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Use of *Yarrowia lipolytica* to Obtain Fish Waste Functional Hydrolysates Rich in Flavoring Compounds

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Abstract: Fishery processing industries generate large amounts of by-products. These by-products come from fish heads, skin, bones, thorns, and viscera. The disposal of these wastes represents an increasing environmental and health problem. Nowadays, there is a growing interest in how to utilize fish materials that are not used for human consumption. Among the different solutions proposed, the use of proteolytic and lipolytic microorganisms represents a green solution for waste valorization. In this work, first we screened several conventional and non-conventional microorganisms for their proteolytic and lipolytic functions. Then, the most promising strains (*Yarrowia lipolytica* YL2, *Y. lipolytica* YL4, *Bacillus amyloliquefaciens* B5M and *B. subtilis* B5C) were tested on a fish waste-based solution. After 72 h incubation at room temperature, the supernatants obtained using the strains of *Y. lipolytica* showed the highest degree of hydrolysis (10.03 and 11.80%, respectively, for YL2 and YL4), the strongest antioxidant activity (86.4% in DPPH assay for YL2) and the highest formation of aldehydes (above 50% of the total volatile compounds detected). Hydrolysates of fish waste obtained with *Y. lipolytica* may be reused in feed and food formulations for their functional and flavoring characteristics.

Keywords: fish waste; *Yarrowia lipolytica*; functional hydrolysate; flavoring compounds

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1. Introduction

Fishing production (extractive and aquaculture) reached a volume of 214 million tons in 2020 and generates large amounts of waste and by-products every year. These include fish heads, viscera, bones, and scales, which may represent up to 70% of processed fish, depending on the size, species, and type of processing. Fish by-products and waste are not put on the market due to low consumer acceptance, natural degradation, or safety reasons. Therefore, they have been usually used for silage, production of fertilizer, or simply discarded, with all the environmental issues resulting from it [1]. In the last two decades, fish waste and by-products received attention as important sources of nutritional compounds [2]. Their composition depends on several factors, such as species, age, nutritional status, sex, season, and health. However, a range of 15–30% is represented by protein followed by fats (0–25%) and moisture (50–80%) [3,4].

Fish proteins have a well-balanced amino acid composition compared to other animal protein sources. Their hydrolysates possess functional properties (antihypertensive, antimicrobial, immune modulatory and antioxidant) [3] and can have several applications in the pharmaceutical, human nutrition, animal nutrition, and cosmetic sectors. Fish hydrolysates are also useful as a nitrogen source in growth media for microorganisms [5]. Fish oils, the second most abundant component, can be extracted and used as food supplements in human diet due to their large quantity of long-chain PUFAs [4]. Alternatively defatting processes are required to obtain higher value ingredients for fish meal [6]. Finally, lipids have been proposed to be used in culture media to induce the production of microbial lipases [7].

Biotechnological approaches represent a promising tool for fish waste valorization [3]. In fact, the use of selected microorganisms that can perform enzymatic activities in situ represents a "greener" and more sustainable approach to obtain several functional compounds [4,5,8,9]. Compared to the use of pure enzymes, fermentation processes may enrich the final functionality and sensorial characteristics of the product [10,11]. For instance, microbial lipolysis and proteolysis can generate free fatty acids and amino acids, respectively, which are precursors of volatile compounds [12]. Therefore, other than the type of substrate (fish species and waste components) [13], the selection of microbial species and strains can impact the quality of the final product and its features [11,14]. Among the most hydrolytic bacteria, the genus *Bacillus* has been widely used for fish waste valorization through the production of protein hydrolysates [15,16], enzymes [17–19], polyhydroxyalkanoate [20] and biosurfactant [21]. For instance, Alcalase, an endoprotease extracted from *B. subtilis*, is widely used to produce fish protein hydrolysates with high solubility and digestibility. Among the yeasts, *Yarrowia lipolytica* has been proposed as a promising tool to valorize agro-food wastes due to its enzymatic functions [22]. Most of the studies performed with *Y. lipolytica* and fish wastes regard the production of single cell oil, lipases, yeast biomasses, or lipid reduction [6,23–25]. However, *Y. lipolytica* also possess proteases that play an important role in food ripening, such as in cheese and fermented sausages [22]. The use of this yeast to obtain fish waste protein hydrolysates has never been explored.

The present work was focused on the selection of proteolytic and lipolytic microorganisms for the valorization of fish waste into functional and flavoring hydrolysates, to be potentially re-used in the food sector. Together with the well-documented Bacilli, the unexplored yeast *Y. lipolytica* was also considered. The most promising strains were then tested in a fish waste solution and the peptide content, functional activity (antioxidant) and volatile molecule compounds were evaluated on the hydrolysates.

2. Materials and Methods

2.1. Microorganisms

All the 8 strains of *Yarrowia lipolytica* (YL1, YL2, YL3, YL4, YL5, YL6, YL7, YL8) and 7 bacteria (*Bacillus subtilis* B5C, B12C, B15C, B28C, B46C, B47C, *B. licheniformis* B1M, and *B. amyloliquefaciens* B5M) applied in this work belong to the culture collection of the Department of Food Science, Alma Mater Studiorum—University of Bologna (Italy) and were isolated from different environments such as: rivers, dairy products, and wine lees. Yeasts were cultured in yeast extract peptone dextrose (YPD) broth for 48–72 h at 30 °C, while the remaining bacteria were grown in brain heart infusion (BHI) broth at 37 °C for 24 h. Microorganisms were refreshed twice in their respective media and then centrifuged at 5000 rpm for 10 min. The pellets were resuspended in the same volume of saline solution (NaCl, 9 g/L) before their use to assess enzymatic properties and perform fermentation of fish waste solution.

2.2. Screening for Enzymatic Properties

2.2.1. Proteolytic Property

Protease activity was determined in different ways. First, 2% skim milk agar (skim milk 2%, agar 1.7%) was spotted with 20 µL of each microorganism and incubated at room temperature for 24 h. A clearance zone around the inoculated site was considered a positive result of proteolytic activity and the diameters were measured by subtracting them from those of the cultures. Second, gelatin solution (gelatin 12%, glucose 1%, peptone 0.5%) was introduced in falcon tubes and inoculated with 50 µL of the different strains. Incubation was performed for 72 h at room temperature and then samples were stored at 4 °C for 30 min to allow the undegraded gelatin to solidify. Samples were compared with uninoculated ones. Positive gelatinase activity was considered if the samples remained liquid. All the trials were performed in triplicate (n = 3).

