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OPTIMIZED HYDROLYSIS IMPROVES sn-2 FATTY ACID PROFILE AND NUTRITIONAL QUALITY OF *Thunnus* sp.

Y. C. E. Silalahi[1,2], Masfria[2,✉], S. M. Sinaga[2] and R. Siburian[3]
[1]Department of Pharmacy, Universitas Sari Mutiara Indonesia, Medan-20123, Indonesia
[2]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Sumatera Utara, Medan-20155, Indonesia
[3]Department of Chemistry, Faculty of Mathematics and Science, Universitas Sumatera Utara, Medan-20155, Indonesia
✉Corresponding author: masfria@usu.ac.id

ABSTRACT
Overall nutritional quality of marine fish oil depends both on the total fatty acid profile and on the specific stereo numbering of fatty acids within the triglyceride molecules. This study integrated bibliometric analysis, enzymatic hydrolysis optimization, and nutritional quality assessment of *Thunnus* sp. oil. Bibliometric mapping using VOSviewer based on Scopus data (2015–2025) revealed three major research clusters related to omega-3 fatty acids, marine lipid processing, and nutritional interventions. Hydrolysis optimization was conducted using Response Surface Methodology, varying pH (7–9), temperature (40–60 °C), and hydrolysis time (6–10 hours). The optimal conditions identified were pH 8.1, 40.4 °C, and 7.8 hours, yielding a predicted hydrolysis percentage of 64.30%, which was experimentally validated with an actual mean hydrolysis of 64.32% and a standard deviation of 9.27%. Gas chromatography analysis revealed that polyunsaturated fatty acids, particularly EPA and DHA, were predominantly located at the sn-2 position. Nutritional Quality Indices based on sn-2 distribution showed a favorable ω-6/ω-3 ratio (0.267), a moderate P/S ratio (0.368), a low Atherogenicity Index (0.993), and a near-optimal Hypocholesterolemic/Hypercholesterolemic ratio (0.934). Although the Thrombogenicity Index (0.777) was slightly above the ideal threshold, the overall lipid profile supports advantages for cardiovascular health. These findings support the nutritional potential of *Thunnus* sp. oil, especially its sn-2-enriched fractions, as a promising functional food ingredient and nutraceutical source for cardiovascular health.
Keywords: sn-2, Fatty Acid, Marine, Design Expert, RSM, Bibliometric, NQI

INTRODUCTION

Marine fish oils are recognized as essential dietary sources of omega-3 fatty acids from the polyunsaturated fatty acids (PUFA) group, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which offer several health benefits, especially in supporting neurological, cardiovascular, and metabolic functions.[1,2] However, the nutritional quality of marine oils is not determined solely by their total fatty acid content; rather, the specific stereo numbering of fatty acids within triglyceride (TG) molecules critically affects their bioavailability and physiological impact.[3,4] In the process of lipid digestion, pancreatic lipase plays a crucial role by specifically hydrolyzing ester bonds at the sn-1,3 positions within the TG molecule. This targeted action ensures the fatty acid (FA) at the sn-2 position remains intact. Consequently, the sn-2 fatty acid remains preserved as a 2-monoacylglycerol and is directly absorbed through the intestinal mucosa without further enzymatic cleavage. This metabolic pathway underscores the biological importance of the sn-2 position: fatty acids located at sn-2 are more efficiently incorporated into plasma lipids and tissue membranes compared to the sn-1,3 positions.[5–8] Oils enriched with essential fatty acids, particularly DHA and EPA, at the sn-2 position thus have greater potential to exert beneficial health effects, enhancing cardiovascular protection, anti-inflammatory responses, and cognitive function.[9,10] Bibliometric mapping of global research trends between 2015 and 2025 using VOSviewer revealed an increasing focus on marine fatty acids, lipid extraction technologies, and nutritional applications.[11,12] Despite this interest, comprehensive studies that integrate bibliometric insights with enzymatic hydrolysis optimization and detailed positional fatty acid profiling, especially for

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tropical marine species such as Thunnus sp., remain scarce.[13,14] Accurate optimization of enzymatic hydrolysis is critical to reliably isolate and characterize the stereospecific of fatty acids, specifically the sn-2 position, thereby providing deeper insights into the true nutritional value of marine-derived oils.[15–17] Given these considerations, this study aims to map the scientific research trends related to marine fatty acids and nutrition through bibliometric analysis, optimize the enzymatic hydrolysis conditions of *Thunnus* sp. oil using Response Surface Methodology (RSM) to maximize hydrolysis efficiency, and evaluate the nutritional quality indices (NQI) based on the fatty acids distribution at the sn-1,3 and sn-2. This integrated approach aims to deliver a thorough understanding of the nutritional potential of *Thunnus* sp. oil, supporting its future development as a functional food ingredient or nutraceutical source. This work supports the advancement of marine oils as sustainable, high-value nutraceuticals, contributing to SDG-3 for Good Health and Well-being.

EXPERIMENTAL

Materials

The study utilized access to the Scopus database for bibliometric data collection, analyzed using VOSviewer software v1.6.20 (Leiden University, The Netherlands). Experimental design and optimization were conducted with Design Expert software v13 (Stat-Ease Inc., USA), and statistical analysis was performed using IBM SPSS Statistics v26 (IBM Corp., USA). Fatty acid analysis was conducted using a Shimadzu GC-14B gas chromatograph (Japan) with an FID detector and a DB-225 capillary column. All solvents and reagents were of analytical grade.

Bibliometric Analysis

Bibliometric data were retrieved from the Scopus database in Research Information System (RIS) format using the keywords "marine" AND "fatty acid" AND "nutrition". The search was limited to English-language articles published between 2015 and 2025. The RIS was imported into VOSviewer, applying a minimum word occurrence threshold of 10 times. The analysis generated a network visualization map, along with overlay visualization and density visualization maps. Additionally, the distance attribute of each value was used to evaluate the "link" and "total link strength" between terms within the research field.[11,12,18]

Extraction of Thunnus sp. Oil

The extraction of *Thunnus* sp. oil was conducted using the solvent n-hexane at a controlled temperature (25 to 30 °C).[19,20] The solvent was eliminated via rotary evaporation, and the oil was preserved at -20 °C until analysis. The extracted oil was characterized through several key analyses, including the measurement of % yield, acid, peroxide, saponification, and iodine value using AOAC standard methods.

