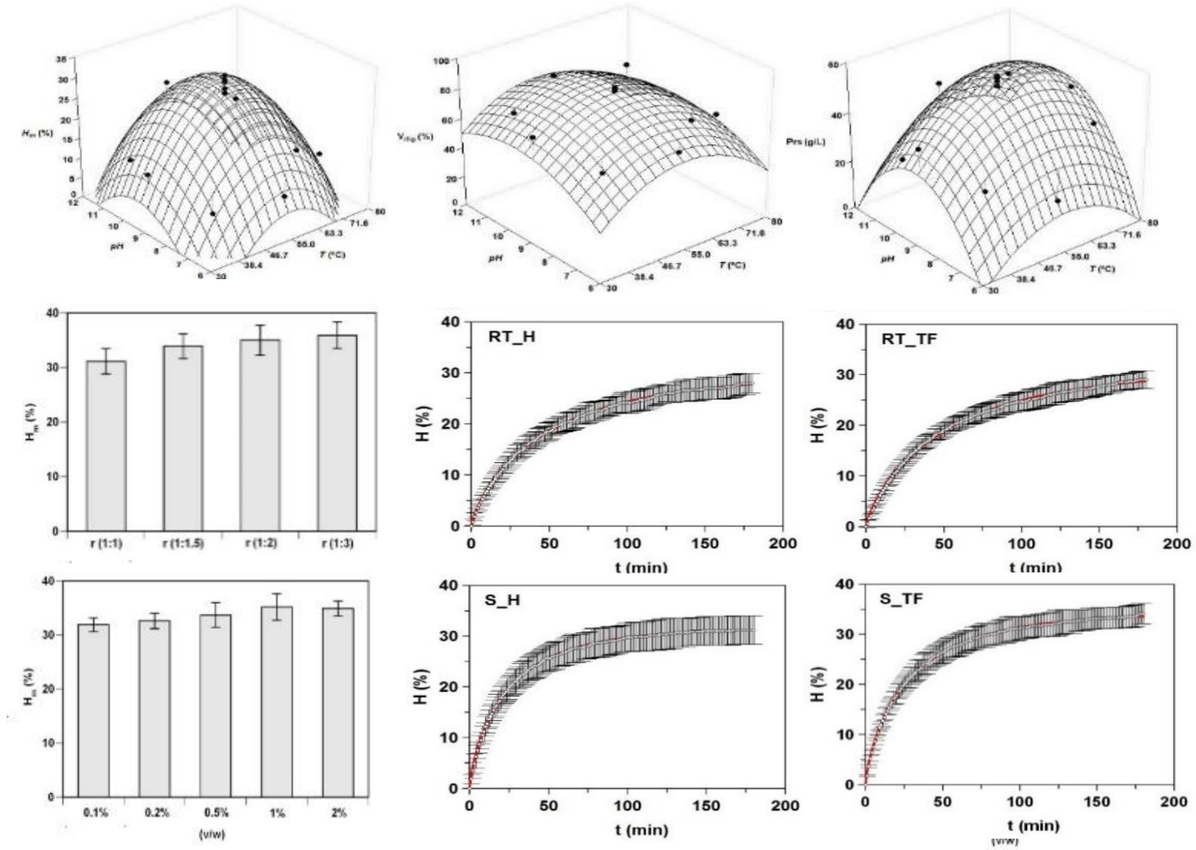
Glass jacket reactor equiped with:

- System of agitation.
- pH measurement and control by means of a pH sensor connected to a pump for dosing of acid or alkali.
- Control of temperature.
- On-line recording and monitoring of T, pH and reagents consumption.

2.1. Production of fish protein hydrolysates (FPH) and fish oils

Different by-products (**heads, trimmings + frames**) from aquaculture **salmon, trout, turbot, seabream** and **seabass** were used as substrates for the production of FPH.

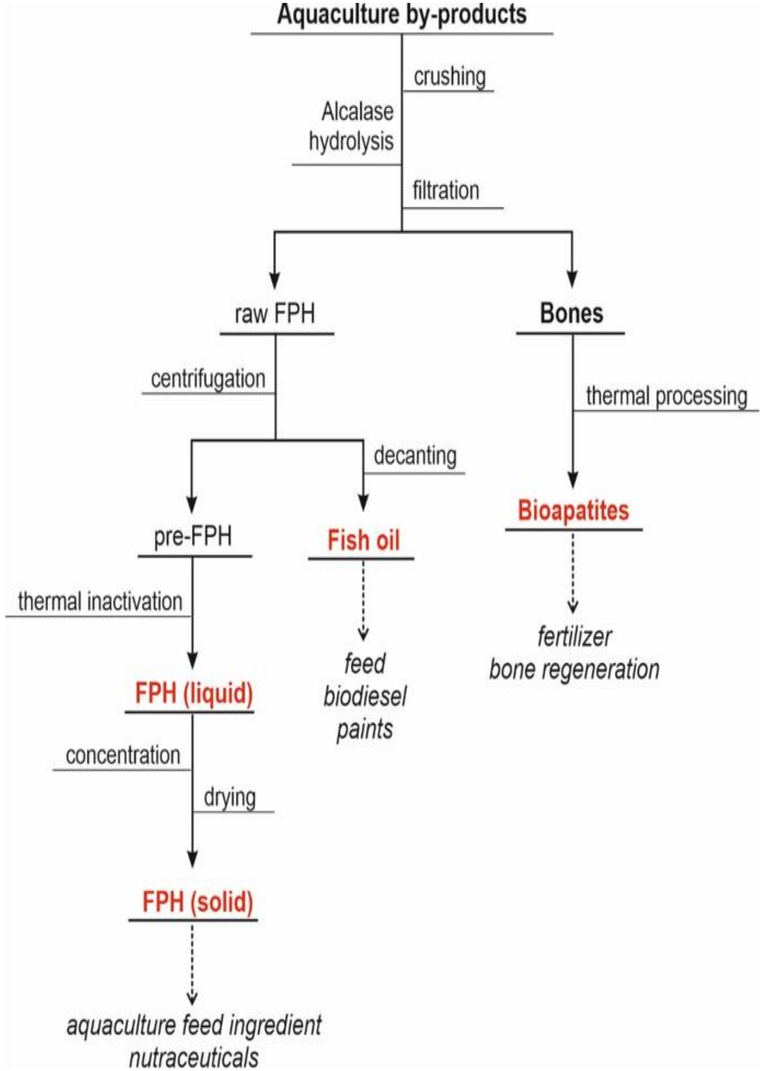
Rainbow trout heads



Optimal values of the most relevant variables to maximize the production of FPH from farming fish by-products. The rest of the conditions of processing were, in all cases, continuous agitation at 200 rpm, r(S:L)=1:1 and 3 h of hydrolysis.

	pH	T (ºC)	[alcalase], % (v/w)
Salmon	8.98	64.2	0.2
Trout	8.27	56.2	0.1
Turbot	8.82	60.3	0.2
Seabream	8.17	57.1	0.2
Seabass	8.46	58.4	0.2

2.1. Production of fish protein hydrolysates (FPH) and fish oils



Steps for FPH production:

- Crushing and homogenization of substrates.
- Enzyme hydrolysis under controlled conditions.
- Filtration for separation of solid (clean bones) and liquid (raw FPH).
- Centrifugation and decanting of oils from raw FPH.
- Thermal inactivation of pre-FPH (liquid FPH) .
- Concentration and drying of FPH (solid FPH).
- Vacuum packaging and cold storage.

2.1. Production of fish protein hydrolysates (FPH) and fish oils

Mass balances and chemical characterization of the products generated by alcalase proteolysis of aquaculture by-products. Y_{dig}: yield of substrate digestion (%, v/w); Y_b: bones recovered (%, w/w); Y_{oil}: oil isolated (%, v/w); H_m: maximum hydrolysis degree (%); Pr: Total soluble protein of FPH (g/L); EAA: essential amino acids content (%, w/w); Dig: in vitro digestibility of FPH (%); Mw: average molecular weight of FPH protein (kDa).