2.2.2. Lipolytic Property

For the lipolytic property, microorganisms were spotted on top of Spirit Blue agar (Sigma-Aldrich, St. Louis, MO, USA) supplemented with the lipase reagent (15 mL) (Sigma-Aldrich, Milan, Italy). Plates were incubated at room temperature and checked after 72 h. Effective lipolysis was determined by halo formation [26]. The diameters were measured by subtracting them from those of the cultures. The trial was performed in triplicate (n = 3).

2.2.3. Protease Activity

Protease activity of selected strains after 72 h growth was measured as described by Sigma's non-specific protease activity assay using casein as a substrate [27]. The protease activity of samples was expressed as Units/mL, in which one Unit is defined as the amount of tyrosine (µmol) equivalent released from casein per minute at 37 °C and pH 7.5. The trial was performed in triplicate (n = 3).

2.3. Production of Fish Waste Hydrolysates

2.3.1. Fish Waste

A mix of fish waste containing head, viscera, skin and bones of anchovies (*Engraulis encrasicolus*), exoskeleton, raptorial claws and telson of mantis shrimps (*Squilla mantis*) and heads of rose shrimps (*Parapenaeus longirostris*) were provided fresh by Ecopesce SRL (Cesenatico, Italy). Samples were brought to the lab, homogenized and stored at −20 °C until their use.

2.3.2. Fermentation Process

10 g of fish waste were thawed and placed in a sterile falcon with 10 mL of sterile distilled water (3% glucose, 4% salt). Then, the selected microorganisms were inoculated with an initial inoculum level of 1% into the fish waste solution. A sample without any added inoculum was used as a control. The fermentation was carried out at 37 °C (for bacteria) or RT (for yeasts) for 72 h. The trial was performed in triplicate (n = 3). An aliquot of the samples was taken for microbial quantification. The remaining hydrolysates were filtered with a piece of gauze, and then centrifuged at 4000 rpm for 10 min. The supernatant was collected and stored at −20 °C. Half of it was used as such in liquid form, while the other half was lyophilized for 96 h using a Drywinner Heto freeze-dryer (Cambridge Biosystems, Cambridge, UK).

2.4. Microbiological Analyses

1 mL of the sample was taken, and serial decimal dilution was performed. After that, all the dilutions were plated with specific media (Oxoid): Yeast extract Peptone Dextrose (YPD) agar with chloramphenicol for the enumeration of yeasts, Brain Heart Infusion (BHI) with cycloheximide to quantify bacilli, Violet Red lactose Bile agar (VRBA) for the enumeration of coliforms and De Man, Rogosa and Sharpe (MRS) agar for lactic acid bacteria. Plates of YPD were incubated for 48–72 h at 30 °C VRBA and BHI at 37 °C for 24 h, MRS at 37 °C for 48 h. Colony-forming units were enumerated and then used to calculate the microbial concentration.

2.5. Protein Analyses

Protein Concentration Assay, Peptide Content and Degree of Hydrolysis (DH)

The protein concentration was measured in the soluble fraction following the Bradford protein assay kit instructions (Bio-Rad; Hercules, CA, USA). The protein calculation was based on a standard curve using bovine serum albumin and expressed as a milligram per milliliter (mg/mL). The peptide content was estimated according to the OPA method, as stated by Church et al. [28], by using serine as a standard for hydrolysis determination. The degree of hydrolysis (DH%) was determined according to the procedure described by Nielsen et al. and Hong et al. [29,30]. All the analyses are the results of three independent replicates (n = 3).

2.6. Antioxidant Activity

2.6.1. DPPH Assay

DPPH radical scavenging activity was determined according to the method from Molyneux [31] with slight modifications. A 100 µL aliquot of fresh hydrolysate (or a solution of 1 mg/mL of lyophilized sample), and ascorbic acid as a positive control, were mixed with 2.9 mL of 0.1 mM DPPH in methanol. The mixtures were incubated in the dark for 30 min and the absorbance was measured at 517 nm. For this method, the free radical scavenging activity was calculated by the following equation:

$$DPPH\ \%\ inhibition\ = \left(\frac{Abs\ control - Abs\ sample}{Abs\ control}\right) \times 100$$

where *Abs control* is the absorbance of DPPH solution except the sample and *Abs sample* is the absorbance of the tested sample with DPPH solution. Antioxidant capacity relative to that of ascorbic acid was calculated using the equation generated with an ascorbic acid standard curve as follows:

$$Ascorbic\ acid\ equivalent\ \left(\frac{mg}{mg}\right)\ = \left(\frac{(DPPH\ inhibition\ \% - b)}{a}\right) \div sample\ concentration.$$

where *a* and *b* are the two coefficients of the ascorbic acid standard curve. Results were reported as the mean of three independent reads (n = 3).

2.6.2. ABTS Assay

The ABTS radical scavenging activity was determined by the decolorization assay described by Re [32] with some modifications. Briefly, the working solution of ABTS was prepared by mixing 7.4 mM ABTS stock solution and 2.6 mM potassium persulfate solution in equal quantities and allowing them to react overnight at room temperature in the dark. Then, the working solution was diluted with distilled water to achieve an absorbance of 1.10 ± 0.02 at 734 nm. After that, 2.85 mL of working solution were added to 150 µL of fresh hydrolysates or a solution of 1 mg/mL of lyophilized sample. The mixture was incubated in the dark for 120 min and absorbance was measured at 734 nm. Trolox was used as a positive control. The percentage inhibition of ABTS+ to ABTS was calculated using the following equation:

$$ABTS\ decoloration\ (\%)\ = \left(\frac{Abs\ control - Abs\ sample}{Abs\ control}\right) \times 100$$

where *Abs sample* is the absorbance of the tested sample with the ABTS solution, while *Abs control* is the absorbance of ABTS solution without sample. Antioxidant capacity relative to that of Trolox was calculated using the equation generated with a Trolox standard curve as follows:

$$Trolox\ equivalent\ \left(\frac{mg}{mg}\right)\ = \left(\frac{(ABTS\ decoloration\ \% - b)}{a}\right) \div sample\ concentration.$$

where *a* and *b* are the two coefficients of the Trolox standard curve. To estimate the µmol Trolox/g of product, the molecular weight of Trolox was used (25,029 g/mol). Results were reported as the mean of three independent reads (n = 3).