Gas Chromatography (GC) Analysis of Fatty Acid

A total of 25 mg of oil was methylated to form fatty acid methyl esters (FAMEs) prior to GC analysis. The GC-14B instrument from Shimadzu, Japan, featuring a FID and a DB-225 capillary column, was utilized for the analysis.[21,22] The operating conditions were standardized with both the injector and detector temperatures set at 260 °C, while nitrogen (N_2) was used as the carrier gas at a pressure of 100 kPa. The column temperature was initially set to stabilize at 200 °C for a duration of 5 minutes, after which it was increased at a rate of 7 °C per minute until it attained 220 °C, where it was held for an additional 10 minutes. Peaks were identified by comparing retention times with known FAME standards, and results were expressed as mol % of total identified fatty acids.

Enzymatic Hydrolysis Optimization

Enzymatic hydrolysis was optimized using Response Surface Methodology (RSM) in Design Expert v13.[17,23,24] For each run, 10 g of oil was mixed into a 125 ml Erlenmeyer flask with 25 ml of 1 M Tris-HCl buffer solution, 10 ml of 2.2% $CaCl_2$, 100 mg lipase, and 20 ml of distilled water. Then vortexed at 240 rpm, then incubated under varying pH, temperature (^oC), and time (hours). The hydrolysis process targeted three critical factors identified as primary influencers: pH (x1), which ranges from 7 to 9; temperature (x2), maintained between 40 and 60 °C; and time (x3), varying from 6 to 10 hours. These parameters are crucial for optimizing the efficiency and effectiveness of the hydrolysis process, highlighting the dynamic relationship between enzymatic activity and environmental conditions. During

incubation, continuous stirring was maintained at a speed of 200 rpm to enhance the reaction kinetics. Hydrolysis was stopped by adding 25 ml of n-hexane and repeating the extraction. After phase separation, the upper layer containing fatty acids was collected and labeled as Filtrate I. The residual aqueous layer was subjected to a second extraction using another 25 ml of n-hexane, yielding Filtrate II after separation. Combined extracts were dried over anhydrous Na_2SO_4 for 10 minutes, and then evaporation. Hydrolysis percentage was calculated using the formula presented below:[25–27]

% Hydrolysis = $\frac{BA_t\text{-}BA_o}{BP_o\text{-}BA_o} x\,100\,\%$, BA_o = Acid value before hydrolysis, BA_t = Acid value after hydrolysis, BP_o = Saponification value before hydrolysis.

The determination of the sn-2 ratio was conducted employing the following formula:[2,28]

$$[\text{sn-2}] = (3 \times [\text{TAG}] - 2\,[\text{sn-1,3}])$$

Nutritional Quality Indices (NQI)

NQI of *Thunnus* sp. oil was assessed based on five key indicators derived from its fatty acid composition, using the following parameters:[29,30]

ratio of ω-6 to ω-3

polyunsaturated/saturated fatty acid (P/S) ratio

indices of atherogenicity (IA)

IA = [(C12:0 + (4 × C14:0) + C16:0)] / (MUFA + n-6 PUFA + n-3 PUFA)

indices of thrombogenicity (IT)

IT = (C14:0 + C16:0 + C18:0 / [(0,5 × MUFA) + (0,5 × n-6 PUFA) + (3 × n-3 PUFA) + (n-3 PUFA/n-6 PUFA)]

hypocholesterolemic/hypercholesterolemic (HH) ratio, was determined according to the formulas proposed by Ulbricht and Southgate (1991).[30,31]

HH = (C18:1 n-9 + C18:2 n-6 + C20:4 n-6 + C18:3 n-3 + C20:5 n-3 + C22:5 n-3 + C22:6 n-3)/(C14:0 + C16:0)

Recommendations for NQI Pertaining to Fatty Acid Composition

The table below presents a comprehensive summary of the Nutritional Quality Index (NQI) parameters, established based on fatty acid composition, to provide guidelines for evaluating the nutritional profile of fish oils. The indices considered include the ω-6/ω-3 ratio, the P/S, the IA, the IT, and the HH ratio. Each of these metrics is associated with specific recommended thresholds indicative of optimal nutritional quality, namely,[30,31]

An ω-6/ω-3 ratio between 1 and 4,

A P/S ratio greater than 0.45,

An IA value lower than 1,

An IT value below 0.5, and

An HH ratio exceeding 1.

The systematic arrangement of these parameters facilitates a thorough evaluation of the health-promoting properties of fish oils by emphasizing key aspects of their fatty acid composition that are particularly relevant to cardiovascular and general health outcomes.

RESULTS AND DISCUSSION

Bibliometric Mapping and Its Relevance to Hydrolysis Optimization and Nutritional Assessment of *Thunnus* sp.

A bibliometric analysis was conducted to systematically explore research trends in the field of marine fatty acids and nutrition. Data were collected from the Scopus database using the keywords "marine," "fatty acid," and "nutrition," restricted to publications in English between 2015 and 2025. The search yielded a total of 511 documents, which were exported in Research Information System (RIS) format for further analysis. This dataset served as the foundation for constructing a comprehensive bibliometric map using VOSviewer version 1.6.20.[11,12,18,32] Upon processing the RIS file, 41,856 terms were initially identified. Applying a minimum occurrence threshold of 10 times, 1,264 terms met the selection criteria.

From these, 758 terms were chosen based on relevance scoring, resulting in a highly refined network visualization that captured the most significant conceptual connections. The bibliometric analysis revealed three major clusters, each representing a distinct thematic research focus.

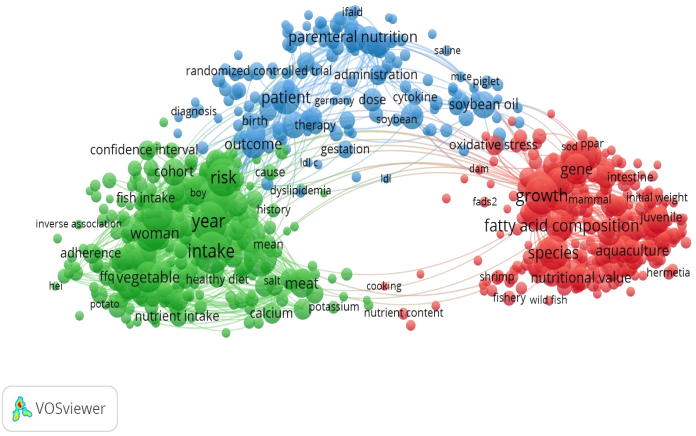

Fig.-1: Bibliometric Network Visualization of Research Trends in Marine Fatty Acids and Nutrition (2015–2025)

