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The Fermentation of a Marine Probiotic Bacterium on Low-Cost Media Formulated with Industrial Fish Gelatin Waterstreams and Collagen Hydrolysates

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Abstract: Chemical effluents generated by the isolation of fish gelatin and collagen hydrolysates produced from the enzyme proteolysis of skin wastes are protein-rich substrates that could be used as nutrients in bacterial bioprocessing. In this study, the suitability of such nutrients in supporting the growth of a marine probiotic bacterium, *Phaeobacter* sp. DIFR 27-4, was studied. Both gelatin effluents and collagen hydrolysates were obtained from the skins of shark, tuna, salmon and turbot. The chemical composition of the substrates included the complete presence of all quantified amino acids. Low-cost marine culture media were formulated with these protein materials alongside a very low concentration of yeast extract and marine water. In batch cultures with gelatin effluents, the growth rates of the strain DIFR 27-4 were somewhat lower than those found in the control marine commercial media. In the case of the hydrolysates, the bacterial production of biomass was similar or higher than that observed in the control, and larger than that observed in the effluents. A simple evaluation of production costs in the different substrates studied indicated that around a 73–125-fold reduction can be achieved when alternative media are used, in comparison to the use of commercial marine broth.

Keywords: *Phaeobacter* sp. DIFR 27-4; marine probiotic bacteria; gelatin effluents; collagen hydrolysates; sustainability; bioconversion

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1. Introduction

Gelatin is a mixture of protein and peptides resulting from the partial hydrolysis of collagen [1]. Because of its biocompatibility, biodegradability, natural origin, and rheological and chemical properties (including its viscoelasticity and gel-forming capacity), gelatin is widely employed in technological applications for cosmetic [2]; pharmacological [3,4]; food-based [5]; nanotechnological [6]; and biomedical [7,8] formulations and devices. Industrial production has traditionally relied on porcine and bovine by-products, such as skin, bones, and hides as the raw materials for gelatin extraction, although alternative sources, including fish, have recently emerged, thereby providing a way to circumvent safety and ethical issues [9]. The basic principles of gelatin extraction are common across tissues and involve the removal of non-collagenous material, typically aided by an acid or alkali, followed by thermal denaturation of the collagen [10]. However, a wide variety of

methods are applied for this purpose, both in terms of the chemical and physical agents used and the parameters of the reaction; this may be, for example, to accommodate the peculiarities of the raw material, and results in gelatin with diverse characteristics.

The scale of the global production of gelatin (exceeding 600 k metric tons annually [11]), and the significant volumes required for the serial extraction steps, result in large amounts of effluent. Each ton of starting material carries up to 1500 m^3 of waste streams [12,13]. With yields of around 20% [14,15], the gelatin industry discards about 4.5 km^3 of effluents globally each year, an area approximately the size of Lake Michigan. These effluents contain suspended matter; high levels of organic compounds (such as lipids, peptides and amino acids, and inorganic species (ammonia, calcium, chloride, etc.) [12,13,16–19]); and extreme pH levels in some cases; therefore, they present considerable pollution potential. Additionally, the rest of the solid skins generated after gelatin extraction are disposed of in processing plants; in ideal cases, they are treated as substrates for fish meal production, and in the majority of cases, they are managed as urban solid waste.

Several approaches exist for the treatment of gelatin effluents. The most conventional strategies revolve around the removal of contaminants from wastewater prior to its release into the environment, typically using aerobic and/or anaerobic biological treatments [12,20], and, after elimination of the solid fraction, coagulation and flocculation (in order to decrease the organic load), and neutralization. This linear approach to handling industrial waste requires transformative efforts in order to become a circular process and increase the sustainability of gelatin production. In this context, effluents may be returned to the gelatin extraction process as inputs, e.g., by recycling acid effluents to recover hydrochloric acid [13]. Often, the organic matter and some inorganic ions present in the gelatin effluents serve as substrates for bioprocessing methods, sometimes accompanied by previous enzymatic degradation, thus leading to the production of biofuels [17]; compost and soil conditioners for agricultural uses [16,18]; and algae cultivation [16,21]. In the case of solid skin remains, enzyme proteolysis is the most valuable solution, enabling a type of collagen hydrolysate rich in proteins (with potential applications in cosmetic formulations and as a potential source of bacterial peptones) to be obtained [22,23].

Microbial fermentation likely represents one of the most logical strategies for dealing with nitrogen-rich waste streams such as those stemming from the gelatin production process. Nitrogen released via the hydrolysis of residues resulting from animal processing has been proven to be capable of supporting the growth of microorganisms of industrial relevance [24–27]. Despite this fact, very little research on gelatin effluents in this context exists; the only approaches are generally restricted to photosynthetic organisms [16,21].

On the other hand, in recent decades, important investigative efforts have been made to further understand and improve the application of probiotic bacteria in aquaculture as substitutes for the antibiotics used to avoid disease and mortality in farming fish [28,29]. Although bacteria from the *Lactobacillales* order have been the most extensively explored [30,31], marine bacteria from the *Proteobacteria* phylum, such as representative of *Pseudomonas*, *Shewanella*, and *Phaeobacter* genera, have shown important probiotic properties that may act to counter pisciculture pathogens [32]. Especially for the latter, several beneficial fish-protecting effects have been reported: (1) increased survival against *Vibrio anguillarum*, and a reduction in mortality rates in turbot larvae [33]; (2) improvement in the seawater and gut microbiota quality of turbot larvae cultures [34]; and (3) enhanced resistance of Atlantic cod larvae to *V. anguillarum* infection [35]. However, the marine medium (MM) recommended for the cultivation of these microorganisms is prohibitively expensive, considering its composition (a small amount of yeast extract and peptone, and various minerals that simulate marine water) [36]. Alternative media formulated with cost-effective nutrients must be proposed as substitutes for commercial media in order to enable high biomass production and their potential incorporation into aquaculture tanks. Waste products from fish gelatin processing—proteins, peptides, and free amino acids—could be interesting and sustainable sources of organic nitrogen that are sufficient to support the growth of marine probiotic bacteria such as *Phaeobacter* sp. DIFR 27-4.

In the present work, we assess the capacity of chemical effluents generated in the production of marine gelatins and collagen hydrolysates from fish skins to act as the main ingredients of strain DIFR 27-4 fermentation in batch cultures. In all cases, the experimental data of biomass production and protein consumption were fitted by two equations in order to compare the ability of the different media proposed. Thus, within a circular blue economy framework, we explored beyond a valorization of fish-skin residues to produce gelatin, to evaluate the utility of process effluents and hydrolysates for potential biotechnological applications in aquaculture.