2.7. Volatile Molecule Profile

The volatile molecule profiles were detected with SPME/GC-MS technique. A DVB/CAR/PDMS fiber (SUPELCO, Bellafonte, PA, USA) was used to perform the Solid phase microextraction (SPME). The samples (3 g) were placed in vials and incubated for 10 min at 45 °C. Then, the fiber was exposed to the vial headspace for 30 min at 45 °C. The volatile molecules adsorbed were desorbed in the gas chromatograph (GC) injector port in splitless

mode at 250 °C for 10 min. The headspace of the volatile compounds was analyzed using Gas-Chromatography (GC) 7890A, Network GC System with mass spectrometry (MS) 5975C (Agilent Hewlett–Packard, Geneva, Switzerland). The column used was J&W CP-Wax 52 CB (50 m × 320 µm × 1.2 µm). The initial temperature was 40 °C for 1 min and then increased by 4.5 °C/min up to 65 °C. After that, the temperature increased by 10 °C/min up to 230 °C and remained at this temperature for 17 min. Compounds were identified by comparison based on NIST 11 (National Institute of Standards and Technology) database. Gas carrier was helium at 1.0 mL/min flow.

2.8. Statistical Analysis

The significance of data (peptide content, degree of hydrolysis, antioxidant activity) was evaluated using ANOVA followed by Tukey HSD Post-Hoc Test at $p < 0.05$ performed with Statistica software (v. 8.0; StatSoft, Tulsa, OK, USA). The volatile molecule profiles were analyzed using a principal component analysis (PCA) with Statistica software.

3. Results

3.1. Screening for Enzymatic Properties

Results related to the proteolytic and lipolytic potential of the different microorganisms tested are reported in Table 1. The bacteria tested showed proteolytic and lipolytic activity in a strain-dependent way. *B. subtilis* B46C, B47C, B28C did not create halos in skim milk or Spirit Blue Agar while they showed a moderate or weak gelatinase activity. On the other hand, strains B5C, B12C, and B15C had good proteolytic (max halo 8 mm) and lipolytic activity (max halo 14 mm), with B5C as the best lipolytic and proteolytic strain. Between the two strains of *B. licheniformis* tested, only B1M showed lipolytic (10 mm) and proteolytic (8 mm) activity. *B. amyloliquefaciens* B5M also possessed a good proteolytic (9 mm) and lipolytic (14 mm) activity. Among the yeasts, 4 strains of *Y. lipolytica* showed proteolytic activity on the skim-milk medium and gelatin. In addition, these strains showed strong lipolytic activity on Spirit Blue Agar medium. Among all the selected strains of *Y. lipolytica*, those with the greatest proteolytic and lipolytic activity (halos of 11 and 15 mm, respectively), were YL2 and YL4.

The protease activity of the most promising strains (namely *Y. lipolytica* YL2 and YL4, *B. amyloliquefaciens* B5M and *B. subtilis* B5C) was also evaluated. Strain B5C showed an activity of 0.17 ± 0.05 U/mL, strain B5M of 0.19 ± 0.07 U/mL, YL2 0.18 ± 0.10 U/mL and YL4 0.30 ± 0.04 U/mL.

Table 1. Results of enzymatic properties of the different tested strains on different substrates. The results are the mean of three replicates ($n = 3$).

Genera and Species	Strain	Origin	Proteolytic Clearing Zone in Skim Milk (mm)	Gelatinase Activity	Lipolytic Clearing Zone (mm)
B. subtilis	B5C	Plant origin	8 ± 1.0	+++	14 ± 0.1
	B12C	Plant origin	6 ± 0.4	+++	10 ± 0.3
	B15C	Plant origin	5 ± 0.3	+++	12 ± 0.5
	B28C	Plant origin	-	+++	-
	B46C	Plant origin	-	+	-
	B47C	Plant origin	-	++	-
B. licheniformis	B1M	Wine lees	8 ± 0.2	-	10 ± 0.3
	B21M	Wine lees	-	-	-
B. amyloliquefaciens	B5M	Wine lees	9 ± 0.3	+++	14 ± 1.1
B. stratosphericus	B18M	Wine lees	-	+	9 ± 0.2

Table 1. *Cont.*

Genera and Species	Strain	Origin	Proteolytic Clearing Zone in Skim Milk (mm)	Gelatinase Activity	Lipolytic Clearing Zone (mm)
Y. lipolytica	YL1	River	9 ± 0.1	+++	14 ± 0.3
	YL2	River	11 ± 0.3	+++	15 ± 0.2
	YL3	River	10 ± 0.1	+++	13 ± 0.5
	YL4	River	11 ± 0.2	+++	15 ± 0.1
	YL5	Dairy product	-	+	-
	YL6	Dairy product	-	+++	-
	YL7	Meat	-	+++	-
	YL8	Smoked ham	-	+++	-

-: No activity; +++: all the gelatin was turned liquid; ++: half of the gelatin was turned liquid; +: $\frac{1}{4}$ of the gelatin was turned liquid.

3.2. Fermentation of Fish Waste

To confirm these activities in a real system, the best-performing strains with stronger activities (namely *Y. lipolytica* YL2 and YL4, *B. amyloliquefaciens* B5M and *B. subtilis* B5C) were assessed on fish waste solution as substrates.

3.2.1. Microbial Characterization and pH

The initial concentrations of the tested strains were 4.9 ± 0.2, 4.1 ± 0.5, 6.9 ± 0.2 and 7.1 ± 0.1 log CFU/mL, for YL2, YL4, B5M, B5C, respectively. Incubation was then performed in agitation (100 rpm), at room temperature for 72 h. At the end of the 72 h incubation, strains B5M and B5C reached both 9.2 ± 0.2 log CFU/mL, while the strains YL2 and YL4 of *Y. lipolytica* reached 7.1 ± 0.1 and 7.2 ± 0.2 log CFU/mL, respectively. In the samples, coliforms and lactic acid bacteria were estimated. At the beginning of incubation, coliforms were below the detection limit and they remained below this limit throughout the incubation time in all the samples. On the other hand, lactic acid bacteria, which were below 2 log CFU/mL at the beginning of incubation, increased after 72 h up to 5.8 log CFU/mL in all the samples.

The pH of the fish waste solution was 6.32 ± 0.12 at the beginning of incubation (Figure 1). After 48 h, the values increased in samples containing the two strains of *Y. lipolytica* up to 7.11 ± 0.14, while they remained constant in sample control and B5M, or they reduced to 5.59 in samples with B5C. After 72 h, pH values reduced to around 5.2 for samples with yeasts, and 4.5 in samples with bacilli and control.

3.2.2. Degree of Hydrolysis (DH) and Peptide Content

The original fish waste contained 1.2 mg/mL of soluble proteins. The DH was evaluated for all the samples, including the control, once incubated for 72 h. As shown in Figure 2, the control sample demonstrated a degree of hydrolysis of 5.0 ± 0.4 %. Both bacterial strains showed similar (B5C, 6.1 ± 0.3%) or lower (B5M, 1.2 ± 0.1%) degrees of hydrolysis. On the other hand, the two strains of *Y. lipolytica* showed a higher degree of hydrolysis with respect to the control, estimated at 10.2 ± 0.2% and 12.3 ± 0.6% for YL4 and YL2, respectively. The peptide content followed the DH results. In fact, the highest content was measured in samples incubated with YL2 (159 mg/mL) and YL4 (179 mg/mL), followed by the control, B5C and B5M (Figure 3).