The green cluster predominantly covers studies related to dietary intake, nutritional epidemiology, and public health. Terms such as intake (355 occurrences, 0.53 relevance), risk (280 occurrences, 0.44 relevance), cohort, and nutrient intake were prominent in this cluster. This area emphasizes the role of marine-derived FAs in promoting health outcomes and preventing chronic diseases, which underlines the importance of evaluating the nutritional quality of specific lipid sources, such as *Thunnus* sp. oil. The blue cluster focuses on clinical applications and therapeutic interventions, highlighted by terms like patient (218 occurrences, 0.61 relevance), therapy, parenteral nutrition, and clinical trial. Research within this cluster investigates the therapeutic roles of fatty acids in disease management, metabolic regulation, and recovery processes. The findings emphasize the need for bioavailable lipid structures, particularly emphasizing the significance of FAs at the sn-2 position, which are more efficiently metabolized. This directly correlates with the present study's objective of optimizing hydrolysis conditions to enrich sn-2 fatty acid content. The red cluster is characterized by biochemical mechanisms, fatty acid profiling, species differentiation, and aquaculture innovation. Important keywords include species (220 occurrences, 0.79 relevance), growth (292 occurrences, 0.62 relevance), fatty acid composition (193 occurrences, 0.82 relevance), fatty acid profile (179 occurrences, 0.67 relevance), and gene (173 occurrences, 1.03 relevance). This cluster demonstrates the increasing scientific interest in understanding species-specific lipid extraction patterns and metabolic pathways, areas directly addressed by characterizing the sn-2 fatty acid distribution in *Thunnus* sp. Integrating these bibliometric findings, the current study is strategically positioned at the intersection of major global research themes: nutritional optimization, clinical nutrition, and marine biochemical profiling. Ultimately, this research not only advances the understanding of marine lipid bioavailability but also contributes to functional food development and supports sustainable aquaculture by enhancing the value of *Thunnus* sp. as a high-quality lipid source for human health.

An overlay visualization was generated using VOS viewer software to illustrate the temporal evolution of research related to marine fatty acids and nutrition. Each node on the map represents a keyword, and its color reflects the average publication year associated with that term, based on a color gradient transitioning from blue (representing previous years) to yellow (indicating more current years). In this visualization, keywords associated with earlier research, such as patient, therapy, parenteral nutrition, and outcome, appear in blue, indicating that these clinical and therapeutic topics were the primary focus around 2019. Conversely, keywords such as fatty acid composition, species, aquaculture, and nutritional value are shaded in yellow, suggesting that biochemical profiling, species-specific fatty acid research, and applications in aquaculture nutrition have gained prominence in more recent years, particularly around 2021.

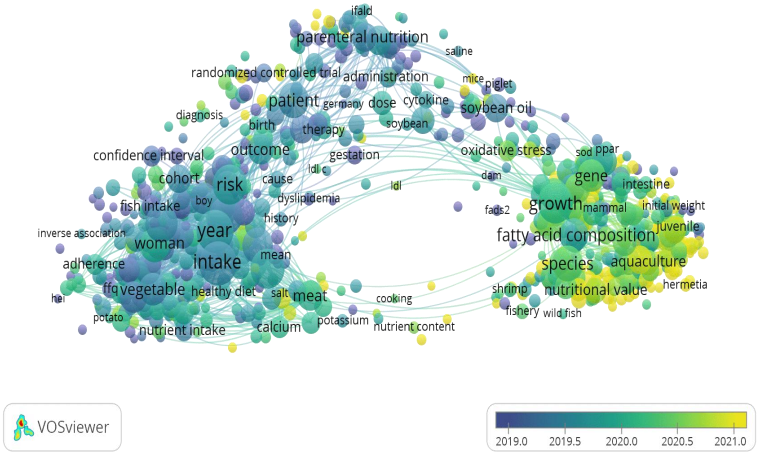

Fig.-2: Overlay Visualization of Research Trends on Marine Fatty Acids and Nutrition

This progression indicates a notable shift in the research landscape, from clinical nutrition interventions toward a deeper exploration of marine species lipid profiles and their implications for human health and sustainable aquaculture. The current study, which focuses on hydrolysis optimization and nutritional assessment based on sn-2 fatty acid distribution in *Thunnus* sp., aligns closely with these emerging research themes. By optimizing the enzymatic hydrolysis process and characterizing the fatty acid distribution, this work addresses key areas of contemporary scientific interest, providing valuable contributions to both marine biochemistry and functional food development.

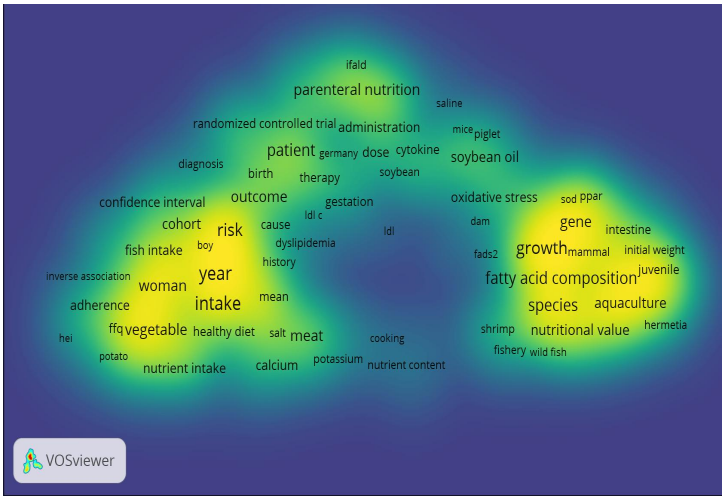

Fig.-3: Density Visualization of Research Topics Related to Marine Fatty Acids and Nutrition (2015–2025)

The density visualization map generated by VOS viewer illustrates the concentration and co-occurrence intensity of research keywords related to marine, fatty acids, and nutrition. In the density map, areas highlighted in yellow represent regions of high term frequency and strong co-occurrence, while green and blue areas indicate progressively lower densities. The most densely populated areas of the map are centered around keywords such as intake, year, risk, patient, growth, fatty acid composition, species, and nutritional value. This indicates that these topics have constituted the main focus of scientific discussions in this research field over the past decade. High-density regions reflect established and intensively studied areas, notably the relationships between dietary fatty acid intake, health risks, patient nutrition management, and fatty acid characterization in marine species. Conversely, keywords located in lower-density (green to blue) areas, such as oxidative stress, shrimp, juvenile, and wild fish, suggest emerging or less extensively explored themes. This pattern underscores an ongoing diversification of research topics, with a growing interest in aquaculture species, oxidative mechanisms, and functional lipid profiles. The current study, which focuses on hydrolysis optimization and nutritional assessment of *Thunnus* sp. based

on sn-2 fatty acid distribution, is well aligned with the high-density topics identified, particularly within the themes of fatty acid composition, species differentiation, and nutritional value. By targeting a critical yet evolving research niche, this study contributes meaningfully to advancing the field of marine lipid science and functional nutrition.

