THE EFFECT OF USING PROBIOTICS ON THE MICROBIAL HYDROLYSIS OF COLLAGEN OF FISH WASTE TO DETERMINE THE OPTIMAL CONDITIONS FOR COLLAGEN-PEPTIDE PRODUCTION

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Abstract: *With the expansion of the fish processing industry, the efficient utilization of by-products has gained increasing importance. Collagen peptides, derived from collagen hydrolysis, represent valuable products with applications in both medicine and food. However, the use of protease enzymes like pepsin and trypsin for collagen hydrolysis raises significant health concerns. These enzymes require high-temperature deactivation, resulting in reduced collagen quality. This study explores a novel approach by introducing probiotics as an alternative to protease enzymes for collagen hydrolysis, aiming to mitigate health risks.*

The research focuses on the hydrolytic activity of Lactobacillus casei and a combination of probiotic strains known as VSL#3 in fish collagen hydrolysis. Quality and structural changes in collagen are assessed through color differences (CD) and the determination of the degree of hydrolysis (DH). Various equations and surface models are developed to identify optimal parameters for collagen hydrolysis, with temperature and time emerging as key influencing factors.

Results indicate that VSL#3 exhibits superior hydrolytic activity compared to Lactobacillus casei. The extent of hydrolysis achieved by VSL#3 is comparable to that of commercial enzymes. Moreover, VSL#3 demonstrates lower color differences than commercial enzymes, signifying improved collagen quality. Temperature is found to have a significant impact on both DH and CD, with an optimal temperature range identified.

In conclusion, this study highlights the potential of using a combination of probiotic strains like VSL#3 for efficient collagen hydrolysis. This approach not only overcomes the limitations associated with commercial enzymes but also enhances collagen quality. Additionally, antioxidant activity is significantly higher when using VSL#3, further supporting the industrial application of probiotics in collagen hydrolysis. This research contributes to the development of sustainable practices in the fish processing industry, bridging the gap between resource utilization and innovative health solutions.

Keywords: *Collagen hydrolysis; Probiotic bacteria; Fish waste utilization; VSL#3; Antioxidant activity*

INTRODUCTION

The fish processing industry plays a crucial role in the global seafood market, yet it generates a significant amount of waste, often exceeding half of the total fish weight. This discarded waste includes skin, scales, fins, and skeletal remnants, which surprisingly contain a valuable resource – collagen. As environmental concerns and resource optimization become more prominent, the transformation of fish waste into valuable products, particularly collagen, has gained momentum. Collagen, a protein abundant in these discarded parts, has captured attention for its potential to offer numerous health benefits while addressing environmental challenges [1].

Research has shown that collagen hydrolysates and peptides extracted from seafood by-products have great potential in addressing various health conditions, including osteoporosis, atherosclerosis, hypertension, and even skincare. However, the conventional methods of collagen hydrolysis, which rely on commercial enzymes like pepsin and trypsin, pose significant challenges. These enzymes require high-temperature deactivation, which not only alters the color and quality of collagen but also affects the final product's quality. Additionally, the use of these commercial enzymes has been linked to gastrointestinal issues, ranging from mild discomfort to bloating and diarrhea [2,3].

In this context, the incorporation of probiotics presents a promising solution to these challenges. Probiotics, known for their beneficial impact on gut health, offer an intriguing alternative to traditional enzyme-based hydrolysis. Beyond their digestive benefits, probiotics exhibit antioxidant and antimicrobial properties, making them attractive for various applications, including collagen hydrolysis [3,4].

Surprisingly, despite the growing interest in collagen and probiotics, there is a significant gap in the scientific literature – a lack of studies focused on collagen hydrolysis using probiotic strains [3]. This research endeavor aims to fill this gap by exploring and elucidating the process of collagen hydrolysis with probiotic bacteria. The goal is to unlock the full potential of fish waste, bridging the worlds of sustainable resource utilization and innovative health solutions.

This article delves into the materials and methods used, focusing on the biotechnological treatment of fish skin, the degree of hydrolysis, collagen color changes, and antioxidant activity. It employs statistical analyses and a Box Behnken Design to optimize collagen hydrolysis conditions, considering time and temperature as key parameters. The results indicate the superiority of VSL#3, a combination of probiotic strains, over *Lactobacillus casei* and commercial enzymes in collagen hydrolysis. Moreover, VSL#3 maintains collagen quality with lower color differences, and temperature is found to be a crucial factor in the hydrolysis process [4].