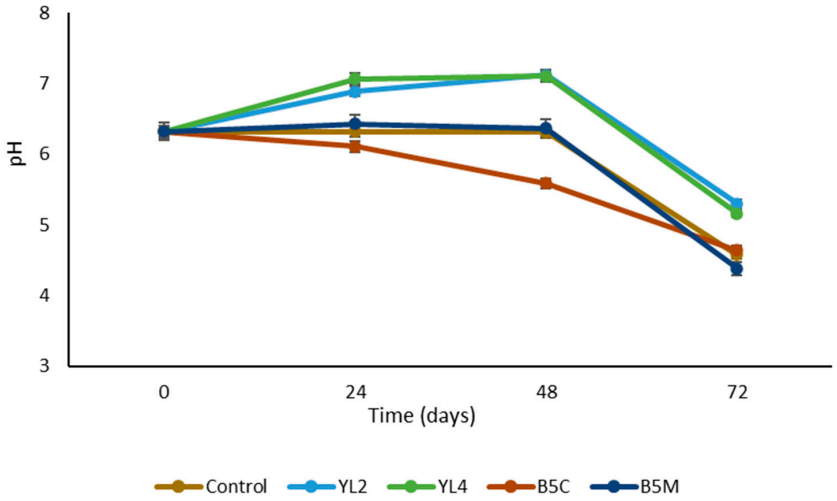

Figure 1. pH profile of the fish waste samples incubated with the different selected strains. Control is fish waste without additional microorganisms. Results are the mean of three repetitions (n = 3).

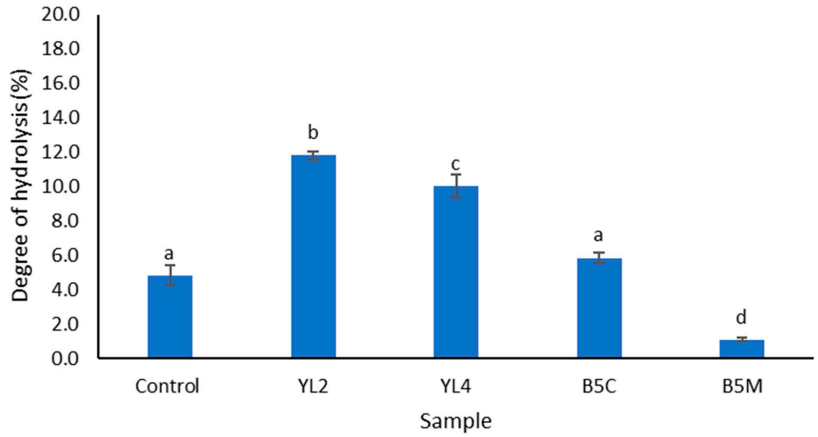

Figure 2. Degree of hydrolysis (DH) expressed in percentage for the samples and measured after 72 h incubation. YL2 and YL4 (*Y. lipolytica*), B5C (*B. subtilis*), and B5M (*B. amyloliquefaciens*). Results are the average of three replicates (n = 3). Different letters mean results are significantly different ($p < 0.05$).

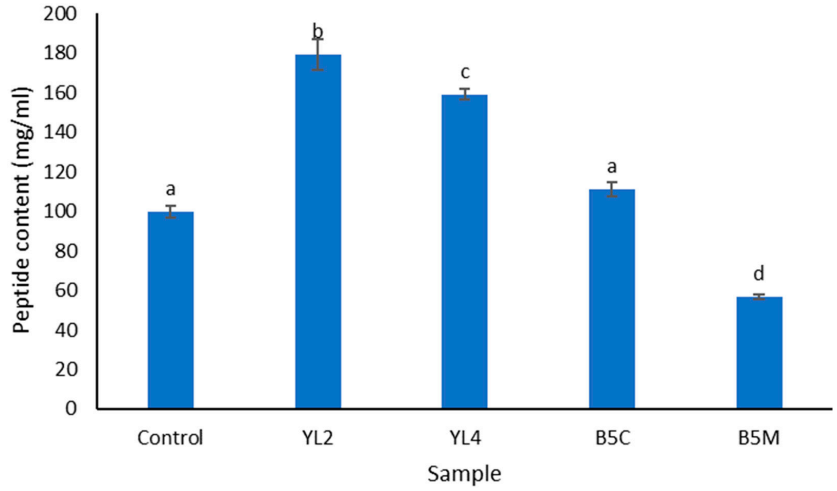

Figure 3. Peptide content expressed in mg/mL of hydrolysate measured after 72 h incubation. YL2 and YL4 (*Y. lipolytica*), B5C (*B. subtilis*), and B5M (*B. amyloliquefaciens*). Results are the average of three replicates (n = 3). Different letters mean results are significantly different ($p < 0.05$).

3.2.3. Antioxidant Activity

The obtained fish hydrolysates were analyzed for their antioxidant power with both DPPH and ABTS assay (Table 2). In DPPH assay, the sample with *Y. lipolytica* YL2 showed the strongest radical scavenging activity (RSA) (86.4 ± 9.4%), followed by *B. subtilis* B5C (72.5 ± 3.7%), while the other samples had similar or lower RSA compared to the control. For instance, the sample with strain B5M with the lower DH showed the lowest RSA. Repeating the DPPH assay with liophylized samples standardizing them for the initial concentration still showed a stronger activity in samples incubated with YL2 and B5C with 34.5 ± 1.5 and 35.1 ± 2.1 mg of ascorbic acid equivalent/mg of sample. In this case, even YL4 presented a significantly higher activity compared to the control.

Table 2. Antioxidant activity of the different fish waste hydrolysates. Results are the average of three replicates (n = 3).

Fish Waste Hydrolysate	DPPH (Radical Scavenging Activity % of Fresh Sample)	DPPH (mg of Ascorbic Acid Equivalent/mg of Lyophilized Samples)	ABTS (Radical Scavenging Activity % of Fresh Sample)	ABTS (Trolox Equivalent μmol/g)
Control 72 h	51.2 ± 15.0 ^{a}	17.0 ± 3.1 ^{a}	12.7 ± 1.2 ^{a}	135.8 ± 22.1 ^{a}
YL2	86.4 ± 9.4 ^{b}	34.5 ± 1.5 ^{c}	34.9 ± 2.5 ^{c}	129.7 ± 12.7 ^{a}
YL4	65.5 ± 2.7 ^{a}	26.9 ± 2.2 ^{b}	45.0 ± 2.3 ^{b}	237.2 ± 33.4 ^{b}
B5C	72.5 ± 3.7 ^{c}	35.1 ± 2.1 ^{c}	39.1 ± 3.3 $^{b, c}$	52.5 ± 10.1 ^{c}
B5M	15.6 ± 2.1 ^{d}	22.8 ± 2.6 $^{a, b}$	0.9 ± 1.1 ^{d}	30.0 ± 11.2 ^{c}

$^{a, b, c, d, e}$ mean significantly different ($p < 0.05$).