2. Materials and Methods

2.1. Chemical Gelatin Effluents and Collagen Hydrolysates

Gelatin waste streams (GE) were generated by isolating gelatin from the skins of blue shark (BS, *Prionace glauca*), turbot (Tu, *Scophthalmus maximus*), salmon (Sa, *Salmon salar*) and yellowfin tuna (YT, *Thunnus albacares*). In Figure 1, a complete scheme of the gelatin processing procedure is shown, including the stages performed for optimal recovery of the gelatin, together with the coproducts and effluents generated. Thus, briefly, portions of skins (the by-product resulting from industrial filleting of the aforementioned species) were initially treated with alkalis (NaOH) and two acids (sulfuric and citric) in a sequential manner [37–39].

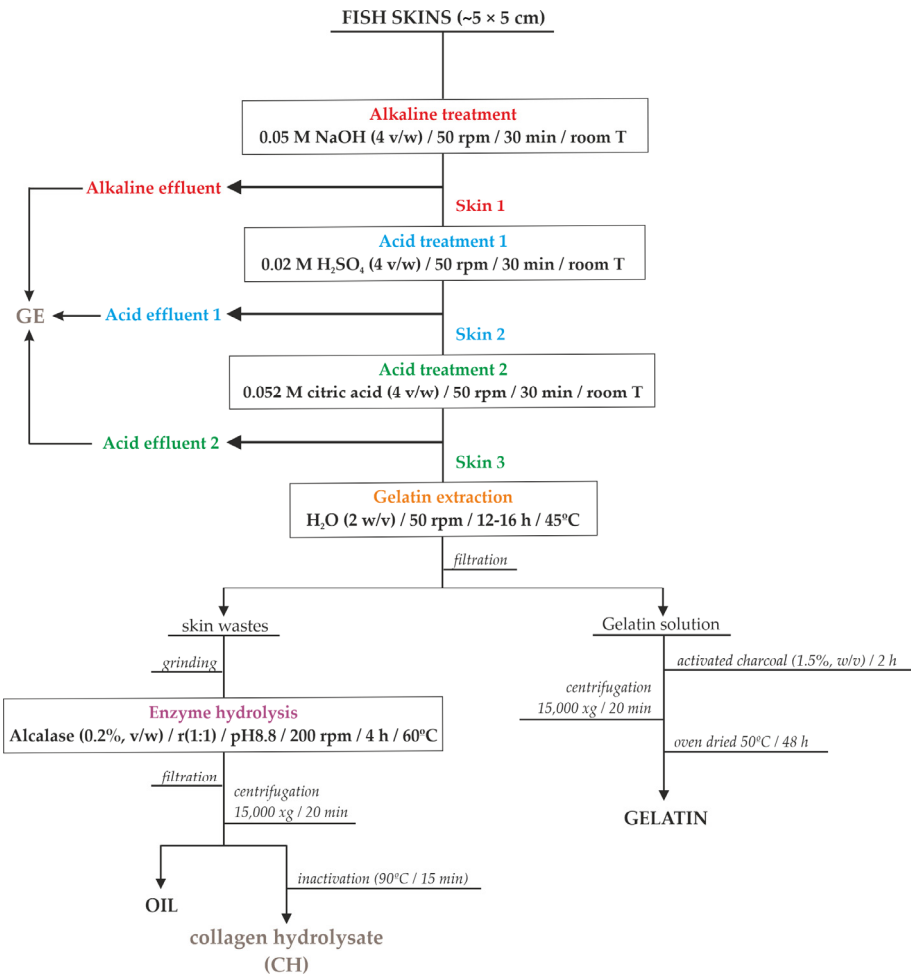

Figure 1. Flowchart showing the steps employed in the production of gelatin and oil from fish skins, together with the concomitant release and production of gelatin chemical effluents (GE) and collagen hydrolysates (CH). Experimental procedures and conditions are indicated at each stage.

Chemical effluents from each treatment were mixed (GE) in proportions of 1/3 for each, and then stored at −18 °C until analysis and use. The resulting skins were soaked in warm water (45 °C) under continuous agitation for thermal extraction of the gelatinous solution, which was purified via adsorption with charcoal or ultrafiltration at 30 kDa and dried. The remaining skins were mixed with water in a ratio of 1:1 for hydrolysis with Alcalase 2.4 L (Novozyme, Nordisk, Bagsvaerd, Denmark) for 4 h [37,38]. The corresponding collagen hydrolysates (CH) were stored at −18 °C until analysis and application. The basic composition of the GE and CH, including their amino acid contents, is summarized in Table 1, Table S1 and Table S2 (Supplementary Materials), respectively.

Table 1. Proximal composition of GE (mean values ± confidence intervals for $n = 3$) and CH. Pr: Total soluble protein; TS: Total sugars; TL: Total lipids. Different superscript letters (a, b, c, d) in each row indicate significant differences between effluents ($p < 0.05$).

SPECIES	SUBSTRATE	pH	Pr (g/L)	TS (g/L)	TL (g/L)
SHARK (BS)	**GE**	6.17 ± 0.31^{a}	2.34 ± 0.50^{a}	0.04 ± 0.03^{a}	0.17 ± 0.15^{a}
TUNA (YT)	**GE**	6.04 ± 0.27^{a}	2.59 ± 0.43^{a}	0.31 ± 0.09^{b}	1.51 ± 0.13^{b}
TURBOT (Tu)	**GE**	5.98 ± 0.19^{a}	4.00 ± 0.26^{b}	$0.12 \pm 0.10^{a,b}$	$1.61 \pm 0.48^{b,c}$
SALMON (Sa)	**GE**	6.50 ± 0.36^{a}	3.96 ± 0.53^{b}	$0.17 \pm 0.14^{a,b}$	2.06 ± 0.38^{c}
SHARK (BS)	**CH**	8.76 ± 0.10^{b}	40.6 ± 1.5^{c}	$0.17 \pm 0.15^{a,b}$	0.19 ± 0.05^{a}
TUNA (YT)	**CH**	8.82 ± 0.20^{b}	42.9 ± 1.3^{c}	$0.15 \pm 0.11^{a,b}$	0.32 ± 0.08^{a}
TURBOT (Tu)	**CH**	8.78 ± 0.08^{b}	46.8 ± 0.4^{d}	$0.12 \pm 0.05^{a,b}$	$0.36 \pm 0.19^{a,d}$
SALMON (Sa)	**CH**	8.85 ± 0.09^{b}	47.6 ± 1.2^{d}	$0.19 \pm 0.11^{a,b}$	0.59 ± 0.08^{d}

2.2. Microbiological Methods, Culture Media, and Analytical Determinations

Phaeobacter sp. DIFR 27-4, the marine bacterium used for the present study, was kindly provided by Dr. Lone Gram (DTU Aqua, Lyngby, Denmark). This microorganism is a member of the *Roseobacter* group [40] and is officially stored in various collections as *Phaeobacter piscinae* 27-4 (German Collection of Microorganisms and Cell Culture, DSM 103509 and BCCM/LMG Bacteria Collection, LMG 29708). Our stock cultures were preserved at −80 °C in a marine medium (MM, Difco, Becton, Dickinson and Company, Sparks, MD, USA) with 25% glycerol. The inocula (1% w/v) consisted of cellular suspensions from 12–16 h cultures on the MM. The alternative media, based on the GE and CH, were formulated with: (1) 1 g/L of yeast extract (Panreac Química, Castellar del Vallés, Barcelona, Spain); (2) filtered seawater; and (3) the volume of each effluent and hydrolysate needed to obtain a final soluble protein concentration of 2.4 g/L in the flasks.