Chemical Characteristics of *Thunnus* sp.

The oil yield from *Thunnus* sp. was 18.074% ± 0.0424, indicating a relatively high extraction efficiency. A high oil yield reflects the significant potential of *Thunnus* sp. as a promising source of marine lipids for both nutritional and industrial purposes. The acid value of the oil was measured at 0.380 ± 0.014 mg KOH/g, reflecting a low concentration of free fatty acids. A low acid value suggests that the oil experienced minimal hydrolytic degradation and maintains good freshness and quality, making it suitable for further processing or direct consumption.[33,34] The saponification value was measured at 205.146 ± 4.430 mg KOH/g, which offers insights into the average molecular weight of the FAs present in the oil. A saponification value in this range is typical for oils rich in long-chain FAs, which are often associated with beneficial nutritional properties, particularly omega-3.[33,35] The peroxide value of 4.553 ± 1.229 meq O_2 /kg indicates the extent of primary oxidation. This value suggests that the oil maintains acceptable oxidative stability, as peroxide values below 5 meq O_2 /kg are generally considered to indicate good quality and low oxidation.[33] Finally, the iodine value recorded at 47.025 ± 2.786 g I_2 /100g reflects a high content of unsaturation in the fatty acid composition. High iodine values are characteristic of oils rich in PUFA, such as EPA and DHA, which are known for their positive effects on human health, including cardiovascular benefits.[35] The chemical characteristics of *Thunnus* sp. Oil, marked by high yield, low acid value, moderate saponification value, acceptable peroxide value, and high iodine value, demonstrates its potential as a high-quality source of marine oils, particularly for applications requiring oils rich in unsaturated fatty acids.

Optimization of Enzymatic Hydrolysis Method Utilizing RSM with Design Expert

The enzymatic hydrolysis of *Thunnus* sp. oil was optimized to achieve maximal hydrolysis efficiency by systematically adjusting critical process variables. Three independent factors were considered: pH, temperature, and reaction time, all of which are known to influence the activity and specificity of lipase enzymes.[24] To efficiently explore the relationships between these factors and the hydrolysis response. RSM is a robust statistical tool that enables the development of predictive models, the evaluation of factor interactions, and the identification of optimal operating conditions with a reduced number of experimental runs. In this study, experimental design and data analysis were conducted using Design Expert Version 13, a specialized software for statistical modeling and optimization.[17,23] Through the implementation of RSM, a series of systematically varied experimental runs were generated, allowing the construction of a second-order polynomial model. This model was then used to predict and validate the conditions that maximize enzymatic hydrolysis, thereby enhancing the understanding of the process behavior. The following sections present the experimental results and the statistical analysis derived from this optimization study, including the response data and the ANOVA outcomes. These provide insights into the significance of each factor and its contribution to the enzymatic hydrolysis efficiency.[17]

Table-1: Optimization of Enzymatic Hydrolysis Parameters (pH, Temperature, Time) for *Thunnus* sp. Oil Using RSM

Run	Factor 1: pH	Factor 2: Temperature (°C)	Factor 3: Time (h)	Response: % Hydrolysis
1	9	40	10	35.295
2	9	47.5	6	32.592
3	8.1	40.4	7.8	64.698
4	9	60	6	28.494
5	7	40	10	38.594
6	8.1	40.4	7.8	60.857
7	7.75	60	6	25.979
8	7.04	51	7.8	60.482
9	7.66747	48.445	6	28.294

10	7	60	10	30.292
11	9	60	8.5	41.977
12	7.04	51	7.8	60.798
13	7	40	6	30.994
14	8.1	51	9.92	36.691
15	7.04	51	7.8	30.964
16	8.1	51	9.92	36.641
17	8.37	53.5	7.28	60.733
18	9	49	8.8	42.886
19	7.88	60	8.72	64.282
20	8.1	40.4	7.8	60.473

Table-1 presents the experimental design and corresponding hydrolysis efficiencies (%) obtained during the enzymatic hydrolysis of *Thunnus* sp. oil using Lipozyme TL IM lipase. The main objective of this investigation was to selectively hydrolyze the triglyceride (TG) molecules at the FA of sn-1,3, enabling the subsequent assessment of fatty acids naturally occupying the sn-2 position. The enzymatic reaction conditions were optimized by varying three parameters: pH (7.0–9.0), temperature (40–60°C), and time (6–10 hours). A total of 20 experimental runs were conducted based on RSM. The percentage of hydrolysis reflects the extent of selective cleavage at the outer glycerol positions (sn-1,3), while leaving the sn-2 position relatively intact. The highest hydrolysis efficiency (64.698%) was recorded under the conditions of pH 8.1, temperature 40.4°C, and hydrolysis time 7.8 hours (Run 3). Other runs, such as Runs 8, 12, 19, and 20, also achieved hydrolysis efficiencies greater than 60%, indicating consistent enzymatic performance under moderately alkaline and moderate thermal conditions.

Table-2: ANOVA for the Enzymatic Hydrolysis Optimization Model of *Thunnus* sp. Oil

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3052.99	9	339.22	3.95	0.0217	significant
A-pH	0.1915	1	0.1915	0.0022	0.9633	
B-Temperature	138.04	1	138.04	1.61	0.2339	
C-Time	24.58	1	24.58	0.2859	0.6045	
AB	2.86	1	2.86	0.0332	0.859	
AC	89.29	1	89.29	1.04	0.3322	
BC	0.6612	1	0.6612	0.0077	0.9319	
A^2	234.85	1	234.85	2.73	0.1294	
B^2	30.98	1	30.98	0.3603	0.5617	
C^2	2253.09	1	2253.1	26.2	0.0005	
Residual	859.86	10	85.99			
Lack of Fit	261.78	5	52.36	0.4377	0.8072	not significant
Pure Error	598.08	5	119.62			
Cor Total	3912.85	19				

Table-2 summarizes the ANOVA for the enzymatic hydrolysis optimization model of *Thunnus* sp. oil. The ANOVA was conducted to assess the statistical significance of the overall model as well as the individual and interaction effects of the independent variables pH, temperature, and time on the response variable, which is the percentage of hydrolysis. The model was statistically significant ($p = 0.0217$) with an F-value of 3.95, indicating that the model can reliably describe the relationship between the studied factors and hydrolysis efficiency. This result confirms that the second-order polynomial model generated through RSM was appropriate for optimizing the enzymatic hydrolysis process. Among the individual factors, none of the main effects (A-pH, B-Temperature, or C-Time) reached statistical significance ($p > 0.05$). The pH variable exhibited a particularly high p-value ($p = 0.9633$), suggesting that pH variations within the selected range had a minimal direct impact on hydrolysis efficiency. Temperature ($p = 0.2339$) and time ($p = 0.6045$) similarly showed no significant individual effects. Similarly, the interaction terms between variables (AB, AC, and BC) were not significant, indicating that combinations of these factors did not produce a synergistic or antagonistic effect on the % hydrolysis within the experimental range.