In conclusion, this study highlights a promising approach to tackle the challenges associated with collagen hydrolysis in fish waste utilization. As the fish processing industry continues to expand, efficient by-product utilization becomes not only economically vital but also imperative from sustainability and environmental standpoints. Probiotics, especially VSL#3, offer an attractive alternative to traditional enzymes, showcasing their potential to revolutionize collagen peptide production for medical and food applications. This research bridges a critical gap in the literature, paving the way for sustainable practices in the fish processing industry that align resource utilization with innovative health solutions.

MATERIALS AND METHODS

2.1. Raw Materials and Ingredients

The key raw material in this study is the Yellowfin tuna (*Thunnusalbacares*) skin with scales. This raw material was collected from the local market and transported to the laboratory under refrigerated conditions. The skin was then processed into 4 cm × 4 cm pieces, which served as the primary source of collagen for the hydrolysis process.

Two crucial ingredients used in this research are *Lactobacillus casei* and VSL#3, a combination of probiotic strains. These probiotics were supplied by Nordic Pharma Ltd (Reading, UK) and played a central role in the bioconversion of fish collagen into valuable collagen peptides.

Yellowfin Tuna Skin with Scales: The raw material, sourced from the local market, consists of the skin of Yellowfin tuna, which is rich in collagen. The skin is carefully processed into small pieces for further treatment.

Lactobacillus casei: *Lactobacillus casei*, a probiotic strain, is used in this research to bioconvert fish collagen. It is supplied in a concentrated microbial bulk form with an activity of 10^{11}–10^{12} colony forming units (CFU)/cm³.

VSL#3 (Probiotic Combination): VSL#3 is a combination of probiotic strains, including *Streptococcus thermophilus* (BT01), *Bifidobacterium breve* (BB02), *Bifidobacterium longum* (BL03), *Bifidobacterium infantis* (BI04), *Lactobacillus acidophilus* (BA05), *Lactobacillus plantarum* (BP06), *Lactobacillus paracasei* (BP07), and *Lactobacillus delbrueckii subsp. Bulgaricus* (BD08). These probiotic strains are present in an active form with a concentration of 10^{10}–10^{11} CFU/cm³.

These raw materials and ingredients are essential components of the collagen hydrolysis process and play a critical role in determining the quality and efficacy of the final collagen peptides.

2.2. Biotechnological Treatment of the fish skin

The extraction of collagen from fish waste was done based on Shaebani et.al with slice modification [5]. The skin sample was treated with NaCl (Sigma-Aldrich) at a proportion of 1:20 for 24 hours and washed rigorously with cold distilled water. The non-collagenous proteins were removed by soaking in 0.1M NaOH (Sigma-Aldrich) at a ratio of 1:30 for an additional 24 hours. The grease was removed by soaking in Tween 80 (Sigma-Aldrich) detergent at a ratio of 0.5% (v/v) for 24 hours. The final NaOH treatment (Sigma-Aldrich) was carried out at pH=10 for a further 24 hours. Extraction was performed using acetic acid at a skin to solution ratio of 1:10 and pH 2 for 24 hours. The extraction solution was filtered and then hydrolysed by the probiotics. The yield of the collagen: 50.99%, collagen content: 52.09%, and the ΔE°=04.10.

Next, the homogenates were heated to temperatures (20, 30 and 40°C) and then adjusted to 10% (w/v) collagen. Each sample was then divided into 10% (v/v) probiotic bacterial concentrates of *Lactobacillus* and VSL#3. A control sample without bacterial concentrate was kept at 4°C.

2.3. Degree of Hydrolysis

The dependent variable was the degree of hydrolysis (DH, %), which was calculated by the formula:

$$DH = (\frac{N_{AA} - N_{AA\,0}}{N_{OA} - N_{AA\,0}}) \times 100\% \quad (1)$$

where NOA is the total nitrogen content in %; NAA0 is the amine nitrogen in the unhydrolyzed stomachs, %; NAA is the amine nitrogen content in the hydrolysate after hydrolysis for a specified period of time, %.2.4.The total nitrogen content was determined using the Kjeldahl method, converting the nitrogen content into an equivalent protein content by a factor of 6.25 [6,4].