	Y_{dig} (%)	Y_b (%)	Y_{oil} (%)	H_m (%)	Pr (g/L)	EAA (%)	Dig (%)
Salmon	86-90	11-12	9-12	30-31	61-70	37-39	93-94
Trout	84-88	9-10	9-11	32-34	48-54	39-43	93
Turbot	83-87	10-17	0-4	37-38	74	35-37	92-94
Seabream	78-90	7-20	4-11	19-22	62-82	42	90-91
Seabass	78-79	11-19	8-14	17-22	63-73	40-42	90-91

Fatty acids	Trout	Salmon	Turbot	Seabream	Seabass
Pentadecenoic acid	4.10±0.11	-	-	0.32±0.06	0.34±0.08
Palmitic acid	6.13±0.09	7.49±0.82	19.80±1.37	15.36±0.90	14.73±1.35
Palmitoleic acid	2.77±0.18	2.09±0.24	6.87±1.49	4.45±0.40	4.59±0.88
Oleic acid	52.85±0.46	44.56±1.31	34.14±5.60	28.59±0.69	31.65±2.69
Linoleic acid	12.06±0.27	22.66±2.20	12.89±2.25	21.28±0.41	23.50±0.95
Linolenic acid	2.04±0.06	5.83±0.85	5.14±0.98	3.33±0.08	3.04±0.86
Eicosenoic acid	1.88±0.68	6.41±2.21	3.15±2.16	1.66±0.50	2.08±0.39
Eicosapentaenoic acid (EPA)	0.63±0.04	0.34±0.08	0.46±0.17	4.80±0.63	3.24±0.71
Nervonic acid	6.72±0.74	-	-	0.24±0.20	0.28±0.19
Docosahexaenoic acid (DHA)	2.14±0.09	3.36±0.23	3.87±2.27	10.72±0.56	5.78±0.63
DHA+EPA (%)	2.77±0.13	3.70±0.27	4.34±2.12	15.52±0.60	9.02±0.67
ω-3 / ω-6 ratio	0.49±0.01	0.49±0.06	0.71±0.18	0.83±0.08	0.51±0.09

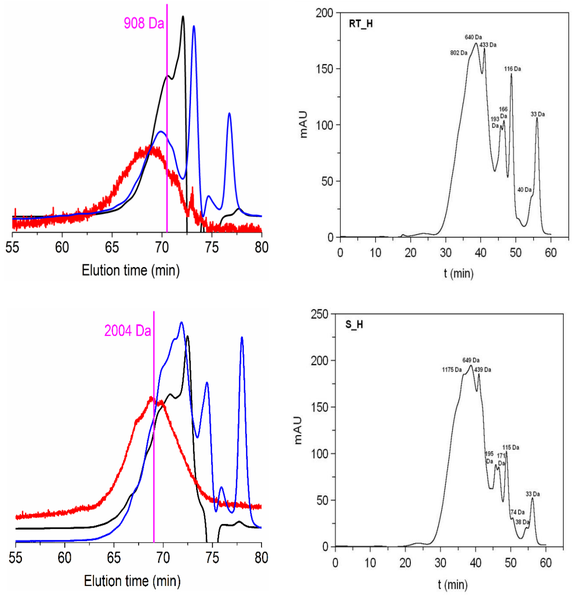
Chemical characterization FPH:

- Almost complete digestion of solid substrates to liquid FPH (Y_{dig}).
- Remarkable production of soluble protein (Pr).
- Complete separation of bones (Y_b) and high recovery of oils (Y_{oil}).
- Highest degree of hydrolysis (H_m) for turbot, trout and salmon.
- Large *in vitro* digestibility (Dig) and high proportion of essential amino acids (EAA) in FPH.

Fatty acid (FA) profiles:

- In oils from all fish species, oleic acid was the predominant FA followed by linoleic acid.
- Low levels of EPA and DHA except in seabass oil and mainly in seabream oil.
- Low omega-3/omega-6 ratio.
- Interesting oils for food and feed supplements and chemicals production (*e.g.*, paints), but low potential for nutraceutical applications.

2.1. Production of fish protein hydrolysates (FPH) and fish oils



- Molecular weights of peptides **mainly lower than 3 kDa**.
- **Prevalence** of molecular weights in the range of **0.5-3 kDa**.
- Average molecular weight (Mw), for all species and substrates, around 1.2-2.0 kDa.
- Number average molecular weight (Mn), for all species and substrates, around 0.8-1.2 kDa.
- High potential of FPH to be incorporated into aquatic diets as substitutes for fish meal.

FPHs	Mn (Da)	Mw (Da)	<1 kDa (%)	1-3 kDa (%)	>3 kDa (%)
Trout	0.9-1.1	1.7-1.9	41.9-48.1	38.0-47.6	10.4-14.0
Salmon	0.9-1.2	1.4-1.9	32.8-48.6	43.3-52.0	8.3-15.0
Turbot	0.8-1.2	1.2-2.1	29.6-61.3	40.3-52.9	4.4-12.1
Seabream	0.8-1.1	1.5-1.9	42.2-62.4	28.3-51.2	6.6-12.5
Seabass	0.8-1.1	1.4-2.0	28.9-65.1	36.2-50.2	7.4-12.2

FPHs	DPPH (%)	ABTS (µg BHT/mL)	Crocin (µg Tr/mL)	I_{ACE} (%)	IC50 (µg Pr/mL)
Trout	48-53	15.0-15.1	8.7-9.0	68-82	509-975
Salmon	45-57	13.1-16.8	7.5-8.5	72-87	479-654
Turbot	36-65	10.0-12.8	7.3-8.0	53-82	213-1274
Seabream	37-52	9.8-15.1	5.0-7.4	37-48	793-1246
Seabass	41-54	10.8-14.5	5.2-6.9	34-50	801-1398

In vitro bioactivities:

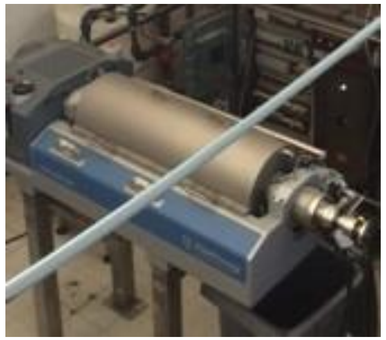
- Overall, modest antioxidant activity (in terms of DPPH, ABTS and Crocin), being salmon FPH slightly higher than the rest.
- Remarkable antihypertensive activity (based on the percentage of inhibition, I_{ACE}).
- FPH from turbot viscera showed the major antihypertensive inhibitory value IC50= 213 µg Pr/mL).

2.1. Production of fish protein hydrolysates (FPH) and fish oils

Scaling-up of FPH production using pilot plant facilities from IIM-CSIC

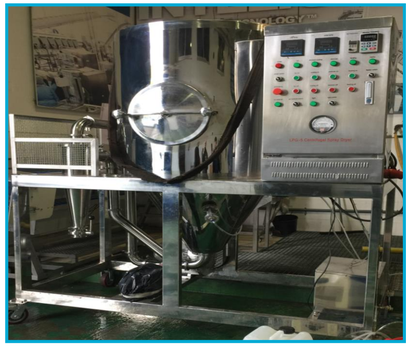
Crushing/homogenization in a grinder

Hydrolysis in a pH-stat reactor (300 L)



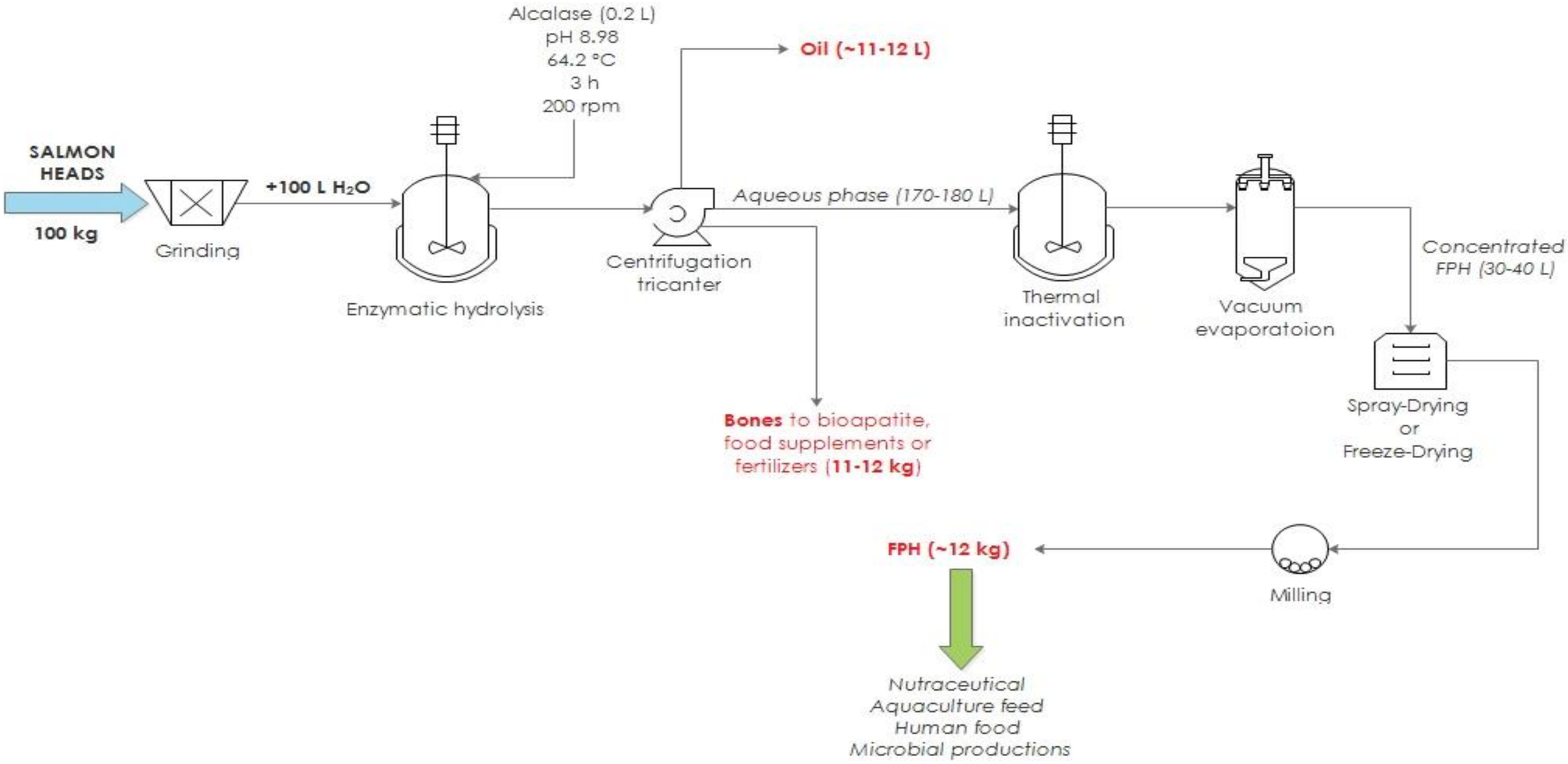
Separation of aqueous FPH and fish oil by centrifugation in a tricanter

