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V1

# D3.5. Technical data sheets of the fish protein hydrolysates obtained

**Project Acronym: SEA2LAND**

**Project full name: Producing advanced bio-based fertilizers from fisheries wastes**

**Grant Agreement No. 101000402**

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# Document Summary

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**Deliverable Lead:** AZTI

**Related Work package:** WP3 Recovering nutrients from fishery and fish processing industry by products

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☒ PU, Public

☐ PP, restricted to other programme participants (including the Commission Services)

☐ RE, restricted to a group specified by the consortium (including the Commission Services)

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**APPROVED BY:** Miriam Pinto (project coordinator, NEIKER), Carlos Bald (WP3 leader, AZTI) (before submission to the EU).

**Grant Agreement Number:** 101000402

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**Project coordinator:** NEIKER-INSTITUTO VASCO DE INVESTIGACION Y DESARROLLO AGRARIO SA

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# Abstract

Within H2020 in SEA2LAND project, and more specifically in WP3, side-streams from fishery and fish and shellfish processing by-products or side-streams will be used as raw materials to produce intermediate products suitable for being formulated into biobased fertilisers. The present document describes the technical specifications of the protein hydrolysates developed at pilot scale that have been obtained from fish viscera from fish processing industries in the Cantabrian Sea pilot study and the hydrolysates from the organic fraction of discarded molluscs combined with fish processing by-products in the Adriatic case study. Two procedures have been hereby employed wit: enzymatic hydrolysis and acid autolysis (fish viscera).


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# 1 Concentrated hydrolysate obtained from enzymatic hydrolysis of rainbow trout viscera

## 1.1 Product description

Concentrated hydrolysate obtained from enzymatic hydrolysis of rainbow trout viscera. Brown colour and molasse texture.

## 1.2 Applications

Intermediate product for the formulation of foliar biofertilisers.

## 1.3 Product composition

### 1.3.1 General composition and metals

| Composition | Units | Result |
|---|---|---|
| pH | - | 6.39 |
| Conductivity | mS/cm | 3.72 |
| Dry matter | % | 52.5 ± 0.5 |
| Total nitrogen | % | 5 |
| Ammonia N | % | 1 |
| Free amino acids | % | 19.5 ± 0.02 |
| Protein | % | 31.4 ± 0.04 |
| Ash | % | 4.1 ± 0.03 |
| $K_2O$ | % | 0.85 |
| Na | % | 1.45 |
| $P_2O_5$ | % | 1.39 |
| $SO_3$ | % | 1.91 |
| Cd | ppm | 0.27 |
| Ni | ppm | 4.04 |
| Cu | ppm | 58.23 |
| Cr | ppm | 4.13 |
| Zn | ppm | 320.37 |
| Hg | ppm | 0.39 |
| Pb | ppm | n.d. |

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### 1.3.2 Amino acid profile

| Amino acid profile | Unit | Free AA |
|---|---|---|
| Thr | % | n.d. |
| Cys | % | 0.01 ± 0.0003 |
| Tyr | % | 1.7 ± 0.03 |
| Val | % | 1.8 ± 0.02 |
| Met | % | 0.9 ± 0.01 |
| Lys | % | 1.7 ± 0.2 |
| Ile | % | 1.4 ± 0.02 |
| Leu | % | 2.2 ± 0.02 |
| Phe | % | 1.2 ± 0.009 |
| Arg | % | 0.6 ± 0.009 |
| Ala | % | 1.9 ± 0.02 |
| Asp | % | 0.7 ± 0.001 |
| Ser | % | 0.02 ± 0.006 |
| Glu | % | 2.3 ± 0.02 |
| Gly | % | 1.1 ± 0.004 |
| Asn | % | 0.5 ± 0.007 |
| His | % | 0.6 ± 0.01 |

### 1.3.3 Molecular size profile

| MW range (Da) | % |
|---|---|
| 50000-27000 | 0.0 |
| 27000-10000 | 0.1 |
| 10000-6500 | 0.2 |
| 6500-3000 | 1.3 |
| 3000-2000 | 1.4 |
| 2000-1000 | 5.0 |
| 1000-500 | 21.2 |
| 500-300 | 6.6 |
| 300-100 | 22.9 |
| 100-1 | 40.1 |

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### 1.3.4 Microbiology

| Species | Concentration |
|---|---|
| Salmonella | Not detected |
| Faecal coliforms | <10 UFC/g |
| E. coli | <10 UFC/g |

## 1.4 References

### 1.4.1 Analytical methods

The proximate composition of the samples was analysed according to the Association of Official Analytical Chemists (AOAC) Official Methods (2007):

Protein content: Kjeldahl nitrogen x 5.7 for fish (method 955.04).