To establish the same concentration of mineral salts in all media (33 g/L), thereby simulating seawater, NaCl was added in the required amounts to each flask. Cultures were performed in three biological replicates using 300-mL Erlenmeyer flasks with 150 mL of the medium in the dark at 22 °C with 200 rpm of orbital shaking. Although the optimal growth temperature for the bacterium is around 31–32 °C, a temperature of 22 °C was selected as this was closer to the optimal culture conditions for the growth of turbot (11–23 °C) and sole (16–23 °C) [41]. The initial pH was, in all cases, adjusted to 7.5 with NaOH or HCl (5 n), and the flasks including the media were sterilized separately at 121 °C for 15 min.

The analyses, carried out in triplicate to determine the basic composition of the GE and CH, were as follows: (a) total lipids, using Soxhlet extraction [42]; (b) total sugars, using the protocol of Dubois et al. [43]; (c) total soluble protein, according to the procedure of Lowry et al. [44]; and (d) amino acid content, employing an amino acid analyzer (Biochrom 30 series, Biochrom Ltd., Cambridge, UK) after a ninhydrin reaction [45]. The protein contents in each of the bacterial post-incubates were also quantified using the method of Lowry et al. [44].

2.3. Bacterial Sampling and Biomass and Cell Analysis

At predetermined times, the flasks were sampled, and the corresponding aliquots were centrifuged at 3270× g for 15 min, from which the concentration of protein (Pr, Lowry method) was determined, and the sediments were then washed and resuspended

in distilled water at an appropriate dilution to measure the optical density (OD) at 700 nm (OD_{700}). Then, the dry weight was estimated from a calibration curve (OD_{700} vs. dry weight). Additionally, in two samples (12 and 30 h), viable cells were also quantified using the plate count technique (N, as colony-forming units per mL, ufc/mL) on the MM agar [36].

2.4. Mathematical Modelling of Strain DIFR 27-4 Cultivation

The growth of marine bacteria, and the production of biomass (X), were predicted using the following logistic equation [46]:

$$X = \frac{X_m}{1 + \exp\left[2 + \frac{4v_X}{X_m}(\lambda_X - t)\right]} \text{ with } X_0 = \frac{X_m}{1 + \exp\left(2 + \frac{4v_X\lambda_X}{X_m}\right)} \quad (1)$$

The consumption of soluble protein was modelled using the following unstructured equation [47]:

$$Pr = Pr_0 - \frac{1}{Y_{x/p}}\left[\frac{X_m}{1 + \left(\frac{X_m}{X_0} - 1\right)\exp\left(-\frac{4v_X}{X_m}t\right)} - X_0\right] - \left(\frac{m_p X_m^2}{4v_X}\right)\ln\left[\frac{X_0\left(e^{\frac{4v_X}{X_m}t} - 1\right) + X_m}{X_m}\right] \quad (2)$$

where X is the growth in terms of biomass production (g/L); X_0 is the initial biomass (g/L); t is the time of culture (h); X_m is the maximum biomass production (g/L); v_m is the maximum growth (biomass production) rate (g L^{-1} h^{-1}); λ_x is the growth lag phase (h); Pr is the protein uptake (g/L); Pr_0 is the initial protein (g/L); $Y_{x/P}$ is the yield factor for biomass formation on protein consumption (g biomass/g protein); and m_P is the maintenance coefficient for protein (g of protein g^{-1} biomass h^{-1}).

2.5. Economy Assessment of Strain DIFR 27-4 Production

A preliminary study of economical sustainability for the strain DIFR 27-4 growth costs was also performed. Taking the market prices of the MM ingredients and the values of X_m summarized in Table 2 as a reference, we calculated the cost of the production of biomass (in EUR/g) in each cost-effective media formulated with the GE and CH, as well as that of the commercial MM.

Table 2. Numerical values and confidence intervals for parameters obtained from experimental data of the strain DIFR 27-4 growth on the GE and CH media, modelled by equations (1–2). R^2 represents the determination coefficients among the experimental and predicted data. Different superscript letters (a, b, c, d) in each row represent significant differences between media ($p < 0.05$). MM: numerical values for the fermentations on the control marine medium. GE: media formulated with chemical gelatin effluents. CH: media formulated with collagen hydrolysates. BS: blue shark. YT: yellowfin tuna. Tu: turbot. Sa: salmon. All cultures were performed in triplicate. NS: not statistically significant.

	X_m	v_x	λ_x	R^2_x	$Y_{x/p}$	m_p	$R^2_{x/p}$
GE_BS	0.654 ± 0.044^{a}	$0.075 \pm 0.029^{a,b}$	2.85 ± 1.90^{a}	0.983	1.30 ± 0.29^{a}	0.030 ± 0.029^{a}	0.985
GE_YT	0.690 ± 0.048^{a}	$0.109 \pm 0.051^{a,b}$	4.71 ± 1.69^{a}	0.982	2.16 ± 0.42^{b}	0.033 ± 0.024^{a}	0.986
GE_Tu	0.667 ± 0.053^{a}	0.046 ± 0.014^{a}	2.44 ± 2.37^{a}	0.985	1.27 ± 0.26^{a}	0.034 ± 0.018^{a}	0.995
GE_Sa	0.680 ± 0.061^{a}	0.064 ± 0.029^{a}	3.24 ± 2.63^{a}	0.976	1.31 ± 0.39^{a}	0.041 ± 0.036^{a}	0.986
CH_BS	1.00 ± 0.13^{c}	0.069 ± 0.015^{a}	4.59 ± 3.18^{a}	0.979	0.969 ± 0.288^{a}	0.028 ± 0.026^{a}	0.991
CH_YT	$1.11 \pm 0.05^{c,d}$	0.129 ± 0.035^{b}	3.70 ± 1.29^{a}	0.992	1.07 ± 0.30^{a}	0.026 ± 0.025^{a}	0.990
CH_Tu	$0.878 \pm 0.137^{b,c}$	0.053 ± 0.028^{a}	2.70 (NS)	0.953	0.869 ± 0.302^{a}	0.013 (NS)	0.980
CH_Sa	$0.949 \pm 0.105^{b,c}$	$0.067 \pm 0.036^{a,b}$	2.20 (NS)	0.979	$1.60 \pm 0.47^{a,b}$	0.038 ± 0.032^{a}	0.982
MM	$0.893 \pm 0.118^{b,c}$	$0.069 \pm 0.042^{a,b}$	0.621 (NS)	0.938	$1.47 \pm 0.38^{a,b}$	0.041 ± 0.036^{a}	0.985