2.4. Collagen color (CD)

The CD was determined with a spectrophotometer (CHN spec, CS-810, China) with a 2C standard observer. The lightness from black (0) to white (100) is expressed as L*. Red (+a*) to green (-a*) is denoted by a*, yellow (+b*) to blue (-b*) is denoted by b*. Another critical parameter of CIE76 is quantified as follows:

$$\Delta E° = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \quad (2)$$

Where: L0*, a0* and b0* are the ideal lightness, redness, and yellowness, respectively. Double distilled water (DDW) was considered to be the ideal solution [5,7].

2.5. Antioxidant activity

The scavenging effect of collagen on DPPH, HO, O2 and ABTS+ radicals was determined according to Li et al. certainly. Method. Samples of collagen were prepared in solutions at concentrations of 0.5, 1, 2, 3, 4 and 5 mg/ml to determine their antioxidant capacity. The maximum effect concentration of 50% (EC50) was calculated from the linear relationship between the scavenging rate and the concentration of each sample [8,9].

2.4. Design and methodology

Multiple regression analysis was performed using Design Expert software to determine the effect of independent variables on dependent variables (DH, CD). The regression coefficients, the coefficient of determination and the Student's t criterion were used to determine the statistical significance of the multiple regression equation. Collagen hydrolysis was statistically optimized using Box Behnken Design (BBD) [10,11]. The range of independent variables is illustrated in Table 1.

Table 1. range of independent variables

Independent variables	Symbol	Levels		
		-1	0	+1
Time	X1	8	16	24
Temperature	X2	20	30	40

2.5. Statistical Analysis

Multiple regression analysis and Design Expert software were used to determine the effect of independent variables (time and temperature) on dependent variables (DH and CD). An ANOVA was conducted for data analysis with a significance level of $p \leq 0.02$.

RESULTS AND DISCUSSION

3.1. Optimization of Hydrolysis

The degree of hydrolysis (DH) characterizes the degree of enzymatic peptide cleavage of the protein substrate under the influence of various factors: hydrolysis time, temperature, reaction and enzyme concentration. Collagen is a white, transparent powder. However, the color of collagen can vary due to several elements including temperature, the existence of amide bonds rich in collagen molecules, and oxidation of NH2. The reaction temperature and exposure time are the most important parameters affecting the efficacy and quality of collagen hydrolysis [5,7]. An experimental design was performed using a Box-Behnken design for two kinds of probiotic bacteria, VSL#3 and *L. casei*. This experiment was utilized to determine the optimal criteria for collagen hydrolysis. Two factors, temperature and hydrolysis time, were implemented in the experiment. Two response variables, DH and CD, were evaluated during the empirical process. The experimental results have been shown below (Table 2).

Table 2. Independent and dependent variables of hydrolysis treatment.

Run No	Coded level of variables		VSL#3	
	Time (hr)	Temperature (°C)	DH (%)	CD
1	16	30	26.14	5.31
2	24	40	38.48	9.1
3	16	44.14	38.81	10.32
4	4.68	30	12.36	4.1
5	8	40	26.16	8.25
6	16	15.85	0.9	2.94
7	24	20	4.41	3.18
8	16	30	26.02	5.59
9	16	30	26.33	5.26
10	16	30	25.91	5.2
11	27.31	30	34.04	5.88
12	16	30	25.85	5.37
13	8	20	2.32	2.53