Ammoniacal N: Kjeldahl method based on UNE-EN 15475

Ash: calcination in furnace overnight at 550 °C (method 942.05).

Dry matter: drying samples at 105ºC until constant weight (method 934.01).

Amino acids were determined by hydrolysis with HCl 6N 110ºC 24h, neutralization with NaOH 6N derivatization separation on a reverse-phase column Poroshell HPH-C18, 4.6 x 100 mm, 2.7 µm in a HPLC 1100 series (Agilent Technologies, USA) with a Diode Array Detector (DAD) at excitation and emission wavelengths of 338 and 390 nm for primary and 262 and 324 nm for secondary amino acids, respectively. Free amino acids are determined previous extraction with HCl 0.6 N.

Hg, Pb, As, Cd, Ni, Cu, Cr and Zn: graphite furnace atomic absorption spectroscopy (GFAAS) previous calcination and acid extraction (AOAC, 999.11).

K: flame Atomic Absorption Spectrometry previous calcination in furnace (AOAC, 969.23).

Total P: spectrophotometric method with molybdovanadate reagent previous calcination (CE 152/2009).

Total S: Determined by ICP-OES after acidic digestion.

Conductivity: conductivity meter after water extraction (ratio 1/5 w/w) (EN 13038, 2011).

Molecular size profile : *Size exclusion high performance liquid chromatography (SEC-HPLC)*.

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Salmonella spp: real time PCR with iQ-Check™ Salmonella II kit from BIO-RAD and iQ-Check™ Enterobacteriaceae Count Plate (AFNOR 3M 01/06-09/97) and Escherichia coli in 3MTM PETRIFILMTM Select E. coli Count Plate (SEC) (AFNOR 3M 01/08-06/01).

### 1.4.2 Applicable legislation

Regulation of the European Parliament and of the Council of 5 June 2019 laying down rules on the making available on the market of EU fertilising products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003

Royal Decree RD 506/2013, of 28 June, on fertilizer products. Spanish Ministry of Presidency.

### 1.4.3 Product proprietary

AZTI Fundazioa/AZTI Foundation
Bizkaiko Zientzia eta Teknologia Parkea, Astondo bidea E609, 48160 Derio (Bizkaia) Spain
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# 2 Concentrated hydrolysate obtained from silage of rainbow trout viscera

## 2.1 Product description

Concentrated hydrolysate obtained from acid autolysis of rainbow trout viscera. Brown colour and molasse texture.

## 2.2 Applications

Intermediate product for the formulation of foliar biofertilisers.

## 2.3 Product composition

### 2.3.1 General and metal composition

| Composition | Units | Result |
|---|---|---|
| pH | - | 4.69 |
| Conductivity | mS/cm | 28.7 |
| Dry matter | % | 36.3 ± 0.5 |
| Total nitrogen | % | 4.3 |
| Ammonia N | % | 0.54 |
| Free amino acids | % | 12.5 ± 0.2 |
| Protein | % | 26.5 ± 0.03 |
| Ash | % | 2.1 ± 0.1 |
| $P_2O_5$ | % | 0.98 |
| $K_2O$ | % | 0.9 |
| Na | % | 0.44 |
| $SO_3$ | % | 1.37 |
| Ni | ppm | 22.74 |
| Pb | ppm | 4.39 |
| Cu | ppm | 36.71 |
| Cr | ppm | 31.65 |
| Zn | ppm | 233.80 |
| Hg | ppm | n.d |
| Cd | ppm | n.d |

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### 2.3.2 Amino acid profile

| Amino acid profile | Unit | Free AA |
|---|---|---|
| Thr | % | 0.81 ± 0.005 |
| Cys | % | 0.1 ± 0.002 |
| Tyr | % | 0.2 ± 0.001 |
| Val | % | 0.9 ± 0.0 2 |
| Met | % | 0.5 ± 0.008 |
| Lys | % | 0.7 ± 0.04 |
| Ile | % | 0.8 ± 0.02 |
| Leu | % | 1.7 ± 0.04 |
| Phe | % | 0.6 ± 0.01 |
| Arg | % | 0.4 ± 0.02 |
| Ala | % | 0.6 ± 0.01 |
| Asp | % | 0.5 ± 0.008 |
| Ser | % | 0.8 ± 0.009 |
| Glu | % | 0.9 ± 0.01 |
| Gly | % | 0.8 ± 0.02 |
| Pro | % | 0.4 ± 0.02 |
| His | % | 0.34 ± 0.004 |

### 2.3.4 Microbiology

| Species | Concentration |
|---|---|
| Salmonella | Not detected |
| Faecal coli | <10 UFC/g |
| E. coli | <10 UFC/g |

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## 2.4 References

### 2.4.1 Analytical methods

The proximate composition of the samples was analysed according to the Association of Official Analytical Chemists (AOAC) Official Methods (2007).