2.6. Numerical Fittings and Statistical Analyses

The fits among the experimental data and parametric estimations were determined by minimizing the sum of quadratic differences between the observed and the models' predicted values, using the non-linear least squares (GRG non-linear) method provided by the macro 'Solver' from the Microsoft Excel spreadsheet. Confidence intervals from the parametric estimates (Student's *t*-test) and the consistency of the mathematical models (Fisher's F-test) were evaluated using the "SolverAid" macro (Levie's Excellaneous website: http://www.bowdoin.edu/, accessed on 15 May 2023). A one-way ANOVA test and a Tukey test were applied to discover the significant differences between the fermentation parameters of the different growth media tested. This procedure was also used to determine the difference in composition between the gelatin effluents and hydrolysates. Statistical significance was also defined as $p < 0.05$.

3. Results and Discussion

3.1. Composition of Gelatin Effluents and Collagen Hydrolysates

The pH of the substrates showed significant differences ($p < 0.05$) between the types of samples (Table 1), but without changes among species ($p > 0.05$). Thus, the range of pH values moved from 6 to 6.5 in the GE and from 8.8 to 8.9 in the CH. The concentration of protein in the gelatin effluents (2.3–4 g/L) was much lower than that found in the collagen hydrolysates (41–48 g/L). In both cases, the amounts in turbot and salmon reached significantly higher levels than those observed in the blue shark and yellowfin tuna species. The total sugar content was negligible (lower than 0.31 g/L), and the presence of lipids in the effluents was generally greater than that in the hydrolysates. The levels of fat in the GE of YT, Tu and Sa were around 1.5–2.1 g/L; meanwhile, in the CH of the same fish, fat levels were only 0.3–0.6 g/L. This difference is due to the fact that separation of the oily fraction from the fish skins was carried out mainly in the first alkaline treatment; this fraction was therefore present in the GE [48–50].

In both materials, the predominant amino acids were glycine (Gly) and glutamic acid (Glu). Aspartic acid (Asp), alanine (Ala), and in some samples, lysine (Lys), were also found in significant percentages (Tables S1 and S2, Supplementary Materials). The data for proline (Pro) and hydroxyproline (OHPro), ranging jointly between 8 and 12%, confirmed the origin of these protein sources to be the collagen present in fish skins [51]. The amount of essential amino acids (TEAA/TAA) in the CH (37–41%) was higher than that determined in GE (23–29%). In general, the eight samples showed the presence of all amino acids, quantified in significant values.

3.2. Phaeobacter Fermentations

The fermentations were performed in media formulated with the GE or CH as a protein source (instead of commercial peptone), and a marine medium (MM) was used as the control. The low amount of yeast extract (1 g/L) in the commercial broth was maintained in the alternative broths, since this ingredient is not only a source of nitrogen and carbon but is also a source of vitamins [52]. The time courses of the strain DIFR 27-4 culture in the GE and MM are displayed in Figures 2 and 3. The experimental data of marine bacterium growth were precisely modelled using a logistic equation (Table 2), and were found to be $R^2 = 0.976–0.985$ in the GE and $R^2 = 0.953–0.992$ in the CH. The data of the strain DIFR 27-4 in the control medium were the worst fitted, with $R^2 = 0.938$. The agreement between the protein consumption data and the profiles predicted by Equation (2) was better, achieving determination coefficients in the range of 0.985–0.995 and 0.980–0.991 for the GE and CH, respectively. Based on Fisher's F-test, the consistency of the fits was always verified ($p < 0.005$), and the numerical parameters of the equations were almost already statistically significant (for $\alpha = 0.05$, Student's *t*-test), but were lower in the case of two lag phases and one coefficient of maintenance.

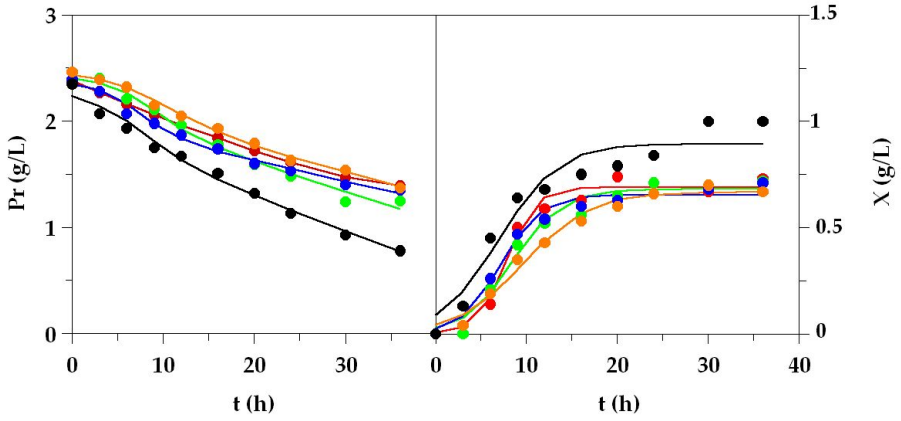

Figure 2. Cultures of the strain DIFR 27-4 on media formulated with the GE of tuna (●), turbot (●), salmon (●) and shark (●), as well as the control MM medium (●). Pr: soluble protein consumption, X: biomass production. Experimental data (points) were fitted to the logistic (1) and unstructured Equation (2) (continuous line). Error bars were omitted for clarity, but the intervals of confidence for the experimental data (for three replicates) were always lower than 20% of the mean values.