FPH concentration by vacuum evaporation

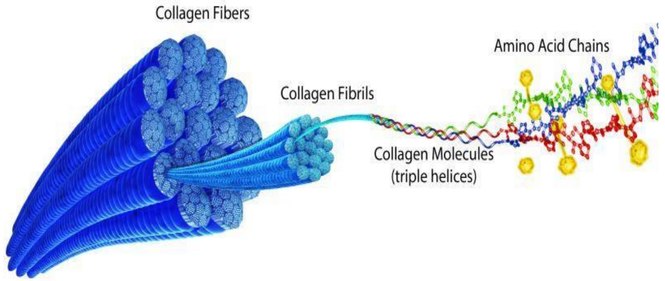


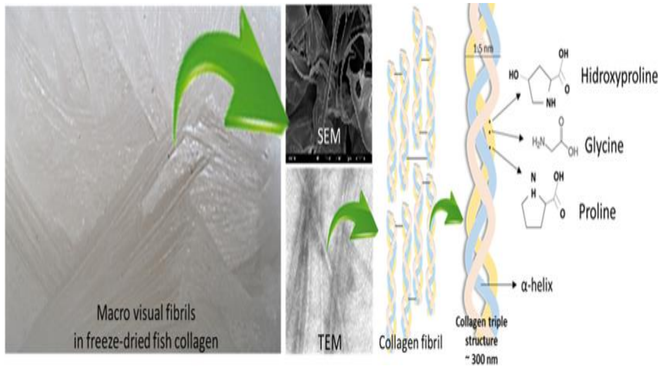
FPH dried using spray-drying or freeze-drying

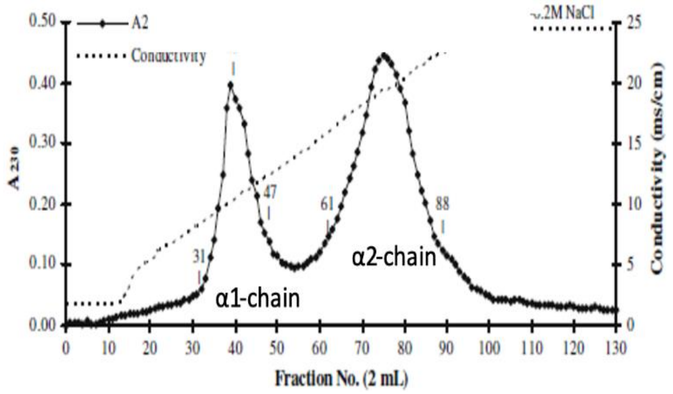
2.1. Production of fish protein hydrolysates (FPH) and fish oils



2.2. Production of collagen and derivatives

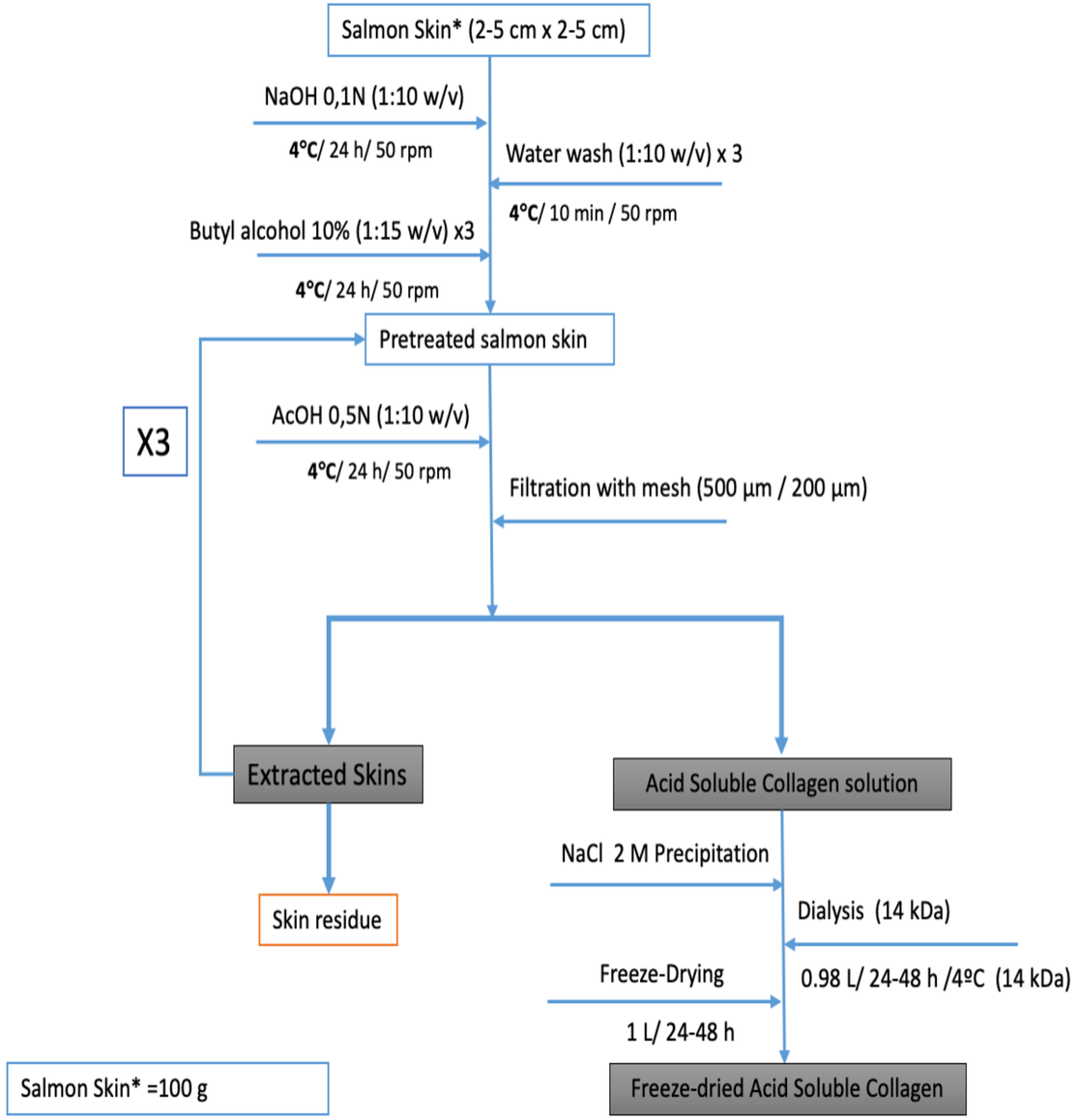






- **Collagen** is the most important fibrous protein present in animals (reaching up to 30% of total body proteins in animals). It is produced by fibroblast.
- **Collagen** is the most abundant extracellular matrix protein of different tissues (skins, bones and connective tissue).
- **Collagen** is a supramolecular and fibrillar structure, is insoluble in water and providing structure, strength, and flexibility to the tissues.
- The basic structure (monomer or tropocollagen) presents a **right-handed triple helix** formed from three left-handed α-chains.
- Glycine, proline and OH-proline are the predominant amino acids in collagen.
- At least 21 types of collagens, type I and II the most abundant in fish.
- **Gelatin** is formed after the partial hydrolysis of collagen.
- **Collagen hydrolysates** are obtained by enzymatic hydrolysis of native collagen or gelatins.
- **Collagen, gelatin and collagen hydrolysates** are compounds with multiple applications in different sectors such as pharmacological (carrier for drug delivery, skin repair, etc.), cosmetic (moisturizers, antiaging), food (gels, thickener), nutraceutical (supplement for bone and cartilage repair) and tissue regenerations (scaffolds and stimulator for osteocytes and chondrocytes growths).