Regarding the ABTS assay, the RSA of fish hydrolysates was higher in samples incubated with YL4 (45.0 ± 2.3%), followed by B5C and YL2 (39.1 ± 3.3% and 34.9 ± 2.5%, respectively). The same analysis repeated with lyophilized samples showed that only samples with YL4 presented a significantly higher antioxidant (237.2 ± 33.4 μmol/g Trolox equivalent) activity compared to the control.

3.2.4. Volatile Molecule Compounds

With SPME/GC-MS analyses of the fish waste hydrolysates, around 102 volatile molecules were detected and identified (Table 3).

Table 3. Volatile molecule profiles (expressed as relative abundance, %) of fish waste solution incubated with *Y. lipolytica* YL2, YL4, *B. subtilis* B5C or *B. amyloliquefaciens* B5M for 72 h. Data are the mean of three different samples. The variability coefficient ranged between 5% and 7%. Results are the average of two replicates (n = 2).

Class of Compounds	Volatile Compound	Control	YL2	YL4	B5C	B5M
Aldehydes	Acetaldehyde	0.04	0.06	0.09	0.08	0.37
	Propanal	0.1	0.83	0.75	0.39	-
	2-propenal	0.17	0.1	-	0.11	0.15
	Butanal	-	0.08	-	0.07	-
	3-methylbutanal	-	0.2	0.17	0.06	-
	Pentanal	0.49	0.7	0.75	0.51	0.37
	2-butenal	0.3	0.48	0.38	0.44	0.11
	2-pentenal	2.56	2.81	3.53	2.68	1.38
	Hexanal	0.99	4.44	0.58	3.58	0.23

Table 3. *Cont.*

Class of Compounds	Volatile Compound	Control	YL2	YL4	B5C	B5M
	Heptanal	1.08	1.71	1.88	1.14	0.63
	2-hexenal	1.48	1.39	5.04	0.93	2.45
	Octanal	1.38	2.11	2.22	1.46	0.63
	2-heptenal	1.06	1.14	0.83	1.17	0.05
	Nonanal	1.54	3.36	4.16	2.1	0.73
	2,4-hexadienal	1.24	2.27	3.62	3.07	0.12
	2-octenal	1.6	3.35	1.74	2.93	-
	Decanal	0.31	0.7	0.73	0.39	0.19
	2,4-heptadienal	7.7	12.13	11.66	12.38	4.7
	2-nonenal	1.55	2.44	2.77	1.96	0.78
	Benzaldehyde	2.79	4.76	5.82	4.24	1.2
	2,6-nonadienal	3.84	1.36	1.06	0.9	0.73
	Undecanal	-	-	0.08	-	-
	2-decenal	1.06	1.99	2.62	1.74	0.37
	2,4-nonadienal	0.1	0.13	0.18	0.18	-
	2-undecenal	0.36	0.67	1.12	0.58	0.16
	2,4-decadienal	0.33	0.75	0.77	0.58	0.25
	Tetradecanal	0.46	0.53	1.14	0.41	0.76
	3-ethylbenzaldehyde	-	0.14	0.16	0.15	-
	5-hydroxymethyl-2-Furaldehyde	1.48	-	-	-	0.86
Ketones	Acetone	-	0.24	0.37	-	0.13
	Methyl isobutyl ketone	0.17	0.09	0.2	0.11	0.14
	1-penten-3-one	1.72	2.2	1.73	2.04	0.83
	2,4-pentanedione	0.12	0.11	0.07	0.14	0.09
	4-methyl-2-hexanone	0.04	-	-	0.04	-
	3-octanone	-	-	-	-	0.38
	3-hydroxy-2 butanone	0.86	-	-	-	8.15
	1-octen-3-one	0.52	1.12	1.75	1.62	-
	2,5-octanedione	0.26	0.35	0.36	0.3	0.4
	1-hydroxy-2-propnaone	0.26	-	-	-	0.15
	2-nonanone	1.21	1.7	1.35	1.64	0.68
	3,5-octadien-2-one	1.41	2.69	0.91	2.41	0.52
	2-undecanone	-	0.25	0.71	0.33	0.21
	2,6-dimethyl-4-heptanone	0.77	0.05	0.19	0.2	0.25
Alcohols	Ethanol	12.16	1.54	2.25	7.82	16.46
	1-penten-3-ol	0.13	0.19	0.18	0.2	0.09
	1-pentanol	1.01	0.18	-	0.1	-

Table 3. *Cont*.

Class of Compounds	Volatile Compound	Control	YL2	YL4	B5C	B5M
	3-methyl-1-butanol	-	-	0.09	-	3.16
	1-pentanol	0.07	0.09	-	0.07	0.25
	2-penten-1-ol	0.1	-	0.34	-	0.11
	1-hexanol	-	-	-	-	0.28
	1-octen-3-ol	0.77	0.83	-	0.51	0.93
	Benzyl alcohol	0.1	0.05	-	0.07	-
	Phenyl ethanol	2.11	0.19	0.14	0.11	3.46
Esters	Ethyl acetate	0.22	-	-	0.06	0.4
	Acetic acid ethenyl ester	0.4	0.29	-	0.16	0.42
	Tetradecanoic acid ethyl ester	0.11	-	-	0.21	0.14
	Hexanoic acid ethylester	0.13	-	-	0.2	0.2
Acids	Acetic acid	11.84	2.16	4	5.23	15.92
	Propanoic acid	0.13	-	0.24	-	0.28
	Octanoic acid	0.32	-	-	-	0.16
	Decanoic acid	0.41	-	-	-	0.24
Furans	2-methyl furan	0.15	1.26	-	1.48	-
	2 -ethyl furan	2.16	2.15	2.16	2.19	2
	Butyl furan	0.04	0.05	-	0.03	-
	2-methyl furan	0.15	1.20	0.00	1.48	0.00
	2-pentyl furan	0.24	-	0.94	-	0.84
	2-(2-pentenyl) furan	1.77	3.66	1.53	4.31	3.06
	2 methoxy furan	0.17	0.38	0.28	0.28	-
	3-pentyl-furan	-	-	-	0.05	-
	2-(2-propenyl) furan	0.17	0.17	-	-	-
	4-methyl-2propyl furan	0.62	0.62	0.54	0.65	0.46
Alkanes, alkenes, alkynes	hexadecane	0.55	0.78	0.64	0.64	0.54
	2,6,10,14 tetramethyl pentadecane	0.64	0.9	1.01	0.71	0.77
	1,3-cyclooctadiene	0.86	1.07	0.72	1.07	0.63
	Heptadecane	2.86	3.95	5.26	3.2	2.96
	1-pentadecene	0.1	0.18	0.59	0.12	-
	2,4-dimethyl-1-heptene	-	-	-	-	0.09
	Decane	0.06	0.06	0.04	0.05	0.08
	1,3-*cis*-5-*cis*-octatriene	0.11	0.22	-	-	-
	1,3-*trans*-5-*cis*-octatriene	0.07	0.15	-	0.16	-
	1-ethyl-1,4-cyclohexadiene	-	-	-	0.11	-