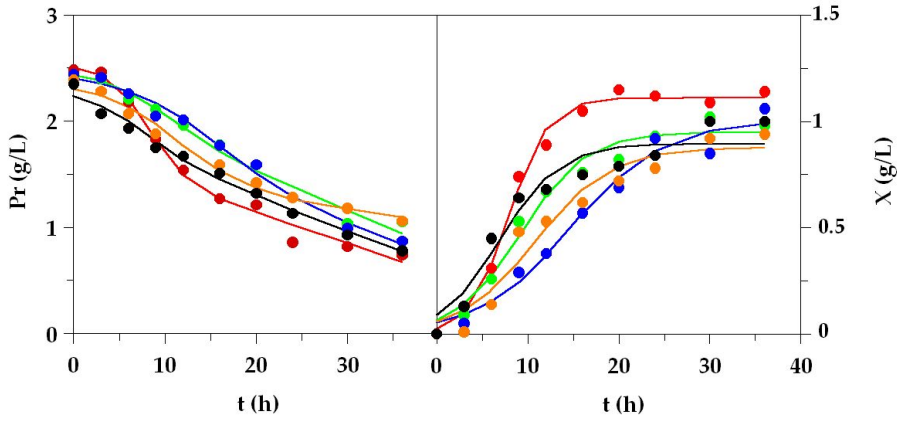

Figure 3. Cultures of the strain DIFR 27-4 on media formulated with the CH of tuna (●), turbot (●), salmon (●) and shark (●), as well as the control MM medium (●). Pr: soluble protein consumption, X: biomass production. Experimental data (points) were fitted to the logistic (1) and unstructured Equation (2) (continuous line). Error bars were omitted for clarity, but the intervals of confidence for experimental data (for three replicates) were always lower than 20% of the mean values.

CH supported the largest maximum biomass production (X_m) in comparison with the GE ($p < 0.05$). BS and YT hydrolysates led to higher growths than those obtained in the MM, Tu, and Sa. In the case of effluents, all alternative media reached similar X_m values between them, but had lower values than the MM. The maximum biomass production rates were reported in both the substrates obtained from tuna fish. The lag phases were shorter than 5 h, and in some samples (CH_Tu, CH_Sa and MM), these short periods of delay were the reason that the numerical estimations were not significant.

These results are in concordance with the cultivation data reported when peptones recovered from fish discards were employed as an ingredient in the culture of marine bacteria [36]. The amount of biomass from strain DIFR 27-4 produced at 30 h achieved values of 1 g/L, 1.11 g/L, 1.16 g/L, and 1.2 g/L in protein sources derived from alcalase hydrolysates of grenadier skin, horse mackerel head, gurnard head and swordfish skins, respectively. Accordingly, peptones from aquaculture wastes (turbot, salmon, trout, etc.) generated from thermal extraction and the enzymatic proteolysis of wastes (heads, trimmings and frames) led to maximum biomass values of around 0.8–1.1 g/L [46]. Other authors, employing a minimal medium based on seawater, vitamins, acetate, ferric EDTA, and thiosulfate, did

not exceed an optical density value (OD_{600}) of 0.5; they found values of 0.25 g/L with a calibration curve of *Phaeobacter* sp. MED 193 isolated from Mediterranean waters [53].

The growth of wild-type *P. inhibens* DSM 17439 (another member of the *Roseobacter* group) in a salt marine medium with casamino acids as a carbon source was similar to the growth of our strain; however, mutants of this bacterium, constructed to enhance the production of tropodithietic acid, doubled the growth capacity of the bacterium [54]. The substrates derived from gelatin processing may also be an adequate material for the bacterial production of that broad-spectrum antibiotic. Nevertheless, we have not found any other article that has addressed the growth of *Roseobacter* group bacteria in different culture media or explored the use of other nitrogen and carbon sources using commercial peptones, new alternative media, or agri-food and fish by-products. The production of secondary metabolites associated with *Roseobacter* strains in waste materials is also unexplored.

The yields of biomass production regarding the protein uptake ($Y_{x/p}$), calculated from Equation (2), varied from 1.27 to 2.16 gX/gPr for the GE and from 0.87–1.60 for the CH, with YT being the most effective substrate for the consumption of proteins. The values of X_0 and Pr_0 are not included in Table 2, since they are not interesting parameters in comparative terms or from a kinetic viewpoint. The coefficients of maintenance for strain DIFR 27-4 metabolism were similar in all cases (0.026–0.041 gPr g^{-1} X h^{-1}), without significant differences among the low-cost media ($p > 0.05$) being observed. The soluble protein uptake was not completed in any situation varying from 1 g/L to 1.7 g/L, leaving between 0.8–1.4 g/L of protein unconsumed by the end of fermentation (Figures 2 and 3). This failure to metabolize all protein content was also reported in broths formulated with fish protein hydrolysates [36]. It is possible that exhaustive protein consumption may be observed when performing fermentations at a constant pH (pH-stat mode) and/or employing fed-batch or continuous-culture performance [55]. Moreover, the use of different carbon to nitrogen ratios (including low concentrations of glucose in the culture media) may be a way to achieve complete depuration of gelatin effluents, as reported by research on other marine bacteria grown in commercial broths [56].

Additionally, viable colonies of bacteria were also counted at 12 and 30 h during the culturing of each media (Figure 4), in order to corroborate the results of OD determinations. The data indicated that the growth response of the viable colonies correlated with the biomass (dry weight) results (Table 2). The number of colony-forming units was higher in CH than in GE, and similar to that found in the MM. Cell growth of around 10^{13} cfu/mL at 30 h also correlated with the viable production of the strain DIFR 27-4 in fish peptones from aquaculture wastes [37]. Such a concentration of probiotic bacteria is various orders of magnitude higher than the number of viable colonies needed for inclusion in turbot cultivation [34].

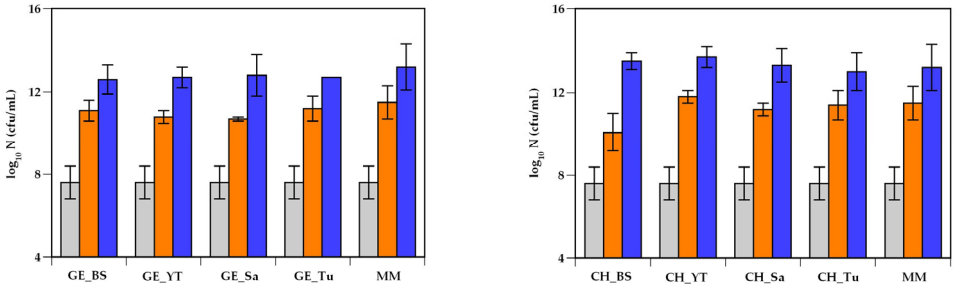

Figure 4. Strain DIFR 27-4 viable counts at different times of culture (inoculum, ■): 12 h (■) and 30 h (■), produced on the gelatin substrates studied. Experimental data are expressed as the logarithm of viable colonies ($\log_{10}$ N). Error bars are the intervals of confidence for the experimental data (for three replicates).