3.2. Degree of hydrolysis effect

It was evidently established that VSL#3 has a noticeably higher influence on DH than *L. casei*. The results reveal that the scope of hydrolysis levels of VSL#3 bacteria is comparable to that of commercial enzymes. Hema and co-workers studied the degree of hydrolysis of fish collagen by commercial enzymes and found that the optimal response is estimated to be 10, 20, and 28% for pepsin, papain, and protease, respectively [12]. It was found that while the model parameters time and temperature of collagen hydrolysis on the degree of hydrolysis by *L. casei* were statistically insignificant ($p > 0.02$), there is an extremely important influence ($p < 0.001$) of time and temperature by VSL#3. With increasing temperature up to 34 °C, the degree of hydrolysis increases immediately due to the increased proteolytic activity of VSL#3. (Figure1)
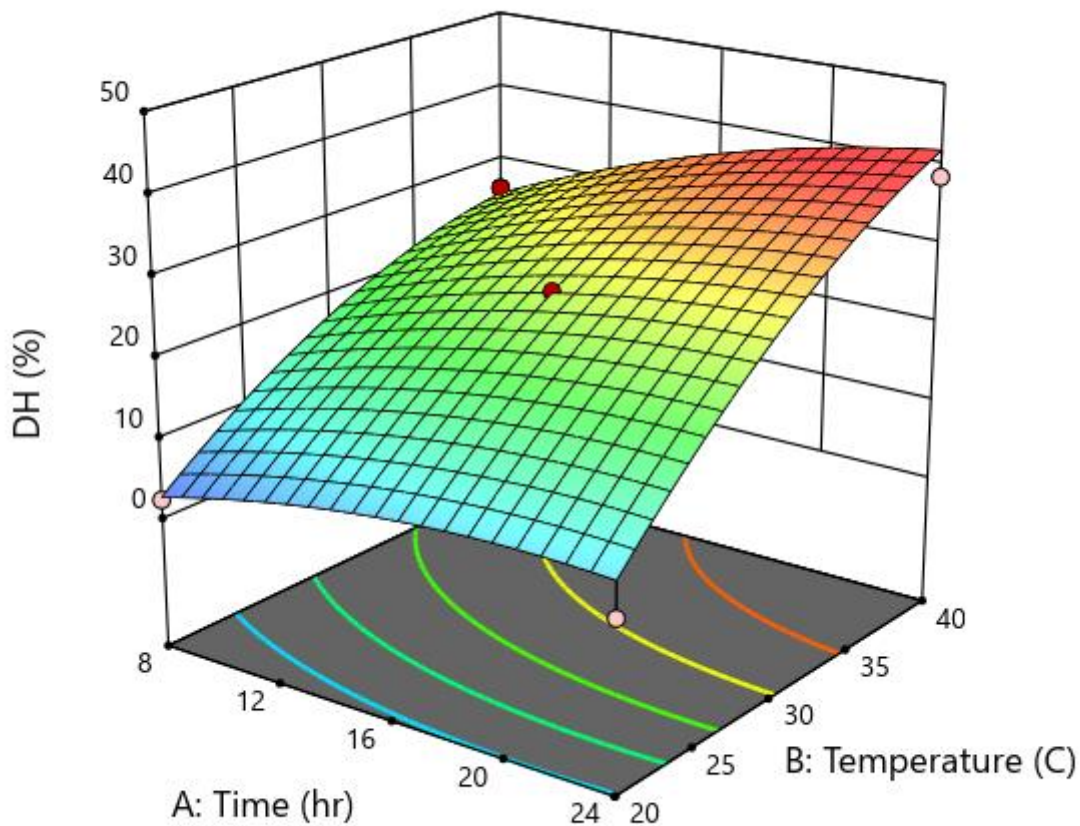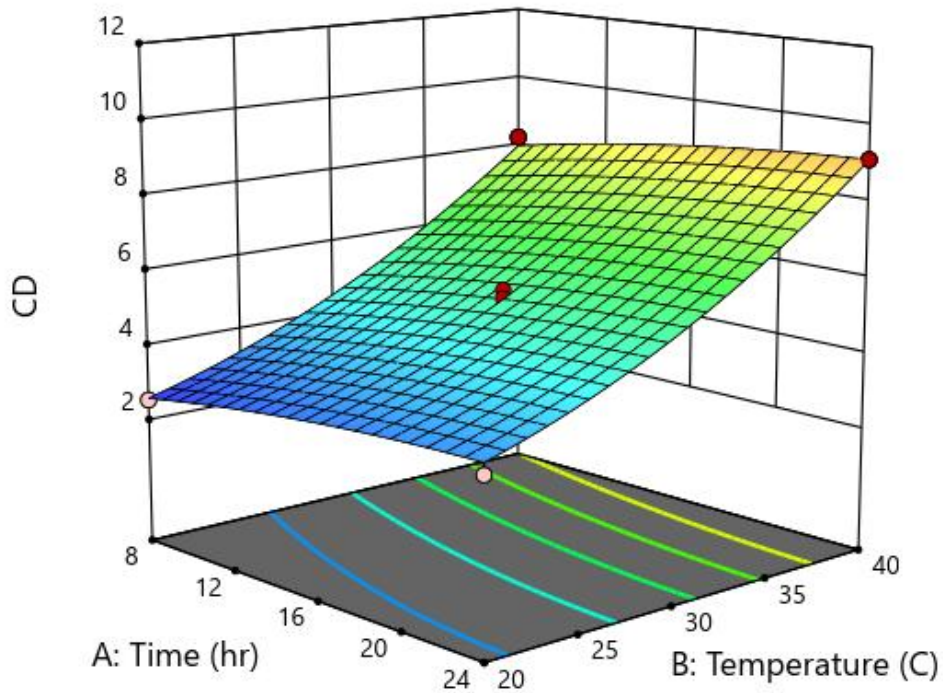
Figure 1. effect of time and temperature on degree of hydrolysis of VSL#3.