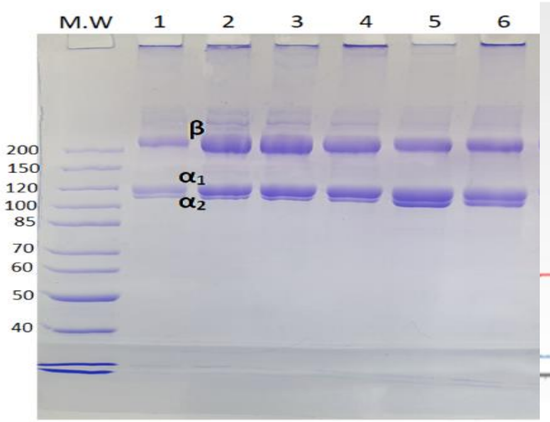
2.2. Production of collagen and derivatives

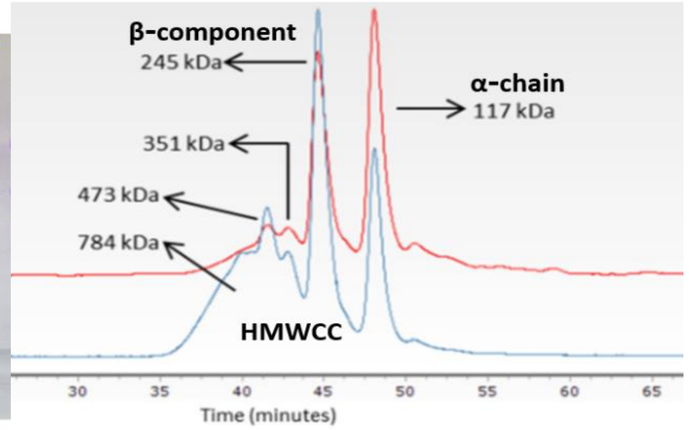


Collagen from salmon skin wastes was produced after (all stages performed at 4°C):

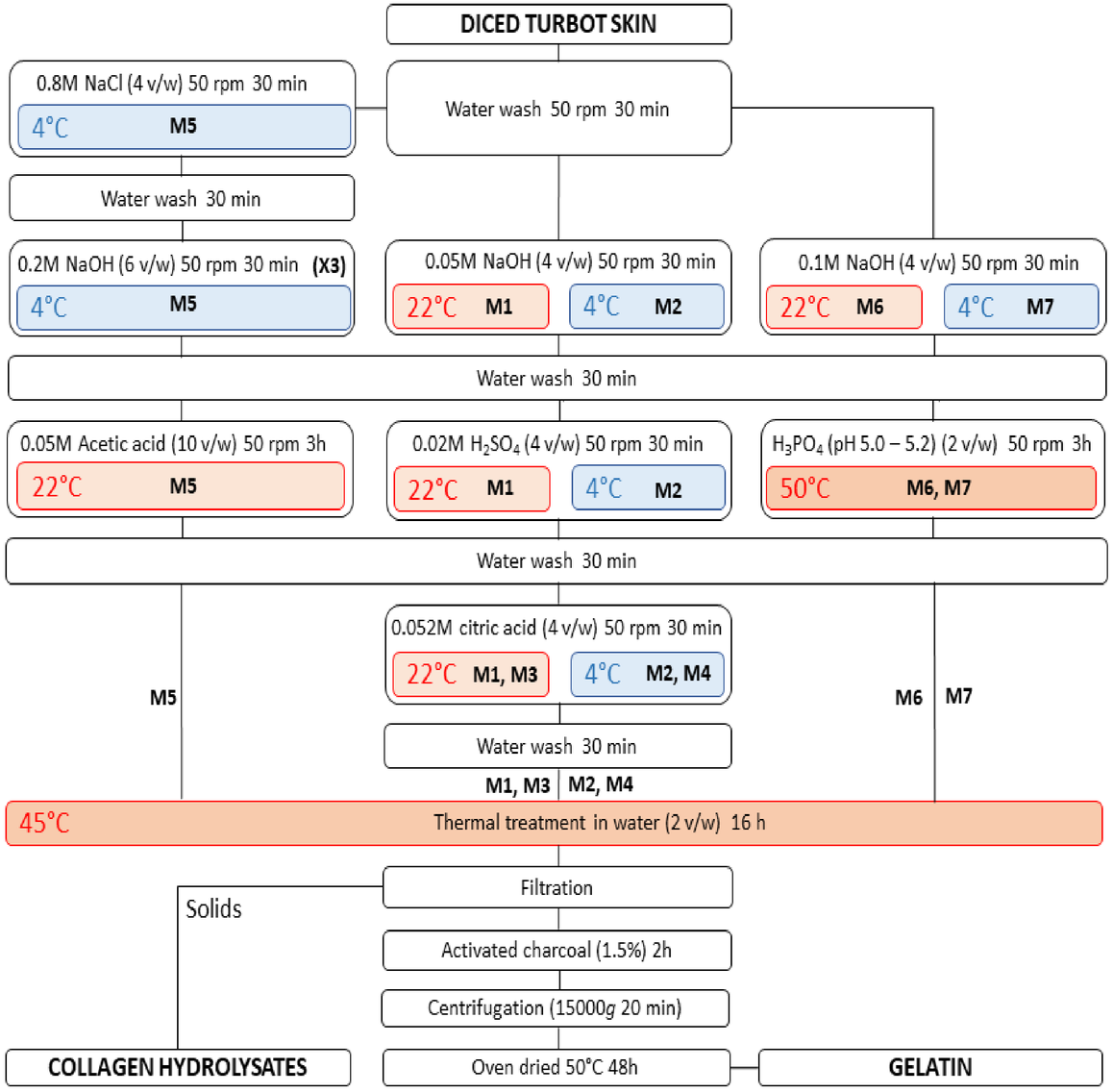
- i) Alkaline treatment with NaOH.
- ii) Defatted using butyl alcohol.
- iii) Precipitation of collagen by acetic acid.
- iv) Filtration of the precipitate.
- v) Repetition of this procedure (x3) with skins remains.
- vi) Saline purification/precipitation of collagen.
- vii) Exhaustive dialysis of saline collagen.
- viii) Drying of acid soluble collagen (ACS) by lyophilization.

High yield and high chemical quality of collagen recovery.





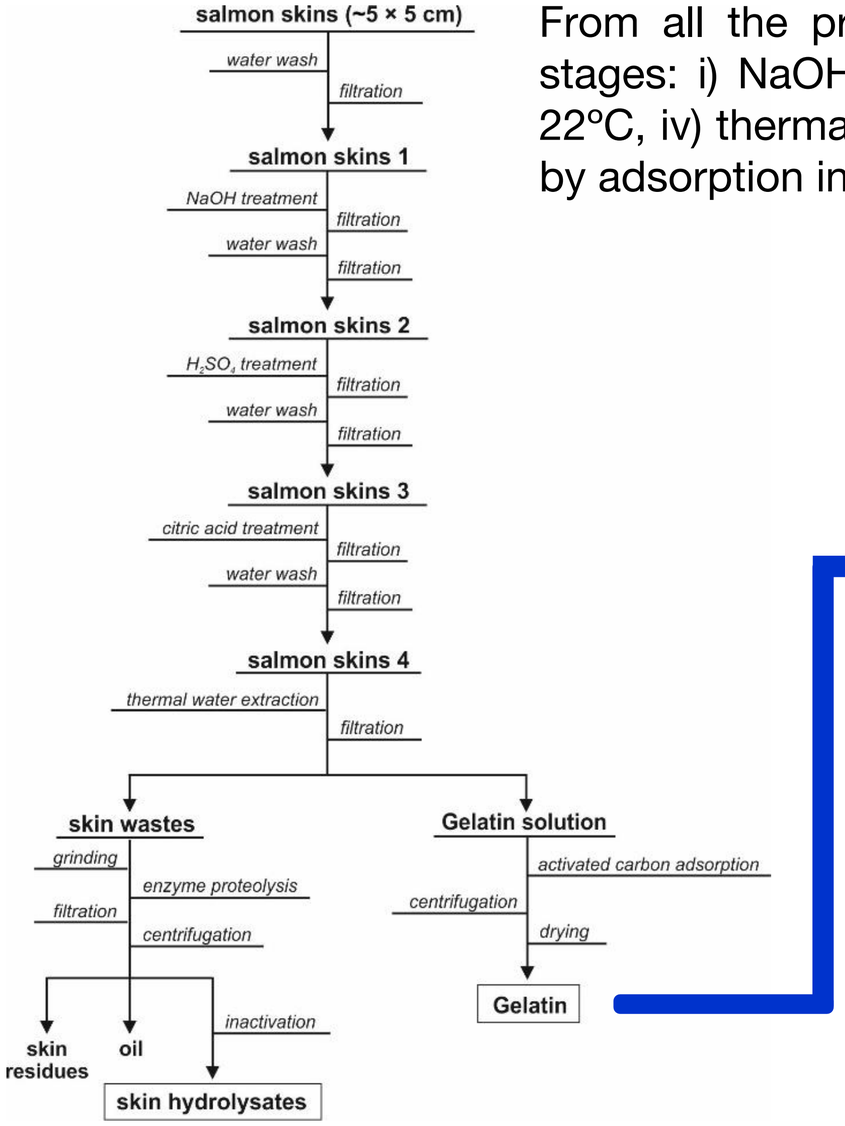
2.2. Production of collagen and derivatives



In GAIN project different processing strategies were studied for the production of **Gelatins** from salmon, turbot, seabream, trout and seabass skin by-products.

- In all cases, skins were cut on portions around 5 x 5 cm.
- Skins were initially water washed to eliminate impurities.
- A set of chemical treatments at 4 or 22ºC (combined alkaline, acidic or saline washes) were applied to the skins before extraction steps.
- Aqueous thermal extraction (at 45ºC) of gelatin solution from treated skins was then performed.
- In parallel, a procedure dealing with acidic water (with fosforic acid) at pH5 was also studied.
- All alternatives were finished with a stage of purification and deodorisation of gelatin solutions by adsorption in activated carbón, and drying of gelatin by forced convection oven.