Protein content: Kjeldahl nitrogen x 5.7 for fish (method 955.04).

Ammoniacal N: Kjeldahl method based on UNE-EN 15475

Ash: calcination in furnace overnight at 550 °C (method 942.05).

Dry matter: drying samples at 105°C until constant weight (method 934.01).

Amino acids were determined by hydrolysis with HCl 6N 110°C 24h, neutralization with NaOH 6N derivatization separation on a reverse-phase column Poroshell HPH-C18, 4.6 x 100 mm, 2.7 µm in a HPLC 1100 series (Agilent Technologies, USA) with a Diode Array Detector (DAD) at excitation and emission wavelengths of 338 and 390 nm for primary and 262 and 324 nm for secondary amino acids, respectively. Free amino acids are determined previous extraction with HCl 0.6 N.

Hg, Pb, As, Cd, Ni, Cu, Cr and Zn: graphite furnace atomic absorption spectroscopy (GFAAS) previous calcination and acid extraction (AOAC, 999.11).

K: flame Atomic Absorption Spectrometry previous calcination in furnace (AOAC, 969.23).

Total P: spectrophotometric method with molybdovanadate reagent previous calcination (CE 152/2009).

Total S: Determined by ICP-OES after acidic digestion.

Conductivity: conductivity meter after water extraction (ratio 1/5 w/w) (EN 13038, 2011)

Molecular size profile : *Size exclusion high performance liquid chromatography (SEC-HPLC)*.

Salmonella spp: real time PCR with iQ-Check™ Salmonella II kit from BIO-RAD and iQ-Check™ Enterobacteriaceae Count Plate (AFNOR 3M 01/06-09/97) and Escherichia coli in 3MTM PETRIFILMTM Select E. coli Count Plate (SEC) (AFNOR 3M 01/08-06/01).

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### 2.4.2 Applicable legislation

Regulation of the European Parliament and of the Council of 5 June 2019 laying down rules on the making available on the market of EU fertilising products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003

Royal Decree RD 506/2013, of 28 June, on fertilizer products. Spanish Ministry of Presidency

### 2.4.3 Product proprietary

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# 3 Concentrated hydrolysate obtained from enzymatic hydrolysis of mollusc and fish by-products

## 3.1 Product description

Concentrated hydrolysate obtained from enzymatic hydrolysis of the organic fraction of discarded molluscs and fish processing by-products. Dark brown colour and molasse texture.

## 3.2 Applications

Intermediate product for the formulation of foliar biofertilisers.

## 3.3 Product composition

### 3.3.1 General composition and metals

| Composition | Units | Result |
|---|---|---|
| pH | - | 5.78 ± 0.01 |
| Conductivity | mS/cm | 21.19 ± 0.23 |
| Dry matter | % | 42.12 ± 1.71 |
| Total nitrogen | % | 4.82 ± 0.17 |
| Ammonia N | % | 1.09 ± 0.01 |
| Free amino acids | % | 3.5 ± 0.17 |
| Total amino acids | % | 13 ± 0.17 |
| Ash | % | 10.15 ± 0.21 |
| $K_2O$ | % | 0.73 ± 0.01 |
| Na | % | 0.97 ± 0.04 |
| $P_2O_5$ | % | 0.74 ± 0.02 |
| $SO_4$ | % | 1.32 ± 0.02 |
| Cd | ppm | n.d. |
| Ni | ppm | n.d. |
| Cu | ppm | 62.55 ± 2.90 |
| Cr | ppm | n.d. |
| Zn | ppm | 269.92 ± 2.96 |
| Hg | ppm | 0.15 ± 0.00 |
| Pb | ppm | n.d. |

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### 3.3.2 Amino acid profile

| Amino acid profile | Unit | Total AA | Free AA |
|---|---|---|---|
| Thr | % | n.d. | 0.0076± 0 |
| Cys | % | 0.509 ± 0.003 (CysH) | 0.142 ± 0.001 (Cys) |
| Tyr | % | 3.59 ± 0.004 | 0.729 ± 0.001 |
| Val | % | 0.474 ± 0.001 | 0.17 ± 0 |
| Met | % | n.d. | 0.089 ± 0.001 |
| Lys | % | 0.418 ± 0.003 | 0.097 ± 0.003 |
| Ile | % | 1.07 ± 0.001 | 0.102 ± 0.001 |
| Leu | % | 1.07 ± 0.006 | 0.245 ± 0 |
| Phe | % | 0.518 ± 0 | 0.181 ± 0.001 |
| Arg | % | 0.1 ± 0.002 | 0.018 ± 0 |
| Ala | % | 0.672 ± 0.001 | 0.409 ± 0.001 |
| Asp | % | 0.243 ± 0 | 0.02 ± 0.002 |
| Ser | % | 0.172 ± 0.001 | 0.013 ± 0.002 |
| Glu | % | 2.24 ± 0.007 | 0.296 ± 0.001 |
| Gly | % | 2.27 ± 0.04 | 0.689 ± 0.002 |
| Pro | % | 0.172 ± 0 | 0.013 ± 0 |
| His | % | 0.139 ± 0 | 0.039 ± 0.001 |
| Trp | % | 0.14 ± 0.022 | 0.271 ± 0.004 |