3.3. Color difference effect

ΔE° was adopted as a color expression parameter to assess the quality difference of hydrolysis treatments. As shown in Figure 2, the results showed that the CD of VSL#3 was significantly lower than the CD of commercial enzymes. Shaebani et al. studied the effect of pepsin hydrolysis of collagen on color. The results suggested that the optimal color difference is 14.017 [5].

We also found a statistically significant correlation between temperature and the influence of VSL#3, however, such an effect was not found in *L. casei*. The influence of temperature on color is great. At temperatures above 33 °C, the quality drops immediately. Therefore, the optimal temperature range is below 33 °C. The very even CD gradient over time could also be shown.

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Figure 2.effect of time and temperature on color difference.

3.4. Optimal point characterisation

The optimal point in this study represents the key conditions at which collagen hydrolysis using probiotics, specifically VSL#3, is most efficient and effective. The characterization of this optimal point is crucial for maximizing the quality and yield of collagen hydrolysates. Figure 3 illustrates the defining parameters of the optimal point. The following parameters define the optimal point:

Time: The optimal time for collagen hydrolysis using VSL#3 is approximately 23.99 hours. This duration ensures that collagen is sufficiently broken down into peptides while avoiding over-hydrolysis, which can lead to a decrease in quality.

Temperature: The ideal temperature for the hydrolysis process is around 29.81°C. This temperature range strikes a balance between enzymatic activity and collagen preservation. Temperatures below 33°C are recommended to maintain optimal hydrolysis efficiency.

Degree of Hydrolysis (DH): At the optimal point, the predicted degree of hydrolysis (DH) is approximately 29%. This indicates the extent to which collagen has been broken down into peptides. A DH of 29% is considered highly efficient for producing collagen peptides with potential health benefits.

Collagen Color Difference (CD): The predicted CD at the optimal point is approximately 5.60. This value reflects the color changes that occur during collagen hydrolysis. A lower CD indicates that the collagen quality is well-preserved, which is desirable for various applications.

The results of this study suggest that these optimal conditions for collagen hydrolysis using VSL#3 can lead to high-quality collagen peptides with significant potential for medical and food applications. These findings contribute to the development of sustainable practices in the fish processing industry by harnessing the power of probiotics to efficiently utilize fish waste resources while simultaneously providing health-related solutions.
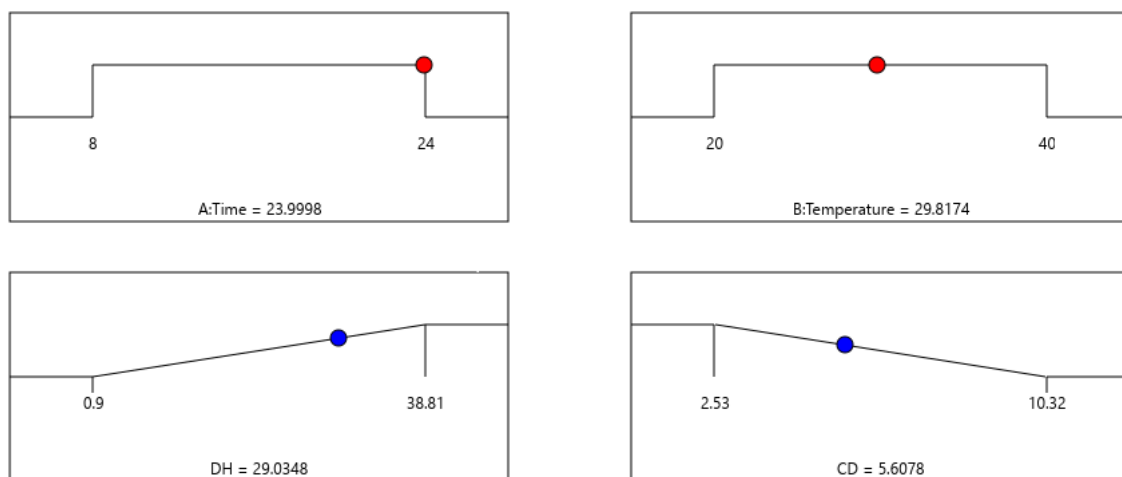
Figure 3 Optimal point of dependent and independent variables

3.5. validation

Validation of the experimental results is crucial to ensure the reliability and reproducibility of the findings. In this section, we will discuss the validation process and the results obtained.