2.2. Production of collagen and derivatives

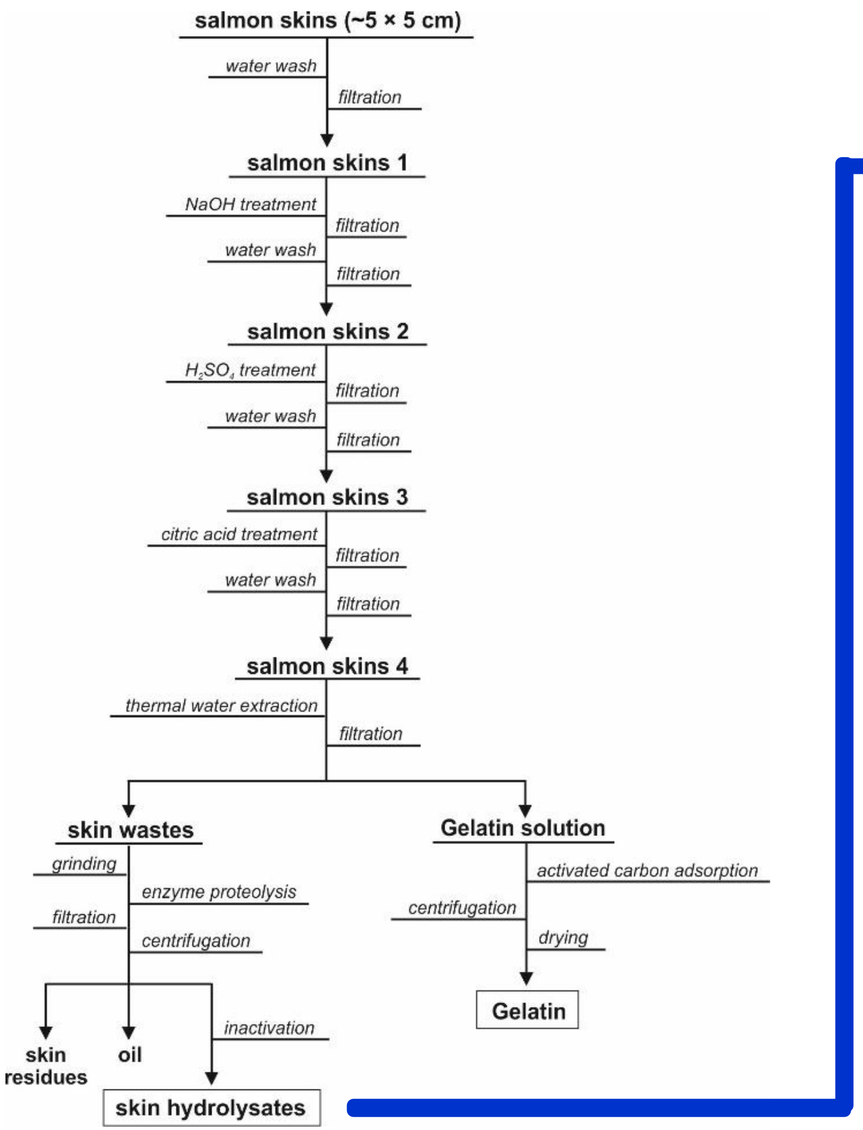


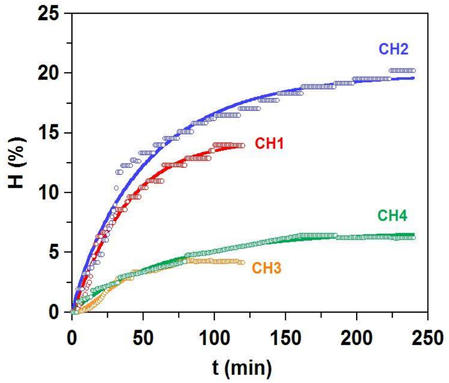
From all the procedures studied, the protocol selected was based on several sequential stages: i) NaOH treatment at 22ºC, ii) H_2SO_4 treatment at 22ºC, iii) citric acid treatment at 22ºC, iv) thermal water extraction at 45ºC of gelatin solution, v) purification and deodorisation by adsorption in activated carbón, vi) drying of gelatin sheets by forced convection oven.

	$Y_{gelatin}$ (%, w/w skin)	Gel strength (g)	Mw (kDa)	Pro+OHPro (%)
Turbot	5.2±0.1	177±6	70-210	19.4±0.5
Trout	1.6±0.2	95±2	60-210	18.2±0.6
Seabass	6.6±0.5	230±13	60-230	19.9±0.2
Seabream	6.8±0.4	181±8	70-220	20.4±0.3
Salmon	4.7±0.8	98±10	80-205	18.7±0.4

- The best **extractive yields** ($Y_{gelatin}$) were found, in the following order: **seabream**, **seabass** and **turbot**.
- Gelatins of **seabass**, **seabream** and **turbot** showed the highest **strengths of gels**.
- The largest content of Proline + OH-Proline were also obtained in gelatins of seabream, seabass and turbot.

2.2. Production of collagen and derivatives





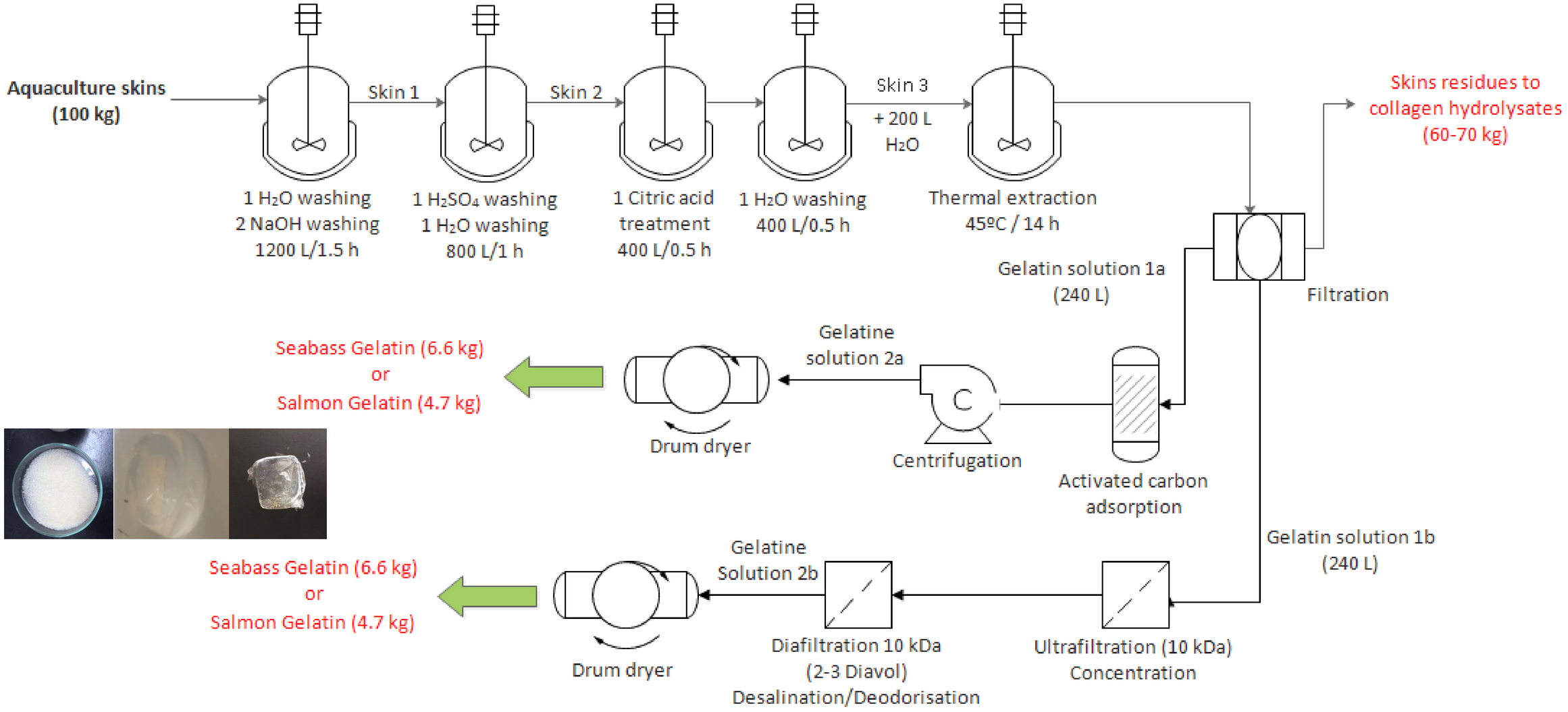
The rest of skins were digested by two proteases (Alcalase or Papain) at two times of hydrolysis (2 or 4 h) under optimal conditions for the production of **collagen hydrolysates**.

- The degree of hydrolysis (H) were perfectly modelled by Weibull equation.

Collagen Hydrolysis (CH)	Protease and hydrolysis time	H (%)	Protein (g/L)	EAA (%)	Mw (kDa)	IC_{50} (μg/mL)
CH1	Alcalase 2 h	14.3	47.6	38.7	2068	189
CH2	Alcalase 4 h	20.0	48.1	41.3	1810	71
CH3	Papain 2 h	4.3	16.2	32.3	8054	732
CH4	Papain 2 h	6.8	26.6	36.0	7587	562

- Largest values of H, soluble protein and essential amino acids were obtained in Alcalase digestions, being 4 h the best option.
- In concordance, lower molecular weights of protein were found at higher degrees of hydrolysis.
- Remarkable antihypertensive activity of collagen hydrolysates generated by Alcalase-4 h.