### 3.3.3 Molecular size profile

| MW kDa | % |
|---|---|
| >6.7 | 45% |
| 6.7-1.7 | 24% |
| 1.7-1 | 13% |
| < 1 | 18% |

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### 3.3.4 Microbiology

| Species | Concentration |
|---|---|
| Salmonella | Not detected |
| Faecal coliforms | <10 UFC/g |
| E. coli | <10 UFC/g |

## 3.4 References

### 3.4.1 Analytical methods

The proximate composition of the samples was analysed according to the Association of Official Analytical Chemists (AOAC) Official Methods (2007):

Ammoniacal N: Kjeldahl method based on UNE-EN 15475

Ash: calcination in furnace overnight at 550 °C (method 942.05).

Dry matter: drying samples at 105°C until constant weight (method 934.01).

Amino acids were determined applying some modifications to AOAC – Official method 994.12, 1997(with HPLC-DAD technique). Acidic hydrolysis of the sample was used for the determination of lysine (Lys), histidine (His), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), Valine (Val), threonine (Thr), arginine (Arg), Alanine (Ala), glycine (Gly), proline (Pro), glutamic acid (Glu), serine (Ser), aspartic acid (Asp), tyrosine (Tyr). Samples were hydrolyzed in 6M HCl for 24 h in water bath at 100°C, followed by neutralization with NaOH. For the determination of sulfur amino acids (Met and Cys), the samples were pre-treated with 1 mL of a mixture of 30% (v/v) hydrogen peroxide and 98% (v/v) formic acid (in the ratio of 1:9 v/v) and were subsequently hydrolyzed in the way mentioned above. For tryptophan determination an alkaline hydrolysis was performed: the sample was hydrolyzed with NaOH 4.2 N for 16 h under N2 flux, and neutralized with HCl. For free amino acids determination, the sample was diluted in borate buffer before derivatization. The HPLC analyses (Agilent 1100 Series HPLC) were performed by automated online pre-column derivatization using an automated liquid sampler and Poroshell 120 column HPH-C18 (3.0 100 mm, 2.7 lm. P/N 695975–502). The standard preparation, derivatization process, LC method used was performed according to Agilent Pub. #5990-4547EN (Pub No. 5990-4547EN, October 8, 2009, Agilent Technologies). The primary amino acids (OPA-derivatized) were monitored at 338 nm. The secondary amino acids (FMOC- derivatized) were monitored at 262 nm. The separation was carried out under gradient elution with two mobile phases. Phase A: 10

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mMol $L^{-1}$ NaH2PO4 + 10mMol $L^{-1}$ Na2B4O7 + 5 mM NaN3, pH 8.2 adjusted with HCl 5 Mol $L^{-1}$, and Phase B: ACN:MeOH:water (45:45:10, v/v/v). The flow rate was 1.00 mL $min^{-1}$, the column temperature 40 °C and injection volume 20µL.hydrolysis with HCl 6N 110ºC 24h, neutralization with NaOH 6N derivatization separation on a reverse-phase column Poroshell HPH-C18, 4.6 x 100 mm, 2.7 µm in a HPLC 1100 series (Agilent Technologies, USA) with a Diode Array Detector (DAD) at excitation and emission wavelengths of 338 and 390 nm for primary and 262 and 324 nm for secondary amino acids, respectively. Free amino acids are determined previous extraction with HCl 0.6 N.

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Total S: Determined by ICP-OES after acidic digestion.

Conductivity: conductivity meter after water extraction (ratio 1/5 w/w) (EN 13038, 2011).

Molecular size profile : *Size exclusion high performance liquid chromatography (SEC-HPLC)*.

Salmonella spp: real time PCR with iQ-Check™ Salmonella II kit from BIO-RAD and iQ-Check™ Enterobacteriaceae Count Plate (AFNOR 3M 01/06-09/97) and Escherichia coli in 3MTM PETRIFILMTM Select E. coli Count Plate (SEC) (AFNOR 3M 01/08-06/01).

### 3.4.2 Applicable legislation

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Italian Legislation D.Lgs 75/2010, of 29 April, on fertilizer products. Italian Ministry of Presidency.

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### 3.4.3 Product proprietary

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# SEA2LAND

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