However, one important observation was that the quadratic term for time (C^2) was highly significant ($p = 0.0005$) with a substantial F-value of 26.2. This finding suggests that the relationship between hydrolysis efficiency and reaction time is nonlinear and that optimizing time is crucial for achieving higher degrees of hydrolysis. On the other hand, the quadratic terms for pH (A^2) and temperature (B^2) were not significant but indicated a moderate contribution to the model. The lack of fit for the model was found to be not significant ($p = 0.8072$), demonstrating that there was no significant deviation between the experimental and predicted values, and confirming that the model fits the experimental data well. Additionally, the relatively low residual and pure error values support the adequacy and precision of the model. The ANOVA results validate the significance of the overall model for predicting the enzymatic hydrolysis of *Thunnus* sp. oil. Although pH, temperature, and time individually did not exert significant direct effects, the quadratic influence of time emerged as a critical factor, emphasizing the importance of carefully controlling the reaction duration to maximize hydrolysis efficiency.

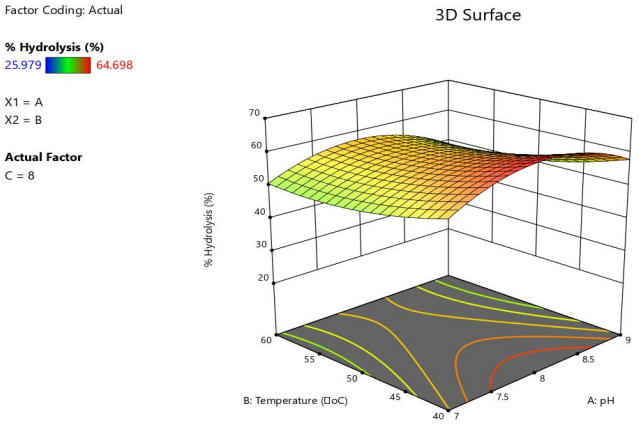

Fig.-4: 3D Response Surface Plot Showing the Effect of pH and Temperature on % Hydrolysis of *Thunnus* sp. Oil at a Constant Time of 8 Hours

Figure-4 illustrates the combined effects of pH (X-axis) and temperature (Y-axis) on the percentage of hydrolysis (Z-axis) during the enzymatic hydrolysis of *Thunnus* sp. oil. The reaction time was held constant at 8 hours, allowing for the assessment of how variations in pH and temperature influence hydrolysis efficiency. The color gradient on the surface plot corresponds to the % hydrolysis, ranging from low values (approximately 25.979%) in blue to high values (up to 64.698%) in red. A clear curvature is observed in the surface, indicating a nonlinear interaction between pH and temperature. However, the interaction term (AC) was not found to be statistically significant. From the plot, it can be observed that the highest hydrolysis percentages occur at a moderate pH (around 8.0–8.3) and lower to moderate temperatures (40–50°C). As the temperature increases beyond 50°C or the pH deviates from the optimal range, the hydrolysis efficiency tends to decrease. These findings highlight the sensitivity of the lipase activity to both pH and thermal conditions, with enzyme deactivation likely occurring at higher temperatures or extreme pH levels.

The surface's relatively smooth shape and the gentle slope changes suggest that while both pH and temperature influence the enzymatic activity, neither factor acts in isolation to alter hydrolysis outcomes drastically within the studied range. Instead, maintaining both parameters within moderate levels ensures optimal enzymatic performance and higher hydrolysis efficiency. The 3D response surface analysis provides visual confirmation of the trends observed in the statistical analysis, reinforcing the conclusion that moderate pH and temperature conditions are critical for maximizing the enzymatic hydrolysis of *Thunnus* sp. oil when the reaction time is fixed at 8 hours.

Figure-5 shows the contour plots representing the relationships between pH (X-axis), temperature (Y-axis), and the outcomes of (a) desirability and (b) percentage of hydrolysis during the enzymatic hydrolysis of *Thunnus* sp. oil. In this optimization study, the reaction time was maintained constant at approximately 8.19 hours, while pH and temperature were varied to identify the optimal conditions for hydrolysis. In the desirability plot (left side), the desirability index ranges from 0 (least desirable) to 1

(most desirable). The plot indicates that the maximum desirability is achieved at a moderate pH (approximately 8.1–8.3) and lower to mid-range temperatures (approximately 40–50°C).

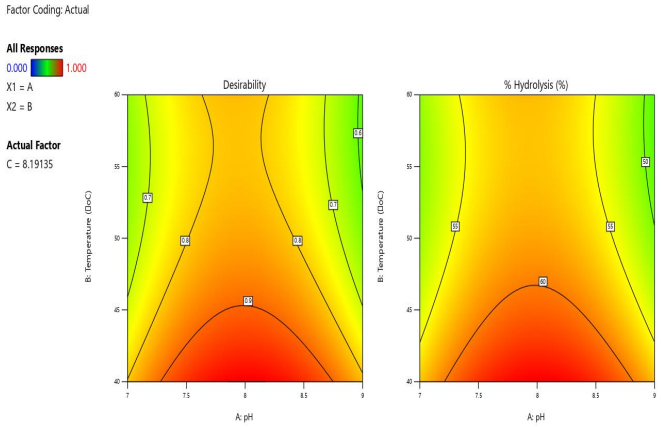

Fig.-5: Contour Plots of Desirability and % Hydrolysis for Enzymatic Hydrolysis of *Thunnus* sp. Oil Based on pH and Temperature at a Fixed Reaction Time of 8.19 Hours

These regions, marked by a more intense red color, correspond to conditions where the optimization objectives — maximizing % hydrolysis — are best satisfied. The % hydrolysis plot (right side) visually supports this trend, showing that hydrolysis efficiency is maximized under similar conditions. The highest hydrolysis percentages occur at pH around 8.1 and temperatures between 40°C and 50°C, as indicated by the red and orange areas of the plot. In contrast, at extreme pH values (below 7.0 or above 9.0) and higher temperatures (above 55°C), hydrolysis percentages significantly decrease, as represented by the green zones. Both contour plots demonstrate that moderate pH and temperature settings are critical to achieving optimal enzymatic hydrolysis of *Thunnus* sp. oil. Moreover, the similarity between the desirability and hydrolysis plots validates that maximizing hydrolysis directly correlates with achieving optimal process desirability. These contour plots provide a clear graphical representation of the optimal processing window, reinforcing that controlled, moderate reaction conditions are essential to enhance hydrolysis efficiency in the enzymatic treatment of *Thunnus* sp. oil.