2.2. Production of collagen and derivatives



2.3. Production of fish peptones for microbial uses

- A **peptone** is a substrate based on proteins, composed by peptides and polipeptides of varied molecular weight, including free amino acids, and small concentration of nucleotides, carbohydrates and lipids.
- **Peptones** are water soluble, not thermally coagulable and are obtained by acid, alkaline, thermal, or enzymatic hydrolysis of raw materials, mainly by-products from animals, vegetal, or microbial origin.
- The name of peptones refers to the origin of raw materials or the enzyme used for producing them, for instance, **Gelatine peptone** is obtained from the pancreatic (pepsin or trypsin) digestion of gelatine and rich in glycine, proline and hydroxyproline, **Meat extract** is produced from bovine residues (skeletal muscle).
- Peptones are the most important and expensive source of organic nitrogen in the microbial culture media.
- **Marine/fish peptones** are peptones generated especifically from fish and seafood by-products, discards and effluents, being aquaculture peptones those obtained from fish farming wastes.

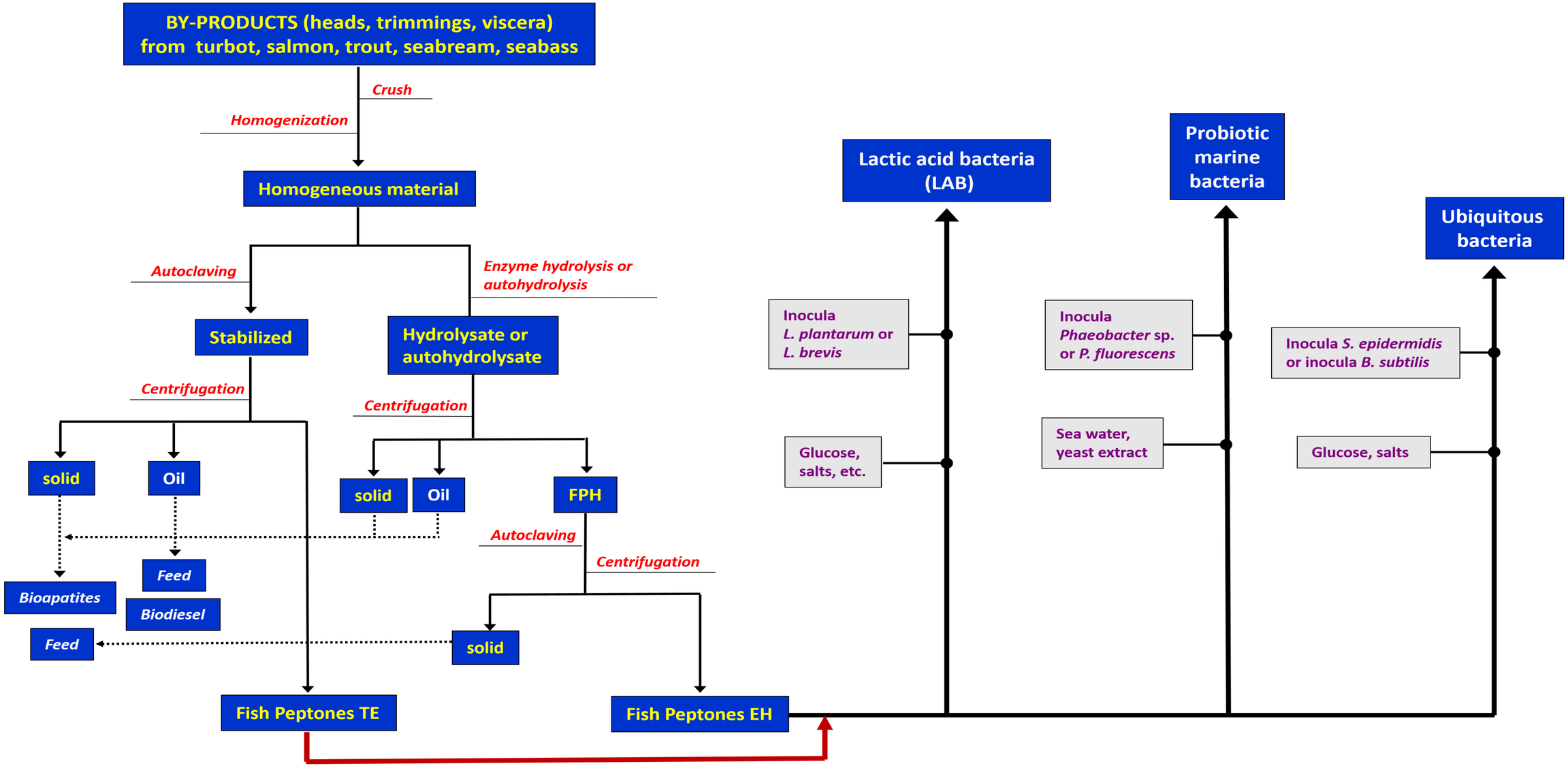
Types of peptones obtaining:

1) **By Acid/alkaline digestion/hydrolysis** of marine substrates using alkalis or acid reagents (NaOH, H_2SO_4, etc.).

2) By Enzyme digestion, using endogenous (autolysis) or exogenous proteases.

3) By Thermal extraction, when an autoclaving process is applied to a fish substrate:water mixture for protein extraction from solid to liquid phase.

2.3. Production of fish peptones for microbial uses



2.3. Production of fish peptones for microbial uses

Range of composition of fish peptones obtained from the enzyme hydrolysis of aquaculture wastes (FP_EH) and from the thermal extraction of aquaculture wates (FP_TE). Pr: soluble total protein. TS: total sugars.

	FP_EH		FP_TE	
	Pr (g/L)	TS (g/L)	Pr (g/L)	TS (g/L)
Salmon	43-70	1.3-1.5	30-48	0.6-1.2
Trout	48-54	1.1-1.4	20-36	0.5-1.4
Turbot	62-74	1.3-1.4	37-73	1.3-1.4
Seabream	38-81	0.8-1.8	19-28	0.7-1.7
Seabass	33-79	0.9-1.7	19-31	0.6-0.9

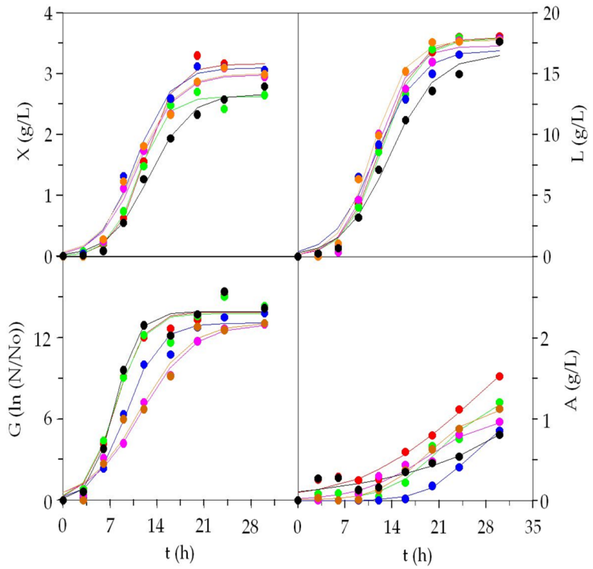
- For all species, the yield of protein recovered in peptones from enzyme hydrolysis (EH) was higher than those achivied by thermal extraction (TE).
- In all situations, the concentration of total sugars (TS) was lower than 2 g/L.
- The average molecular weights of the peptides present in EH were around 900–1800 Da with 5% of peptides larger than 3 kDa.
- In the case of TE, those values ranged mostly between 50–640 kDa since no specific protein hydrolysis was applied.

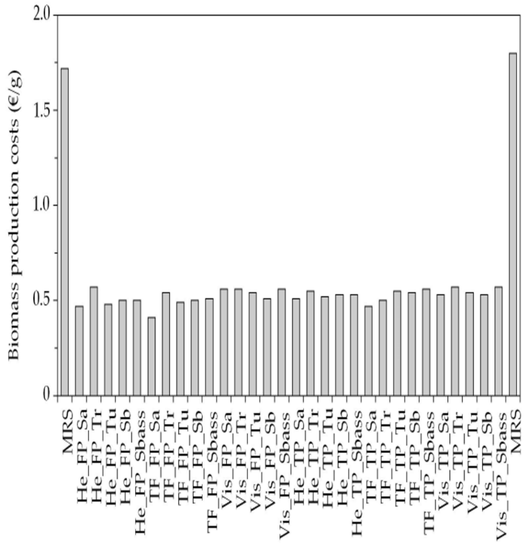
2.3. Production of fish peptones for microbial uses

Commercial MRS medium	
MEAT EXTRACT	8 g/L
BACTOPEPTONE	10 g/L
Yeast extract	4 g/L
$MnSO_4$	0.05 g/L
MgSO4	0.2 g/L
K_2HPO_4	2 g/L
Tween 80	1 g/L
Sodium acetate	5 g/L
Ammonium citrate	2 g/L
Glucose	20 g/L

MRS-fish peptone medium	
FISH PEPTONE	10-12 g/L *Lowry protein*
Yeast extract	4 g/L
$MnSO_4$	0.05 g/L
$MgSO_4$	0.2 g/L
K_2HPO_4	2 g/L
Tween 80	1 g/L
Sodium acetate	5 g/L
Ammonium citrate	2 g/L
Glucose	20 g/L

Lactobacillus plantarum





batch cultures (30°C/200 rpm/without O_2)