Table 3. *Cont.*

Class of Compounds	Volatile Compound	Control	YL2	YL4	B5C	B5M
	3-ethyl-2-methyl-1-pentene	-	-	-	0.03	-
	Dodecane	-	0.15	-	0.24	-
	Tridecane	0.47	0.46	0.5	0.32	0.51
	Tetradecane	0.11	0.17	-	0.07	-
	2,4-dimethyl-1,3-pentadiene	-	-	-	0.04	-
	3,5,5-trimethyl-2-hexene	0.9	1.02	0.21	0.72	0.43
	Pentadecane	14.35	16.6	13.23	14.7	11.48
	Cyclooctane	0.26	0.13	0.29	0.32	0.16
Others		2.02	0.87	3.09	0.74	3.71
Total area [1]		1894	2555	1818	2668	1759

-: under the detection limit. [1]: Arbitrary units (×1,000,000).

The control sample after fermentation was characterized by alcohols (16.4%), aldehydes (34.0%) and alkanes, alkenes and alkynes (21.3%) (Figure 4). The sample incubated with *B. amyloliquefaciens*, B5M, showed a higher abundance of alcohols (24.7%) and ketones (11.9%) compared to the control. The addition of both the two strains of *Y. lipolytica* (YL2 and YL4) improved the abundance of aldehydes (50.6 and 53.8%, respectively) and reduced the content of acids (2.1 and 4.2 compared to 12.6%). Even B5C demonstrated a similar behavior to the two strains of yeast but to a lower extent.

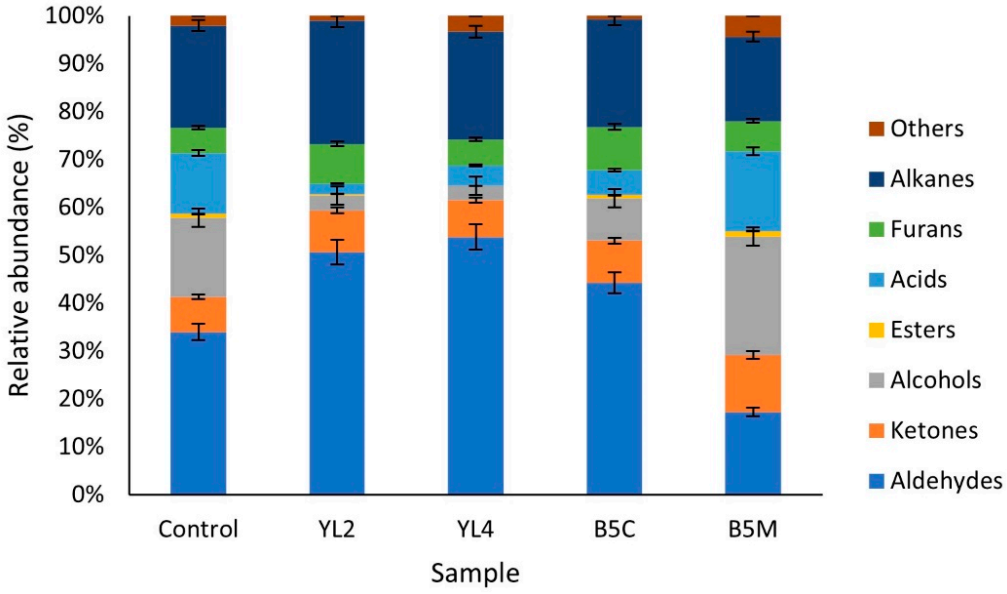

Figure 4. Classes of compounds detected and identified in the samples of fish waste incubated with the selected microorganisms. YL4 and YL2 (*Y. lipolytica*), B5C (*B. subtilis*) and B5M (*B. amyloliquefaciens*). Results are expressed as relative abundance (%). Control: fish waste without any microorganisms.

Analyzing these results with PCA (Figure 5), it was possible to observe that factor 1 explained 49.50% of the variance while factor 2 explained 27.64% of the variance. The control was in the fourth quadrant while all the other samples were distributed in the other three. The negative values characterizing PC1 were explained by aldehydes such as 2,4-heptadienal, benzaldehyde, 2-nonenal, 2-octenal and 2-butenal, while the positive values

were characterized by acids and alcohols (ethanol, 1-hexanol, phenyl ethanol). Positive values of PC2, instead, were characterized by aldehydes (i.e., 2-hexenal, tetradecanal) and ketones (1-pentadecene and 2-undecanone), while negative values were characterized by alcohols (benzyl alcohol, 1-pentanol), ketones (2,4-pentanedione, 4-methyl-2-hexanone) and esters (acetic acid ethenyl ester).