Table-3: Confirmation Test of Predicted and Actual Hydrolysis Percentages at Optimal Conditions

Run	pH	Temperature (°C)	Time (h)	Actual % Hydrolysis	Predicted % Hydrolysis	Deviation (Actual-Predicted)
1	8.1	40.4	7.8	65.84	64.30	1.54
2				63.59		-0.71
3				64.58		0.28
4				63.82		-0.48
5				64.11		-0.19
6				63.98		-0.32
Average Actual				64.32	64.30	0.02
Predicted Mean					64.30	
Predicted Median					64.30	
Standard Deviation				9.27		
Standard Error Prediction					6.34	
95% PI Low					50.17	
95% PI High					78.43	
Validation Result						Model Confirmed

Table-3 presents the results of the confirmation test comparing the predicted hydrolysis percentage with the actual experimental values obtained under the optimized conditions (pH 8.1, temperature 40.4 °C, and time 7.8 hours) for the enzymatic hydrolysis of *Thunnus* sp. oil. Six independent experimental runs were conducted to validate the predictive model created using RSM. Actual hydrolysis percentages obtained

ranged from 63.59% to 65.84%, with an average actual hydrolysis of 64.32%. The predicted mean hydrolysis percentage was 64.30%, closely matching the observed mean value with a deviation of only +0.02%. The model's standard deviation across the six runs was 9.27, indicating acceptable experimental reproducibility. The standard error of prediction (SE Pred) was calculated at 6.34, and the 95% prediction interval ranged from 50.17% to 78.43%, encompassing the observed data mean. The high degree of agreement between the predicted and actual experimental values, together with the low deviation and acceptable standard error, substantiates the robustness and predictive capability of the established model for optimizing the enzymatic hydrolysis of *Thunnus* sp. oil. Thus, the model can be reliably used for process optimization and further application studies involving marine oil hydrolysis.

Table-4: Regression Coefficients and Significance Levels for the Hydrolysis Optimization Model

	Intercept	A	B	C	AB	AC	BC	A^2	B^2	C^2
% Hydrolysis	58.553	-0.138	-3.734	1.632	-0.741	-4.002	0.349	-7.601	2.723	-23.836
p-values		0.963	0.234	0.605	0.859	0.332	0.932	0.129	0.562	0.0005

Table-4 presents the regression coefficients and corresponding p-values for the model describing the enzymatic hydrolysis efficiency of *Thunnus* sp. oil. The coefficients represent the estimated effects of each independent variable (pH = A, Temperature = B, Time = C), their interactions (AB, AC, BC), and their quadratic terms (A^2, B^2, C^2) on the response (% hydrolysis). The intercept value of 58.553 indicates the baseline hydrolysis percentage when all coded factors are at their center points. Among the linear terms, none (A, B, C) demonstrated statistical significance ($p > 0.05$), suggesting that simple linear changes in pH, temperature, or time individually did not have strong direct effects on the hydrolysis percentage within the studied range. Among the interaction terms (AB, AC, BC), no significant interactions were observed, as indicated by their high p-values ($p > 0.05$). However, among the quadratic terms, the C^2 (Time squared) term was highly significant ($p = 0.0005$, in bold), indicating that the reaction time had a strong nonlinear effect on the hydrolysis efficiency. The negative coefficient of -23.836 for C^2 suggests that excessively long or short reaction times reduce hydrolysis efficiency, and an optimal time range exists to maximize the process. The regression analysis confirms that while individual linear and interaction effects were not significant, the quadratic effect of time was critical, influencing the hydrolysis outcome in a nonlinear fashion. This emphasizes the importance of carefully optimizing the reaction time to achieve maximum enzymatic hydrolysis efficiency in *Thunnus* sp. oil. The study by Monte et al. (2015) utilized Lipozyme TL IM as the biocatalyst, and notable methodological differences exist between this research and previous work on diacylglycerol (DAG) production via glycerolysis of fish oil. In the earlier study, the primary optimization factors included temperature, reaction time, glycerol-to-oil molar ratio, and enzyme loading, whereas pH was not considered a critical parameter.[36] This omission is significant, as pH plays a crucial role in influencing lipase activity, stability, and overall hydrolysis efficiency, particularly in aqueous or partially aqueous systems. In contrast, the present study incorporated pH as one of the major optimization variables, enabling a more comprehensive assessment of enzymatic performance under controlled conditions.[37]

Overall, the optimization of enzymatic hydrolysis conditions for *Thunnus* sp. oil using Response Surface Methodology and Design Expert Version 13 successfully identified the key parameters influencing hydrolysis efficiency. The reaction time emerged as the most critical factor, exhibiting a significant quadratic effect on the hydrolysis outcome. The model demonstrated a strong predictive ability, as confirmed by the tight correlation between predicted and actual experimental results during validation. These findings underscore the necessity of optimizing enzymatic hydrolysis parameters to enhance the release and characterization of naturally occurring sn-2-positioned fatty acids in *Thunnus* sp. oil. The established model provides a robust foundation for further applications in marine oil processing and functional food development.

Specificity of Fatty Acids at sn-1,3 and sn-2 in Triglyceride

Characterization of fatty acid composition is fundamental to understanding the nutritional value of marine oils.[31,38] The total fatty acid profile of *Thunnus* sp. oil was determined, with a specific focus on the distribution of FAs at the sn-1,3 and sn-2 positions within the triglyceride. This positional analysis is

essential, as the configuration of fatty acids influences both their metabolic fate and their bioavailability during digestion.[5] The detailed fatty acid distribution results are displayed in Table-5.