Finally, and based on the numerical data of X_m included in Table 2 and the prices of the ingredients present in the MM, the costs of biomass (in EUR/g) production were

calculated for each alternative media (Table 3). Thus, the cost of the gelatin substrates in comparison to the MM were reduced by around 73–78-fold and 99–125-fold in the GE and CH, respectively. This bioconversion of effluents and skin remains is vital for the integral valorization of fish skin waste. In future studies, the scaling-up of fermentations must be carried out (for example, in 2 L bioreactors) in order to reach larger dry-weight values of strain DIFR 27-4. Furthermore, all the various production processes for fish gelatin and collagen hydrolysates, including lifecycle assessment and CO_2-footprint analysis, must be considered for a complete evaluation of the circular economy.

Table 3. Costs of strain DIFR 27-4 biomass production in the commercial marine medium an in the media formulated with the GE and CH. GE: media formulated with chemical gelatin effluents. CH: media formulated with collagen hydrolysates. BS: blue shark. YT: yellowfin tuna. Tu: turbot. Sa: salmon.

Substrate	Culture Media	Biomass (EUR/g)
commercial	MM	10.14
GE_BS	Medium formulated with GE_BS	0.138
GE_YT	Medium formulated with GE_YT	0.130
GE_Tu	Medium formulated with GE_Tu	0.135
GE_Sa	Medium formulated with GE_Sa	0.132
CH_BS	Medium formulated with CH_BS	0.090
CH_YT	Medium formulated with CH_YT	0.081
CH_Tu	Medium formulated with CH_Tu	0.103
CH_Sa	Medium formulated with CH_Sa	0.095

4. Conclusions

A viable alternative for the biotechnological valorization of residues generated in the recovery of gelatin from turbot, shark, tuna, and salmon skins was proposed. Given the increasing necessity of developing sustainable procedures in fishery, canning, and aquaculture industry applications, wastewaters from chemical skin treatment and collagen hydrolysates were successfully used as the main protein ingredient in the biomass production of a marine probiotic bacterium, *Phaeobacter* sp. DIFR 27-4. This was confirmed in biomass and cell viability quantifications. From an economical perspective, the replacement of the marine broth with the proposed substrates led to a 73–125-fold reduction in the production cost of marine probiotic biomass. The procedures evaluated herein, following the concepts of a marine biorefinery, are essential to the integral recovery of biomass from fish wastes and align with the aims of the circular bioeconomy. Nevertheless, additional calculations using mass flows, energy demand, lifecycle assessment (LCA), and CO_2 footprint should be considered to determine the real sustainability of the present processes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr11082397/s1, Table S1: Amino acids content of GE, Table S2: Amino acids content of CH.

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References

1. Alipal, J.; Mohd Pu'ad, N.A.S.; Lee, T.C.; Nayan, N.H.M.; Sahari, N.; Basri, H.; Idris, M.I.; Abdullah, H.Z. A review of gelatin: Properties, sources, process, applications, and commercialisation. *Mater. Today Proc.* **2021**, *42*, 240–250. [CrossRef]
2. Chen, T.; Hou, H. Protective effect of gelatin polypeptides from Pacific cod (*Gadus macrocephalus*) against UV irradiation-induced damages by inhibiting inflammation and improving transforming growth factor-beta/Smad signaling pathway. *J. Photochem. Photobiol. B* **2016**, *162*, 633–640. [CrossRef] [PubMed]
3. Kang, M.G.; Lee, M.Y.; Cha, J.M.; Lee, J.K.; Lee, S.C.; Kim, J.; Hwang, Y.S.; Bae, H. Nanogels derived from fish gelatin: Application to drug delivery system. *Mar. Drugs* **2019**, *17*, 246. [CrossRef] [PubMed]
4. Hermida-Merino, C.; Cabaleiro, D.; Lugo, L.; Valcarcel, J.; Vázquez, J.A.; Bravo, I.; Longo, A.; Salloum-Abou-Jaoude, G.; Solano, E.; Gracia-Fernández, C.; et al. Characterization of tuna gelatin-based hydrogels as a matrix for drug delivery. *Gels* **2022**, *8*, 237. [CrossRef]
5. Ding, M.Z.; Zhang, T.; Zhang, H.; Tao, N.P.; Wang, X.C.; Zhong, J. Gelatin molecular structures affect behaviors of fish oil-loaded traditional and Pickering emulsions. *Food Chem.* **2020**, *309*, 125642. [CrossRef]
6. Leyva-Jiménez, F.J.; Manca, M.L.; Manconi, M.; Caddeo, C.; Vázquez, J.A.; Lozano-Sánchez, J.; Escribano-Ferrer, E.; Arráez-Román, D.; Segura-Carretero, A. Incorporation of *Lippia citriodora* microwave extract into total-green biogelatin-phospholipid vesicles to improve its antioxidant activity. *Nanomaterials* **2020**, *10*, 765. [CrossRef]
7. Negrini, N.C.; Bonnetier, M.; Giatsidis, G.; Orgill, D.P.; Farè, S.; Marelli, B. Tissue-mimicking gelatin scaffolds by alginate sacrificial templates for adipose tissue engineering. *Acta Biomat.* **2019**, *87*, 61–75. [CrossRef]
8. Alves, A.L.; Carvalho, A.C.; Machado, I.; Diogo, G.S.; Fernandes, E.M.; Castro, V.I.B.; Pires, R.A.; Vázquez, J.A.; Pérez-Martín, R.I.; Alaminos, M.; et al. Cell-laden marine gelatin methacryloyl hydrogels enriched with ascorbic acid for corneal stroma regeneration. *Bioengineering* **2023**, *10*, 62. [CrossRef]
9. Dille, M.J.; Haug, I.J.; Draget, K.I. Chapter 34—Gelatin and collagen. In *Woodhead Publishing Series in Food Science, Technology and Nutrition*; Phillips, G.O., Williams, P.A.B.T.-H., Third, E., Eds.; Woodhead Publishing: Sawston, UK, 2021; pp. 1073–1097. ISBN 978-0-12-820104-6.
10. Boran, G.; Regenstein, J.M. Chapter 5—Fish Gelatin. In *Advances in Food and Nutrition Research*; Taylor, S.L.B.T.-A., Ed.; Academic Press: Cambridge, MA, USA, 2010; Volume 60, pp. 119–143. ISBN 1043-4526.
11. EFSA Panel on Biological Hazards (BIOHAZ); Koutsoumanis, K.; Allende, A.; Bolton, D.J.; Bover-Cid, S.; Chemaly, M.; Davies, R.; De Cesare, A.; Herman, L.M.; Hilbert, F.; et al. Potential BSE risk posed by the use of ruminant collagen and gelatine in feed for non-ruminant farmed animals. *EFSA J.* **2020**, *18*, e06267.
12. Maree, J.P.; Cole, C.G.B.; Gerber, A.; Barnard, J.L. Treatment of gelatine factory effluent. *Water SA* **1990**, *16*, 265–268.
13. Wang, G.; Yu, N.; Guo, Y. A novel process to recycle the highly concentrated calcium and chloride ions in the gelatin acidification wastewater. *J. Clean. Prod.* **2018**, *188*, 62–68. [CrossRef]
14. Ahmad, T.; Ismail, A.; Ahmad, S.A.; Abdul Khalil, K.; Awad, E.A.; Akhtar, M.T.; Sazili, A.Q. Recovery of gelatin from bovine skin with the aid of pepsin and its effects on the characteristics of the extracted gelatin. *Polymers* **2021**, *13*, 1554. [CrossRef]
15. Fatimah, S.; Sarto, S.; Fahrurrozi, M.; Budhijanto, B. Characterization and development of gelatin from cow bones: Investigation of the effect of solvents used for soaking beef bones. *Appl. Sci.* **2023**, *13*, 1550. [CrossRef]
16. Ghatnekar, S.D.; Kavian, M.F.; Sharma, S.M.; Ghatnekar, S.S.; Ghatnekar, G.S.; Ghatnekar, A.V. Application of vermi-filter-based effluent treatment plant (pilot scale) for biomanagement of liquid effluents from the gelatine industry. *Dyn. Soil Dyn. Plant* **2010**, *4*, 83–88.
17. Tawfik, A.; Ni, S.-Q.; Awad, H.M.; Ismail, S.; Tyagi, V.K.; Khan, M.S.; Qyyum, M.A.; Lee, M. Recent approaches for the production of high value-added biofuels from gelatinous wastewater. *Energies* **2021**, *14*, 4936. [CrossRef]
18. Awasthi, M.K.; Pandey, A.K.; Bundela, P.S.; Wong, J.W.C.; Li, R.; Zhang, Z. Co-composting of gelatin industry sludge combined with organic fraction of municipal solid waste and poultry waste employing zeolite mixed with enriched nitrifying bacterial consortium. *Bioresour. Technol.* **2016**, *213*, 181–189. [CrossRef]
19. Rai, K.; Maheshwari, A. Removal of pollutants from industrial effluents using plastic clay and pyrophyllite. *Asian J. Chem.* **2002**, *14*, 739–745.
20. Badrinath, S.D.; Kaul, S.N.; Deshpande, V.P.; Gadkari, S.K. Ossein wastewater characterization and treatability study. *Water Res.* **1991**, *25*, 1439–1445. [CrossRef]