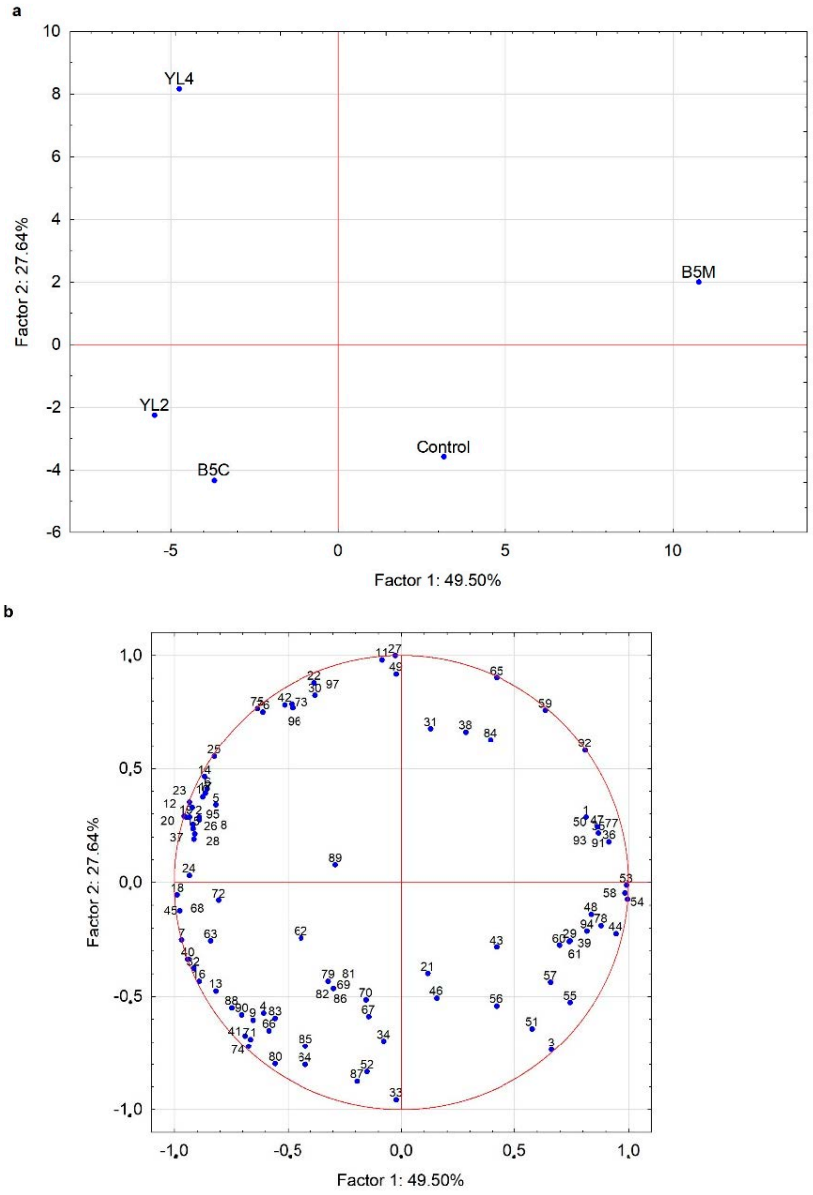

Figure 5. Plot of cases (**a**) and variables (**b**) obtained by PCA elaboration of the total volatile molecules characterizing the fish waste-based compounds obtained in relation to the strain employed. 1: acetaldehyde; 2: propanal; 3: 2-propenal; 4: butanal; 5: 3-methyl-butanal; 6: pentanal; 7: 2-butenal; 8: 2-pentenal; 9: hexanal; 10: heptanal; 11: 2-hexenal; 12: octanal: 13: 2-heptenal; 14: nonanal; 15: 2,4-hexadienal; 16: 2-octenal; 17: decanal; 18: 2,4-heptadienal; 19: 2-nonenal; 20: benzaldehyde; 21: 2,6-nonadienal; 22: undecanal; 23: 2-decenal; 24: 2,4-nonadienal; 25: 2-undecenal; 26: 2,4-decadienal: 27: tetradecanal; 28: 3-ethylbenzaldehyde; 29: 2-Furaldehyde, 5-(hydroxymethyl); 30: acetone; 31: methyl isobutyl ketone; 32: 1-penten-3-one; 33: 2,4-pentanedione; 34: 4-methyl-2-hexanone; 35: 3-octanone; 36: 3-hydroxy-2-butanone; 37: 1-octen-3-one; 38: 2,5-octanedione; 39: 1-hydroxy-2-propnaone; 40: 2-nonanone; 41: 3,5-octadien-2-one; 42: 2-undecanone; 43: 2,6-dimethyl-4-heptanone;

44: ethanol; 45: 1-penten-3-ol; 46: 1-pentanol; 47: 3-methyl-1-butanol; 48: 1-pentanol; 49: 2-penten-1-ol; 50: 1-hexanol; 51: 1-octen-3-ol; 52: benzyl alcohol; 53: phenylethyl alcohol; 54: ethyl acetate; 55: acetic acid ethenyl ester; 56: tetradecanoic acid ethyl ester; 57: hexanoic acid ethyl ester; 58: acetic acid; 59: propanoic acid; 60: octanoic acid; 61: decanoic acid; 62: 2-methyl furan; 63: 2-ethyl furan; 64: butyl furan; 65: 2-pentyl furan; 66: 2-methyl furan; 67: 2-(2-pentenyl) furan; 68: 2-methoxy furan; 69: 3-pentyl furan; 70: 2-(2-propenyl) furan; 71: 4-methyl-2-propyl furan; 72: hexadecane; 73: pentadecane-2,6,10,14-tetramethyl; 74: 1,3-cyclooctadiene; 75: heptadecane; 76: 1-pentadecene; 77: 2,4-dimethyl-1-heptene; 78: decane; 79: 1,3-cis-5-cis-octatriene; 80: 1,3-trans-5-cis-octatriene; 81: 1-ethyl-1,4-cyclohexadiene; 82: 3-ethyl-2-methyl-1-pentene; 83: dodecane; 84: tetradecane; 85: tridecane; 86: 2,4-dimethyl-1,3-pentadiene; 87: 2-hexene-3,5,5-trimethyl; 88: pentadecane; 89: cyclooctane; 90: 2-ethyl-phenol; 91: 1,5,6,7-tetrahydro-4-indolone; 92: Benzene, 1,3-bis(1,1-dimethylethyl); 93: 2-ethyl-3,5-dimethyl-pyrazine; 94: hydroxy-gamma-butyrolactone; 95: thiazole; 96: indole; 97: trimethylamine.

4. Discussion

Proteolytic and lipolytic microorganisms are constantly being researched for their possible application in several sectors, such as environmental remediation, detergent manufactory, biomedicine, the food industry and, recently, for food waste recovery and valorization. In this work, yeasts and bacteria, known for their proteolytic and lipolytic properties, were screened in vitro using common substrates, namely skim milk, Spirit Blue Agar and gelatin. Among the microorganisms tested, the best performing ones were *B. subtilis* B5C, *B. amyloliquefaciens* B5M and two strains of *Y. lipolytica*, YL2 and YL4. Regarding bacterial strains, the genus *Bacillus* contains several species with documented proteolytic and lipolytic activities [33,34]. For instance, *Bacillus subtilis* and *B. amyloliquefaciens*, both Gram-positive bacteria with a generally recognized safe (GRAS) status, can break down many types of carbohydrates, proteins, and lipids due to their enzymes [35,36]. Therefore, it is not surprising that the *Bacillus* species performed well in the different assays (skim milk, gelatin and Spirit Blue Agar). *Y. lipolytica* is one of the most extensively studied yeast species, after *S. cerevisiae*, which contributes to food ripening thanks to its proteolytic and lipolytic properties [37–39]. *Y. lipolytica* possesses the "qualified presumption of safety" (QPS) and GRAS status for production purpose and it has been defined as "novel food" by the European Food Safety authority (EFSA) [40]. Due to its properties, this yeast has been proposed as a promising tool to recover and valorize food wastes and by-products [22]. Concerning proteolytic activity, strain YL4 possessed the strongest, followed by Bacilli and YL2. The values obtained were higher compared to those reported by Zou et al. [14], but in line with other studies [41]. It is possible that the lower activity described by Zou et al. [14] also depends on the very poor substrate (marine broth) applied to grow the strain tested. At the same time, higher proteolytic activities were reported when optimal conditions were selected [42].