Table-5: Fatty Acid Distribution at sn-1,3 and sn-2 Positions in *Thunnus* sp. Oil (n = 3)

Fatty Acid	Total FA	Ratio sn-1,3	Ratio sn-2
C 10:0		0.136 ± 0.005	0.271 ± 0.010
C 12:0		0.292 ± 0.002	0.585 ± 0.005
C 14:0	3.974 ± 0.605	2.713 ± 0.011	6.495 ± 1.791
C 15:0	1.245 ± 0.033	1.084 ± 0.004	1.565 ± 0.107
C 16:0	33.107 ± 0.076	36.568 ± 0.003	26.186 ± 0.233
C 17:0	2.445 ± 0.088		
C 18:0	13.811 ± 0.156	10.744 ± 0.036	19.946 ± 0.394
C 20:0	0.586 ± 0.006		1.757 ± 0.019
C 21:0	0.524 ± 0.022		1.571 ± 0.066
C 24:0		0.268 ± 0.011	0.536 ± 0.022
SFA	55.690 ± 0.388	51.804 ± 0.005	58.910 ± 1.493
C 15:1		0.135 ± 0.001	0.269 ± 0.003
C 16:1	4.167 ± 0.130	4.341 ± 0.009	3.819 ± 0.409
C 17:1	0.988 ± 0.059	1.050 ± 0.002	0.863 ± 0.180
C 18:1	18.706 ± 0.457	18.054 ± 0.011	20.010 ± 1.393
C 20:1	0.932 ± 0.029	1.744 ± 0.035	0.693 ± 0.017
C 22:1	2.447 ± 0.095	3.023 ± 0.016	1.295 ± 0.316
C 24:1	0.530 ± 0.021	0.780 ± 0.026	0.030 ± 0.011
MUFA	27.769 ± 0.240	29.127 ± 0.048	26.977 ± 0.857
C 18:2	1.640 ± 0.103	1.389 ± 0.001	2.141 ± 0.308
C 18:3	0.976 ± 0.047	0.438 ± 0.042	2.052 ± 0.059
C 20:2		0.151 ± 0.001	0.301 ± 0.001
C 20:3		0.135 ± 0.001	0.270 ± 0.001
C 20:4	2.990 ± 0.127	2.543 ± 0.029	3.883 ± 0.437
C 20:5	0.664 ± 0.001		1.992 ± 0.004
C 22:2		0.145 ± 0.001	0.291 ± 0.002
C 22:6	10.273 ± 0.350	14.269 ± 0.066	2.280 ± 1.182
PUFA	16.542 ± 0.629	19.069 ± 0.053	13.210 ± 1.987

Table-5 illustrates the detailed fatty acid composition and distribution of the sn-1,3 and sn-2 within triglyceride molecules in *Thunnus* sp. oil. SFA were found to be the predominant group, accounting for 55.690% of the total fatty acids, with a lower concentration observed at the sn-1,3 positions (51.804%) contrast to sn-2 (58.910%). MUFA represented 27.769% of the total fatty acids, with a relatively balanced between sn-1,3 (29.127%) and sn-2 (26.977%). Oleic acid (C18:1n-9) was the major MUFA detected across both positions. PUFA, which includes essential fatty acids (EFAs), constituted 16.542% of the total fatty acids. Notably, the PUFA content at the sn-2 position (13.210%) was lower than that at the sn-1,3 positions (19.069%), contrary to the typical enrichment observed in other marine oils. Nevertheless, linoleic acid (C18:2n-6) and DHA, C22:6n-3, were prominently detected at the sn-2 position. DHA, a critical n-3 fatty acid linked to cardiovascular and cognitive health benefits, demonstrated significant positioning at the sn-2 site, which is advantageous because fatty acids located at the sn-2 are preferentially absorbed during the digestive process. The structural distribution of FAs, especially the location of essential FAs at the sn-2 position, is crucial for determining the nutritional quality of *Thunnus* sp. oil. This configuration directly impacts the calculation of Nutritional Quality Indices (NQI), which assess the health-promoting potential of lipids based on their fatty acid profiles.[30,31] By analyzing the positional distribution, particularly at the sn-2 position where bioavailability is maximized, the NQI values derived will provide a more accurate assessment of *Thunnus* sp. oil's nutritional quality. This step is essential for positioning *Thunnus* sp. oil as a valuable resource in the development of functional foods and nutraceutical formulations aimed at promoting human health. A more careful examination reveals that the stearic acid (C18:0) content at the sn-2 position in the present study was considerably higher

(19.946%) compared to the value reported by Zhang et al. (2018) (3.26%). This notable difference suggests a divergent lipid metabolic pattern, possibly due to species-specific characteristics, environmental factors, or differences in the analytical or enzymatic hydrolysis methods used. While Zhang *et al.* reported a higher content of PUIFA, especially DHA and EPA, at the sn-2 position, the present study found a predominance of SFA, particularly palmitic (C16:0) and stearic acids (C18:0), at the sn-2 position of *Thunnus* sp. oil. This finding highlights potential variations in the nutritional properties and metabolic bioavailability profiles of tuna oils depending on the specific species and processing methods. The present study involved the optimization of enzymatic hydrolysis conditions, including pH, temperature, and time, to maximize the release of sn-2 fatty acids using RSM. In contrast, Zhang et al. (2018) directly analyzed triacylglycerol compositions using HPLC with APCI-MS without optimizing hydrolysis parameters. While their method focused on TAG molecular identification, the approach used in this study more closely simulates biological digestion, offering deeper insight into the bioavailability of FAs at the sn-2 position.[34]

Nutritional Quality Indices (NQI) Analysis Based on Fatty Acid Distribution

To further evaluate the nutritional potential of *Thunnus* sp. oil, Nutritional Quality Indices (NQI) were calculated based on the FAs at the sn-2 and sn-1,3 positions. These indices provide a comprehensive assessment of the balance between saturated and unsaturated fatty acids, along with their associated implications for cardiovascular health based on fatty acid distribution. The calculated values are summarized in Table-6.