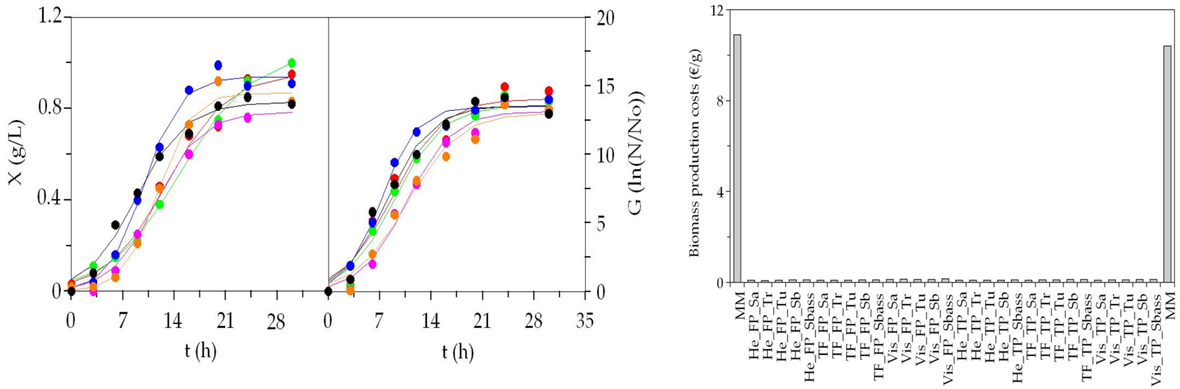
- In both lactic acid bacteria, ***Lactobacillus brevis*** and ***Lactobacillus plantarum***, similar or higher production of biomass (as dry weight) were obtained in media formulated with fish peptones, from EH or TE, than observed in commercial medium used as control (MRS).
- Similar growths (as viable cells) in all media tested including MRS.
- Similar production of lactic acid in all media tested.
- Largest production of acetic acid in medium containing trout_TE.
- Highest productive yields (growths and metabolite production per nutrient consumptions) in media formulated with salmonid peptones.
- **Reduction of production costs using fish/aquaculture peptones: 4 times in biomass, 3 times in lactic acid and 3.5 times in acetic acid.**

2.3. Production of fish peptones for microbial uses

Commercial MARINE medium (DIFCO)	
PEPTONE	5 g/L
Yeast extract	1 g/L
NaCl	19.5 g/L
$MgCl_2$	5.9 g/L
$CaCl_2$	1.8 g/L
KCl	0.55 g/L
KBr	0.08 g/L
$NaHCO_3$	0.16 g/L
Na_2SO_4	3.24 g/L
Citrato férrico	0.1 g/L
$SrCl_2$	34 mg/L
$MnSiO_4$	4 mg/L
Na_2HPO_4	8 mg/L
$(NH_4)NO_3$	1.6 mg/L
NaF	2.4 mg/L
H_3BO_3	22 mg/L

FISH-REVAL-IIM medium	
FISH PEPTONE	2.6 g/L *Lowry protein*
Yeast extract	1 g/L
Filtered seawater	

Phaeobacter sp.



batch cultures (22ºC/200 rpm/without O_2)

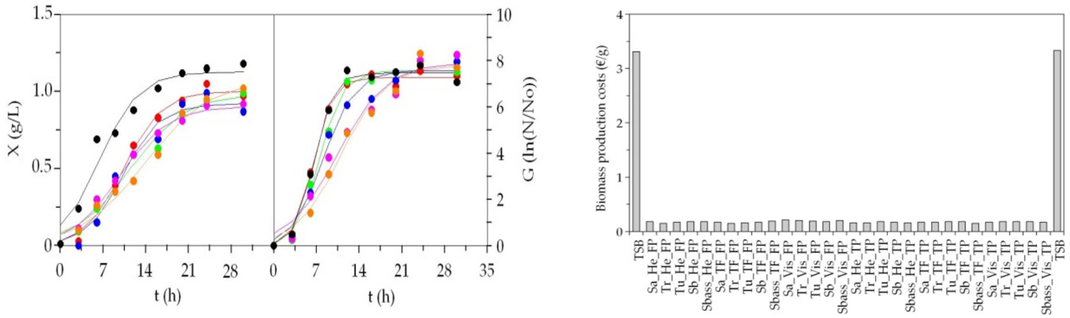
- For both marine probiotic bacteria, ***Phaeobacter sp.*** and ***Pseudomonas fluorescens***, higher or similar production of biomass (as dry weight) were obtained in media formulated with fish peptones, from EH or TE, than observed in commercial medium used as control (Marine medium).
- Similar growths (as viable cells) in all media tested including Marine medium.
- Highest growth yields (regarding protein consumption) found in commercial media. This efficiency was also greater for EH peptones than TE peptones.
- **Reduction of production costs using fish/aquaculture peptones in comparison with commercial marine medium was around 70-130 times for both types of growth quantified (biomass and cells).**

2.3. Production of fish peptones for microbial uses

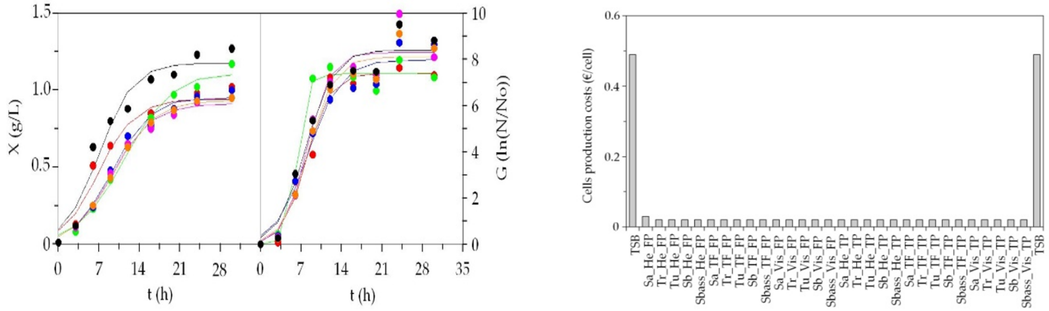
Commercial TSB medium	
CASITONE	17 g/L
SOY PEPTONE	3 g/L
NaCl	5 g/L
K_2HPO_4	2.5 g/L
Glucose	2.5 g/L

MRS-fish peptone medium	
FISH PEPTONE	11 g/L *Lowry protein*
NaCl	5 g/L
K_2HPO_4	2.5 g/L
Glucose	2.5 g/L

Bacillus subtilis



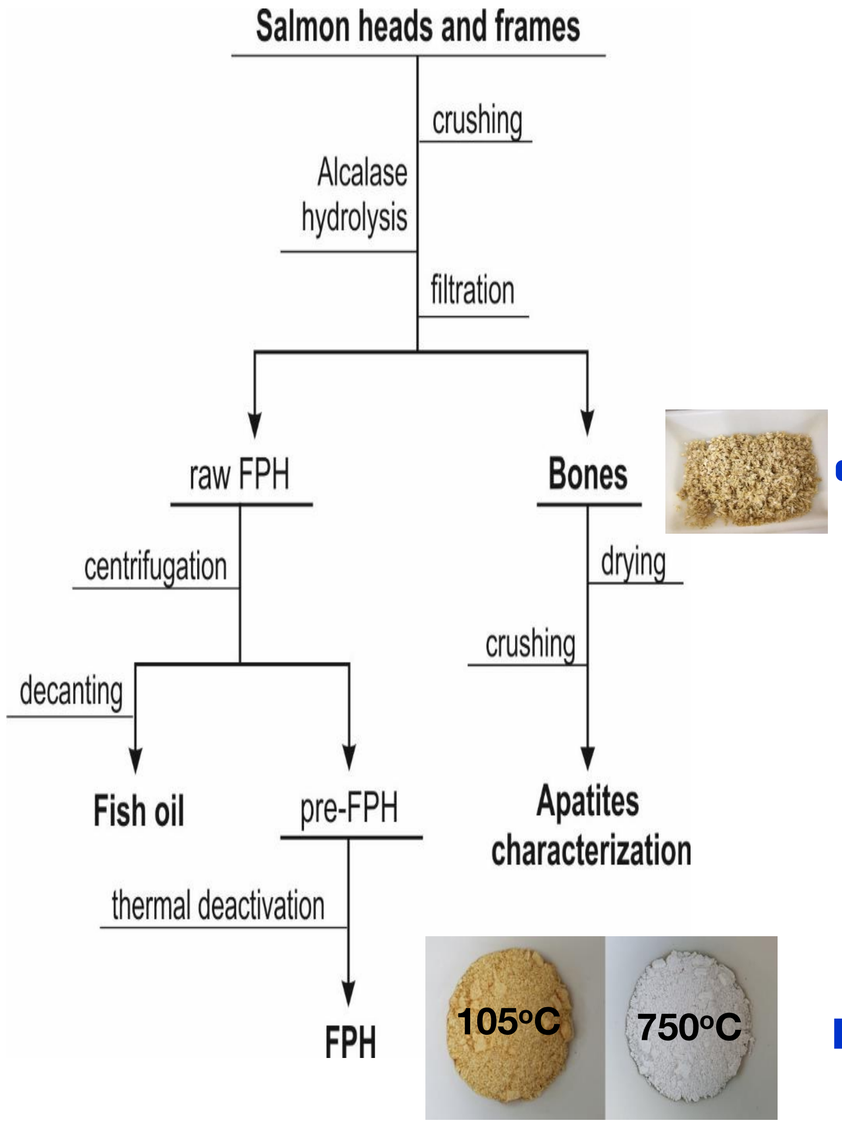
Staphylococcus epidermidis



batch cultures (30 or 37ºC/200 rpm/without O_2)