The 4 proteolytic and lipolytic microorganisms were then tested in a fish waste solution as substrates. As already performed by Zou et al. [43], samples were incubated for 72 h in order to observe differences in hydrolysis. All the tested strains grew in the fish waste solution with B5M, B5C, and YL4 increasing by around 2.2 log CFU/mL, while YL2 increased by 3.1 log CFU/mL. The pH increased during the first 48 h with *Y. lipolytica*, while it remained stable with the two species of *Bacillus*, as well as the control. After 72 h, instead, the pH decreased in all samples due to the growth of indigenous lactic acid bacteria present in the matrix that reached a final concentration of 5.8 log CFU/mL. Looking at the degrees of hydrolysis, the two strains of *Y. lipolytica* were the best-performing ones. The values obtained are in line with the data reported by Zou et al. [43] when brown crab processing side streams were fermented with *Pseudoalteromonas* strains after 72 h. In our case, the two yeasts strains performed better than the two bacteria strains. The use of *Y. lipolytica* to produce protein hydrolysates for fish waste has never been reported. In fact, most of the literature is focused on the production of single cell proteins, single cell oils, citric acid, polyols and enzymes from food waste [22], or single cell oils and lipid reduction from fish waste [6,23]. Alternatively, protein hydrolysates have been used as

substrates for *Y. lipolytica* to produce oils [44,45]. Only Rossi et al. [46] reported the use of *Y. lipolytica* RO25 to obtain cricket powder hydrolysates richer in health-promoting molecules, while Dabrowska et al. [47] described the use of purified *Y. lipolytica* proteases to reduce the antigenic response to bovine milk allergens. Regarding the functionality of the obtained hydrolysates, those of *Y. lipolytica* showed the strongest antioxidant activity both in DPPH and ABTS assay. To some extent, the higher antioxidant activity of the fresh samples was correlated with the higher degree of hydrolysis and peptide content present in those samples. This aspect has been reported in several publications where protein hydrolysates are assessed for their radical scavenging activity [43,48–50]. Surprisingly, *B. subtilis* B5C, which showed a lower degree of hydrolysis and a lower peptide content, formed hydrolysates with a good radical scavenging activity. Similar values on DPPH assay were reported by Manni et al. [51] using shrimp waste hydrolysates obtained with crude protease from *Bacillus cereus*. After lyophilization, only hydrolysates obtained with YL2 and B5C maintained their stronger activity in DPPH assay, while only YL4 showed good radical scavenging activity in ABTS assay, with respect to the control. Lyophilization reduced the difference of the hydrolysates, since similar amounts of powders were compared. This means that other compounds present in the hydrolysates, other than peptides, may have played a role in the radical scavenging activity.

The production of volatile compounds upon microbial fermentation is well documented in the literature. For instance, the fungal fermentation of Fuzhuan brick-tea demonstrated an increase in volatile compounds, especially aldehydes (51%), such as 2-pentenal, 2-hexenal, 1-penten-3-ol and 2,4-heptadienal [52]. Zhao et al. [53] obtained an increase in 2-methylbutanal and 3-methylbutanal after the fermentation of fish sauce made with freshwater fish by-products. In our work, samples fermented with the two strains of *Y. lipolytica* showed a higher abundance of aldehydes (more than 50%). In particular, the most represented ones were 2,4-heptadienal (12.1 and 11.6%), benzaldehyde (4.7 and 5.8%), nonanal (3.3–4.1%), 2-pentenal (2.8 and 3.5%), 2-hexenal (1.3 and 5.0%) and 2-nonenal (2.4 and 2.7%) in YL2 and YL4 samples, respectively. However, even pentanal, heptanal, octanal and nonanal were more abundant in samples fermented with yeast. These aldehydes have specific aromatic characteristics: 2,4-heptadienal is described as fatty, green, oily, aldehydic, vegetable, cake, and cinnamon at 1%; benzaldehyde has fruity, almond, nutty and creamy notes; nonanal is described as fatty, citrussy and green; 2-pentenal is characterized by a strawberry, fruity flavor; 2-nonenal has earthy, fishy, cucumber and green notes [54]. Moreover, only the two samples incubated with *Y. lipolytica* developed 3-methyl-butanal (around 0.2%), which is responsible for the taste and characteristic smell of fermented fish sauces [53]. Aldehydes can be produced during various biochemical reactions starting from free amino acids, and especially free fatty acids [55,56]. The fact that their abundance was higher in samples containing *Y. lipolytica* than in the control sample may depend on the stronger lipolytic properties of this yeast on this substrate. The presence of aldehydes in fish waste hydrolysates makes them an interesting source of flavor and aroma compounds that can be reused in food preparation. It is also interesting to mention that aldehydes from oxidized fatty acids, including pentanal, 2-heptenal and 2-nonenal, can react with DPPH [57]. This could explain the stronger antioxidant activity of the fresh hydrolyzed samples compared to the lyophilized ones. In fact, according to Dong et al. [58], freeze-drying can significantly decrease the varieties of total volatile compounds. At the same time, the fact that lyophilized samples of YL2 and YL4 maintained the strongest radical scavenging activity in DPPH and ABTS assay, respectively, suggests that the remaining non-volatile compounds had an impact on their antioxidant power.

5. Conclusions

Most of the studies performed on *Y. lipolytica* and fish wastes concern the production of single cell oil, yeast biomass, and enzymes or lipid reduction [6,23,24,59]. To the best of our knowledge this is the first work that describes the use of wild-type strains of *Y. lipolytica* to produce antioxidant hydrolysates containing peptides and flavor/aroma compounds

(reach in aldehydes) from fish waste that could be exploited in feed or food formulations. From one point of view, it would be interesting to assess the behavior of this yeast on different types of fish waste as substrates. At the same time, due to the higher peptide content observed, other technological properties impacting feed and food formulation could be also evaluated (for instance, solubility, surface activity, foaming and emulsifying properties) in the samples.

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