21. Blanco, G.C.; Stablein, M.J.; Tommaso, G. Cultivation of *Chlorella vulgaris* in anaerobically digested gelatin industry wastewater. *Water Supply* **2020**, *21*, 1953–1965. [CrossRef]
22. Valcarcel, J.; Hermida-Merino, C.; Piñeiro, M.M.; Hermida-Merino, D.; Vázquez, J.A. Extraction and characterization of gelatin from skin by-products of seabream, seabass and rainbow trout reared in aquaculture. *Int. J. Mol. Sci.* **2021**, *22*, 12104. [CrossRef]
23. Al-Nimry, S.; Dayah, A.A.; Hasan, I.; Daghmash, R. Cosmetic, biomedical and pharmaceutical applications of fish gelatin/hydrolysates. *Mar. Drugs* **2021**, *19*, 145. [CrossRef]
24. Vázquez, J.A.; Pedreira, A.; Durán, S.; Cabanelas, D.; Souto-Montero, P.; Martínez, P.; Mulet, M.; Pérez-Martín, R.I.; Valcarcel, J. Biorefinery for tuna head wastes: Production of protein hydrolysates, high-quality oils, minerals and bacterial peptones. *J. Clean. Prod.* **2022**, *357*, 131909. [CrossRef]
25. Vázquez, J.A.; Durán, A.I.; Menduíña, A.; Nogueira, M.; Gomes, A.M.; Antunes, J.; Freitas, A.C.; Dagá, E.; Dagá, P.; Valcarcel, J. Bioconversion of fish discards through the production of lactic acid bacteria and metabolites: Sustainable application of fish peptones in nutritive fermentation media. *Foods* **2020**, *9*, 1239. [CrossRef] [PubMed]
26. Ummadi, M.; Curic-Bawden, M. Use of protein hydrolysates in industrial starter culture fermentations. In *Protein Hydrolysates in Biotechnology*; Pasupuleti, V.K., Demain, A.L., Eds.; Springer: Dordrecht, The Netherlands, 2010; pp. 91–114. ISBN 978-1-4020-6674-0.
27. Barragán, P.J.; Sánchez, Ó.J.; Martínez, L.J. Towards valorization of bovine blood plasma: Optimal design of a culture medium based on bovine blood plasma with enzymatically hydrolyzed proteins for the growth of a probiotic bacterium by submerged fermentation. *Waste Biomass Valor.* **2022**, *13*, 1143–1155. [CrossRef]
28. Hjelm, M.; Bergh, Ø.; Riaza, A.; Nielsen, J.; Melchiorsen, J.; Jensen, S.; Duncan, H.; Ahrens, P.; Birkbeck, H.; Gram, L. Selection and identification of autochthonous potential probiotic bacteria from turbot larvae (*Scophthalmus maximus*) rearing units. *Syst. Appl. Microbiol.* **2004**, *27*, 360–371. [CrossRef] [PubMed]
29. Sonnenschein, E.C.; Jimenez, G.; Castex, M.; Gram, L. The *Roseobacter*-group bacterium *Phaeobacter* as a safe probiotic solution for aquaculture. *Appl. Environ. Microbiol.* **2021**, *87*, e02581-20. [CrossRef]
30. Ringø, E.; van Doan, H.; Lee, S.H.; Soltani, M.; Hoseinifar, S.H.; Ramasamy, H.; Song, S.K. Probiotics, lactic acid bacteria and Bacilli: Interesting supplementation for aquaculture. *J. Appl. Microbiol.* **2020**, *129*, 116–136. [CrossRef]
31. Ringø, E.; van Doan, H.; Lee, S.; Song, S.K. Lactic acid bacteria in shellfish: Possibilities and challenges. *Rev. Fish. Sci. Aquacult.* **2020**, *28*, 139–169. [CrossRef]
32. Ringø, E.; Li, X.; van Doan, H.; Ghosh, K. Interesting probiotic bacteria other than the more widely used lactic acid bacteria and bacilli in finfish. *Front. Mar. Sci.* **2022**, *9*, 848037. [CrossRef]
33. Planas, M.; Pérez-Lorenzo, M.; Hjelm, M.; Gram, L.; Uglenes Fiksdal, I.; Bergh, Ø.; Pintado, J. Probiotic effect in vivo of *Roseobacter* strain 27-4 against *Vibrio* (*Listonella*) *anguillarum* infections in turbot (*Scophthalmus maximus* L.) larvae. *Aquaculture* **2004**, *255*, 323–333. [CrossRef]
34. Prol-García, M.J.; Pintado, J. Effectiveness of probiotic *Phaeobacter* bacteria grown in biofilters against *Vibrio anguillarum* infections in the rearing of turbot (*Psetta maxima*) larvae. *Mar. Biotechnol.* **2013**, *15*, 726–738. [CrossRef]
35. D'Alvise, P.W.; Lillebø, S.; Wergeland, H.I.; Gram, L.; Bergh, Ø. Protection of codlarvae from vibriosis by *Phaeobacter* spp.: A Comparison of Strains and Introduction Times. *Aquaculture* **2013**, *384–387*, 82–85. [CrossRef]
36. Vazquez, J.A.; Duran, A.; Nogueira, M.; Menduina, A.; Antunes, J.; Freitas, A.C.; Gomes, A.M. Production of marine probiotic bacteria in a cost-effective marine media based on peptones obtained from discarded fish by-products. *Microorganisms* **2020**, *8*, 1121. [CrossRef]
37. Vázquez, J.A.; Hermida-Merino, C.; Hermida-Merino, D.; Piñeiro, M.M.; Johansen, J.; Sotelo, C.G.; Pérez-Martín, R.I.; Valcarcel, J. Characterization of gelatin and hydrolysates from valorization of farmed salmon skin by-products. *Polymers* **2021**, *13*, 2828. [CrossRef]
38. Valcarcel, J.; Fraguas, J.; Hermida-Merino, C.; Hermida-Merino, D.; Piñeiro, M.M.; Vázquez, J.A. Production and physicochemical characterization of gelatin and collagen hydrolysates from turbot skin waste generated by aquaculture activities. *Mar. Drugs* **2021**, *19*, 491. [CrossRef]
39. Sousa, S.C.; Vázquez, J.A.; Pérez-Martín, R.I.; Carvalho, A.P.; Gomes, A.M. Valorization of by-products from commercial fish species: Extraction and chemical properties of skin gelatins. *Molecules* **2017**, *22*, 1545. [CrossRef]
40. Sonnenschein, E.C.; Phippen, C.B.W.; Nielsen, K.F.; Mateiu, R.V.; Melchiorsen, J.; Gram, L.; Overmann, J.; Freese, H.M. *Phaeobacter piscinae* sp. nov., a species of the *Roseobacter* group and potential aquaculture probiont. *Int. J. Syst. Evol, Microbiol.* **2017**, *67*, 4559–4564. [CrossRef]
41. FAO. 2023. Available online: https://www.fao.org/fishery/en/culturedspecies/ (accessed on 5 May 2023).
42. Bligh, E.G.; Dyer, W.J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917. [CrossRef]
43. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350–356. [CrossRef]
44. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275. [CrossRef]
45. Moore, S.; Spackman, D.H.; Stein, W.H. Chromatography of amino acids on sulfonated polystyrene resins. An improved system. *Anal. Chem.* **1958**, *30*, 1185–1190. [CrossRef]