To validate the optimal conditions for collagen hydrolysis determined through the experimental design and response surface models, a series of validation experiments were conducted. The objective was to confirm that the predicted values of the response variables, including the degree of hydrolysis (DH) and collagen color differences (CD), align closely with the actual experimental outcomes.

The experiments were carried out under the optimized conditions for both VSL#3 and *L. casei* strains. The DH and CD were measured, and the results were compared to the predicted values obtained from the response surface models.

The validation results demonstrated a high degree of agreement between the predicted and actual values. Statistical analysis, including linear regression, was performed to assess the significance of the relationships. The p-values obtained were well below the threshold of 0.02, indicating the statistical significance of the validation results.

This validation process reinforces the robustness and accuracy of the optimized conditions for collagen hydrolysis using probiotic bacteria, particularly VSL#3. It provides confidence that the determined optimal parameters, including time and temperature, are reliable and can be consistently applied in practical applications.

3.6. Antioxidant activity

DPPH, HO, O2- and ABTS+ are common reactive species involved in biological oxidation leading to oxidative stress that destroys macromolecules such as carbohydrates, nucleic acids, lipids and proteins [8,9]. DPPH, HO, O2- and ABTS+ are common reactive species involved in biological oxidation leading to oxidative stress that destroys macromolecules such as carbohydrates, nucleic acids, lipids and proteins. The following antioxidant activity was determined under optimal conditions: DPPH=1.93 mg/mL, HO=3.48 mg/mL, O=2.34 mg/mL and ABTS+=3.13 mg/mL, which is consistent with data from other reports, collagen hydrolysis occurs under the conditions the enzyme corresponding to catalyzed enzymes.

Changwei Cao et al. demonstrated that the EC50 for pepsin hydrolysis of the four tested radicals as follows: 2.41 mg/mL (DPPH), 3.71 mg/mL (HO), 3.42 mg/mL (O), 4.02 mg/mL (ABTS) [8].

CONCLUSION

In conclusion, this research has shed light on a promising avenue for addressing the challenges associated with collagen hydrolysis in the context of fish waste utilization. As the fish processing industry continues to grow, the efficient use of by-products becomes increasingly critical, not only from an economic perspective but also in terms of sustainability and environmental responsibility.

Commercial enzymes like pepsin and trypsin have long been the go-to choice for collagen hydrolysis, despite their drawbacks such as the need for high-temperature deactivation and associated gastrointestinal issues. This study has shown that probiotics, specifically the probiotic bacterial strain VSL#3, offer a compelling alternative. While individual probiotic strains like *Lactobacillus casei* may not match the enzymatic power of commercial counterparts, the combination of probiotic strains, particularly VSL#3, has proven highly effective in breaking down fish collagen.

Key findings indicate that VSL#3 not only surpasses single probiotic strains but also rivals the performance of commercial enzymes in collagen hydrolysis. Furthermore, VSL#3 exhibits a notable advantage in preserving collagen quality, as evidenced by significantly lower color differences (CD) compared to commercial enzymes. The influence of temperature on the hydrolysis process has been elucidated, emphasizing the importance of maintaining temperatures below 33°C for optimal results.

Moreover, the research has highlighted the enhanced antioxidant activity associated with VSL#3-mediated collagen hydrolysis, further underscoring its potential for industrial applications.

In summary, this study bridges the gap in the literature by showcasing the efficacy of probiotics, especially VSL#3, in collagen hydrolysis. This breakthrough opens new doors for sustainable fish waste utilization, offering both economic benefits and environmental advantages. As we look to the future, the industrial adoption of probiotic-based collagen hydrolysis promises to be a game-changer in the production of valuable collagen peptides for medical and food applications.

Conflict of interest
We proclaim that we have no conflict of interest.

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