- In both ubiquitous bacteria, ***Bacillus subtilis*** and ***Staphylococcus epidermidis***, the productions of biomass (as dry weight) in alternative peptones were slightly lower than found in commercial medium used as control (TSB).
- Similar or slightly lower growths (as viable cells) were defined in fish peptone media in comparison to TSB.
- Largest growth yields (regarding glucose and protein consumption) in commercial media. This efficiency was also greater for EH peptones than TE peptones.
- **Reduction of production costs using fish/aquaculture peptones in comparison with TSB medium around 16-26 times for both types of growth quantified (biomass and cells).**

2.4. Recovery of bioapatites from fish bones



The non-digested material after alcalase hydrolysis was mainly rest of skeletons (bones) composed majoritarily by a mineral fraction – $Ca_{10}(PO_4)_6(OH)_2$ and similars– and an organic fraction (protein). The characterisitics of this protein fraction, in terms of amino acid content, for farming species were:

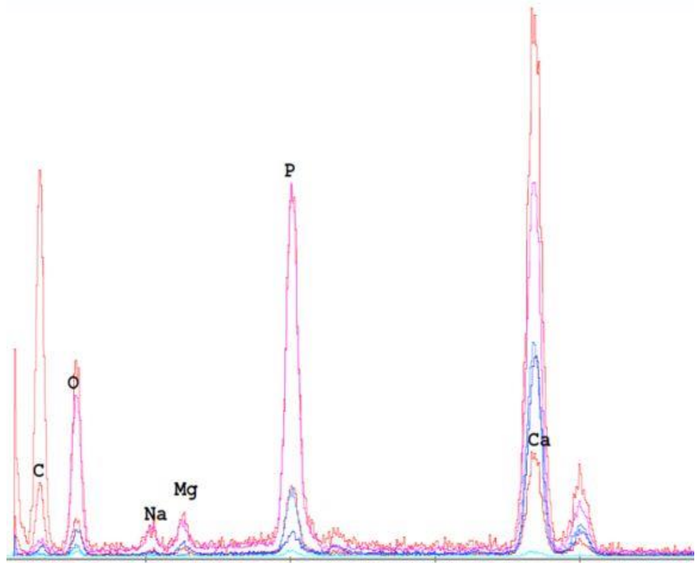
	Protein (% w/w bones)	Glycine (% w/w aa)	Proline (% w/w aa)	OH-Proline (% w/w aa)	EAA (% w/w aa)
Trout	27	21.2	8.9	9.0	26.4
Salmon	29	18.8	10.3	8.9	27.5
Turbot	21	19.5	9.5	8.0	26.9
Seabream	41	21.5	10.2	9.1	25.4
Seabass	25	18.4	10.8	9.2	26.8

- More than 21% in bones weight are protein reached 41% in seabream.
- **Glycine, Proline** and **OH-Proline** are the most abundant amino acids (aa).
- In all cases, **Proline+OH-Proline** is higher than 17%: potential source for the production of collagen.

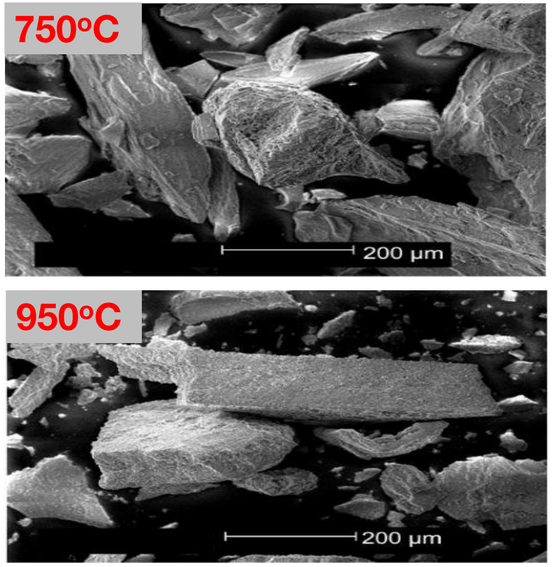
Bones were used as substrates for the production of **bioapatites**, firstly dried at 105ºC and crushed, and then calcinated at 750ºC and 950ºC.

2.4. Recovery of bioapatites from fish bones

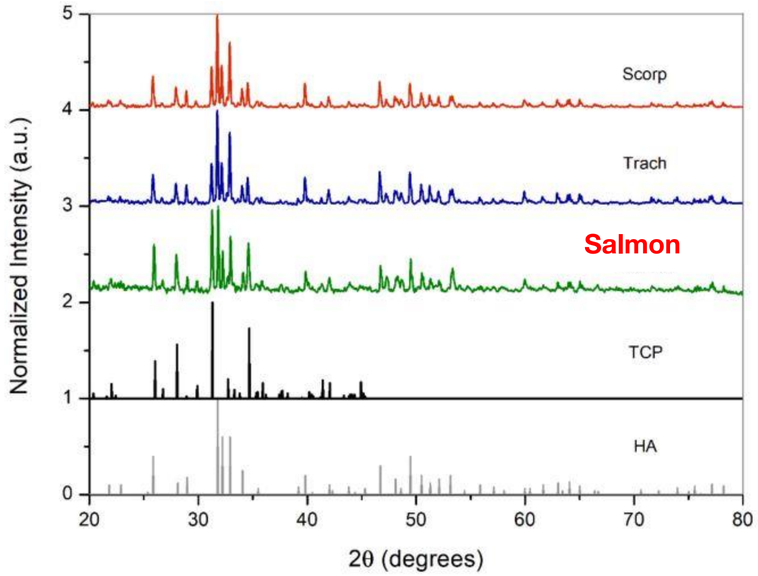
Energy dispersive spectroscopy (EDS)



Scanning electron microscopy (SEM)



X-ray powder diffraction (XRD)



- Presence of **Ca** and **P** as main mineral components in salmon samples.
- SEM images reveal **porous-shaped structures** without significant differences between the two temperatures.
- **Granules of irregular morphologies** can be also found in the powder samples of salmon bones.
- XRD patterns were compared with that of commercial synthetic hydroxyapatite (HA) and tricalcium phospate (TCP).
- This comparison indicates that even though **HA** was the **major component** of the resultant powders, there was a small amount of TCP.

2.4. Recovery of bioapatites from fish bones

Inductively coupled plasma optical emission spectroscopy (ICP-OES)

ICP-OES	Mg (ppm)	P (ppm)	K (ppm)	Ca (ppm)	Mn (ppm)	Se (ppm)	Pb (ppm)	Ca/P (molar)
Trout	4781	114230	2269	248900	41	0.65	0.07	1.68
Salmon	5109	105750	2426	227100	40	1.44	0.02	1.66
Turbot	4157	90830	1661	197980	159	0.51	0.03	1.69
Seabream	5072	121370	1784	275200	32	0.56	0.08	1.75
Seabass	5012	117340	1858	269000	38	0.87	0.09	1.77

- In all cases, the presence of **Mg** and **K** was very remarkable as well as the levels of **Se**.
- Although the table only describes the concentration of Pb present in the bioapatites, all the heavy metals analysed (Cd, Sn, Pb and Hg) showed values much lower than the maximum legal concentration.
- The **molar Ca/P ratio** was quite similar in all samples and also identical or slightly higher than that of stoichiometric HA (1.67).

In summary, we have obtained biogenic calcium phosphate (**bioapatites**) from aquaculture bones contained a biphasic material **HA-TCP** of potential application in organic **bone implants**. They are also doped with other elements as Mg, K, etc., which are very beneficial for bone metabolism and cell adhesion if aquaculture bioapatites are used in the formulation of tissue regenerative scaffolds.

It is also a substrate of potential use as **adsorbent**, ingredient of **fertilizers** or as **food supplement** rich in Ca and P.

3. Conclusions

In **GAIN project**, at the **IIM-CSIC** we have developed different strategies for the valorization of aquaculture wastes beyond the well-known fish meal and silage production:

1) The production of **fish protein hydrolysates (FPH)** of high quality –in terms of protein and amino acids contents, digestibility, molecular weight, etc.–, from different wastes of five aquaculture species has been optimized. **Fish oils** were also recovered in the same productive process.
2) Various of these FPH were also produced to pilot plant scale in order to be applied in the formulation of aquaculture feed replacing fish meal.
3) A protocol for the extraction of **native collagen soluble in acid (ACS)** from salmon and turbot skin wastes was established.
4) A sustainable process was also developed for the integral valorization of skin wastes of five aquaculture species yielding the joint production of **gelatin** (adequate for food and pharma applications) and **collagen hydrolysates** (of potential nutraceutical uses).
5) **30 fish peptones** were obtained from different fish farming species, by-products and types of production.
6) These peptones were successfully employed as organic nitrogen sources for the growth of several bacteria of technological interest. Additionally, various of those peptones were also validated for the industrial production of commercial human probiotic bacteria by a Biotech company Bialactis.
7) Clean bones recovered from the production of aquaculture FPH were studied as source of valuable **bioapatites** with potential utilization in bone tissue regeneration, Ca/P supplements, and even fertilizers.

THANKS A LOT FOR YOUR ATTENTION

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