46. Vázquez, J.A.; Durán, A.I.; Menduíña, A.; Nogueira, M. Biotechnological valorization of food marine wastes: Microbial productions on peptones obtained from aquaculture by-products. *Biomolecules* **2020**, *10*, 1184. [CrossRef] [PubMed]
47. Kedia, G.; Vázquez, J.A.; Pandiella, S.S. Evaluation of the fermentability of oat fractions obtained by debranning using lactic acid bacteria. *J. Appl. Microbiol.* **2008**, *105*, 1227–1237. [CrossRef] [PubMed]
48. Aryee, A.N.A.; Simpson, B. Comparative studies on the yield and quality of solvent-extracted oil from salmon skin. *J. Food Eng.* **2009**, *92*, 353–358. [CrossRef]
49. Haq, M.; Ahmed, R.; Cho, Y.-J.; Chun, B.-S. Quality properties and bio-potentiality of edible oils from Atlantic salmon by-products extracted by supercritial carbon dioxide and conventional methods. *Waste Biomass Valor.* **2017**, *8*, 1953–1967. [CrossRef]
50. Ferdosh, S.; Sarker, Z.I.; Norulaini, N.; Oliveira, A.; Yunus, K.; Chowdury, A.J.; Akanda, J.; Omar, M. Quality of tuna fish oils extracted from processing the by-products of three species of neritic tuna using supercritical carbon dioxide. *J. Food Proc. Preservat.* **2015**, *39*, 432–441. [CrossRef]
51. Nitsuwatm, S.; Zhang, P.; Ng, K.; Fang, Z. Fish gelatin as an alternative to mammalian gelatin for food industry: A meta-analysis. *LWT Food Sci. Technol.* **2021**, *141*, 110899. [CrossRef]
52. Tao, Z.; Yuan, H.; Liu, M.; Liu, Q.; Zhang, S.; Liu, H.; Jiang, Y.; Huang, D.; Wang, T. Yeast extract: Characteristics, production, applications and future perspectives. *J. Microbiol. Biotechnol.* **2023**, *33*, 151–166. [CrossRef]
53. Muthusamy, S.; Baltar, F.; González, J.M.; Pinhassi, J. Dynamics of metabolic activities and gene expression in the *Roseobacter* clade bacterium *Phaeobacter* sp. strain MED193 during growth with thiosulfate. *Appl. Environ. Microbiol.* **2014**, *80*, 6933–6942. [CrossRef]
54. Will, S.E.; Neumann-Schaal, M.; Heydorn, R.L.; Bartling, P.; Petersen, J.; Schomburg, D.; Virolle, M.-J. The limits to growth—Energetic burden of the endogenous antibiotic tropodithietic acid in *Phaeobacter inhibens* DSM 17395. *PLoS ONE* **2017**, *12*, e0177295. [CrossRef]
55. Goldman, J.C.; Dennett, M.R. Growth of marine bacteria in batch and continuous culture under carbon andnitrogen limitation. *Limnol. Oceanogr.* **2000**, *45*, 789–800. [CrossRef]
56. Jiménez-Mercado, A.; Cajal-Medrano, R.; Maske, H. Marine heterotrophic bacteria in continuous culture, the bacterial carbon growth efficiency, and mineralization at excess substrate and different temperatures. *Microb. Ecol.* **2007**, *54*, 56–64. [CrossRef]