Table-6: Nutritional Quality Indices (NQI) Based on FA at sn-2 and sn-1,3 (n = 3)

NQI	Total FA	Ratio sn-1,3	Ratio sn-2	Recommendation
ω 3	11.912 ± 0.399	14.706 ± 0.024	6.324 ± 1.245	
ω 6	4.630 ± 0.230	3.932 ± 0.028	6.024 ± 0.745	
ω 6/ω 3	0.389 ± 0.006	0.267 ± 0.001	0.953 ± 0.071	1 – 4
P/S	0.297 ± 0.013	0.368 ± 0.001	0.224 ± 0.039	> 0.45
AI	1.106 ± 0.066	0.993 ± 0.001	1.326 ± 0.226	< 1
TI	0.934 ± 0.029	0.777 ± 0.001	1.441 ± 0.194	< 0.5
HH	0.951 ± 0.022	0.934 ± 0.001	0.990 ± 0.079	> 1

Table-6 presents the NQI calculated based on the FA compositions at the sn-2 and sn-1,3, as well as the total FA profile of *Thunnus* sp. oil. The indices assessed include ω-3 and ω-6 fatty acid contents, the ω-6/ω-3 ratio, the P/S, the AI, the TI, and the HH ratio. The ω-3 FA was highest at sn-2 position (14.706 ± 0.024%), indicating a strong enrichment of essential fatty acids at a metabolically favorable site for absorption. Meanwhile, the total fatty acid content for ω-3 was 11.912 ± 0.399%, and the lowest value was recorded at the sn-1,3 position (6.324 ± 1.245%). This distribution suggests that *Thunnus* sp. oil has a high potential for delivering bioavailable ω-3 FAs beneficial for cardiovascular and cognitive health. In contrast, the ω-6 fatty acid was higher at the sn-1,3 position (6.024 ± 0.745%) compared to the sn-2 position (3.932 ± 0.028%), contributing to a favorable ω-6/ω-3 ratio of 0.267 ± 0.001 at sn-2 — substantially lower than the sn-1,3 ratio (0.953 ± 0.071). The sn-2 ω-6/ω-3 ratio meets the recommended value range of 1–4, promoting an anti-inflammatory nutritional profile. The P/S ratio at sn-2 was 0.368 ± 0.001, slightly below the recommended threshold (>0.45), while at sn-1,3 it was even lower (0.224 ± 0.039). Despite this, the high sn-2 ω-3 enrichment compensates for cardiovascular health promotion. Regarding cardiovascular risk indices, the Atherogenicity Index (AI) at sn-2 was 0.993 ± 0.001 (meeting the ideal recommendation <1), and the Thrombogenicity Index (TI) was 0.777 ± 0.001, slightly above the optimal threshold (<0.5) but still acceptable. These findings suggest that *Thunnus* sp. oil has a relatively low atherogenic and thrombogenic risk, especially when considering the ω-3 abundance at sn-2. Lastly, the Hypocholesterolemic/Hypercholesterolemic (HH) ratio at sn-2 was 0.934 ± 0.001, approaching the recommended value (>1), reflecting a positive effect on lipid metabolism. The present study differs from Tilami et al. (2018) and Eugênia et al. (2016), although all three address the assessment of FA composition and NQIs in fish species. Both Tilami et al. and Eugênia et al. analyzed the total lipid profiles and calculated NQIs such as ω-6/ω-3 ratio, PUFA/SFA ratio, IA, and IT ratio to evaluate the general nutritional value of freshwater fish. Their approaches involved direct fatty acid profiling without

positional analysis or hydrolysis treatment.[31,39] Overall, the results demonstrate that *Thunnus* sp. oil, particularly through its sn-2 fatty acid distribution, exhibits excellent nutritional properties and holds significant potential for application in functional foods aimed at promoting human health.

Relevance to SDG-3

By enhancing the bioavailability of omega-3 fatty acids through optimized enzymatic processing and demonstrating favorable NQI values, this study contributes directly to SDG 3 for Good Health and Well-being. The findings support the development of sustainable, health-promoting marine-derived products to aid in the prevention of noncommunicable diseases.

CONCLUSION

This study comprehensively evaluated the hydrolysis optimization and nutritional quality of *Thunnus* sp. oil through an integrated approach involving bibliometric mapping, enzymatic hydrolysis optimization, and fatty acid nutritional assessment. The bibliometric analysis using VOSviewer revealed the global research trends on marine fatty acids and nutrition between 2015 and 2025. Three primary research clusters were identified: (1) the exploration of omega-3 PUFAs and their health benefits, (2) advances in lipid extraction and processing technologies, and (3) nutritional interventions involving marine-derived functional foods. These findings provided a solid foundation for framing the relevance and novelty of the present study within the current scientific landscape. Optimization of the enzymatic hydrolysis process was successfully achieved using RSM with Design Expert. The optimal conditions were determined to be a pH of 8.1, a temperature of 40.4 °C, and a reaction time of 7.8 hours, yielding a predicted hydrolysis efficiency of 64.30%. Experimental confirmation validated the model, with an actual hydrolysis mean of 64.32% and a minimal deviation of 0.02%, demonstrating the robustness and accuracy of the developed optimization model. Nutritional assessment of *Thunnus* sp. oil based on the stereospecific of fatty acids revealed that essential fatty acids, particularly DHA, C22:6n-3, were favorably positioned at the sn-2 site of triacylglycerols, which is known to enhance bioavailability during digestion. Although the total PUFA content was slightly lower at the sn-2 position compared to sn-1,3, the distribution of key essential FAs at sn-2 supports the nutritional value of *Thunnus* sp. oil. Nutritional Quality Indices (NQI) including ω-6/ω-3 ratio (0.953 %), PUFA/SFA ratio (0,224 %), Atherogenicity Index (1.326 %), Thrombogenicity Index (1.441 %), and Hypocholesterolemic/Hypercholesterolemic (0.990 %) ratio. Further confirmed the favorable nutritional profile of the oil, with most indices aligning with recommended healthy ranges. In conclusion, *Thunnus* sp. oil demonstrates promising potential as a high-value marine lipid source for application in functional food development. The integration of bibliometric insights, optimized hydrolysis processing, and detailed nutritional quality assessment underscores the scientific and practical relevance of this study for future marine biotechnology and nutraceutical applications. In line with SDG 3 in Good Health and Well-being, these findings support the utilization of Thunnus sp. Oil in functional foods and nutraceuticals to promote cardiovascular health and reduce the risk of non-communicable diseases. Future research should explore the feasibility of large-scale processing, oxidative stability during storage, and clinical validation of health benefits to accelerate product development and commercial application.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

The present work is related to *SDG-3: Good Health and Well-being*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

Yosy Cinthya Eriwaty Silalahi https://orcid.org/0000-0001-6641-1433
Masfria https://orcid.org/0000-0001-6087-4349

Siti Morin Sinaga https://orcid.org/0000-0001-6641-1433
Rikson Siburian https://orcid.org/0000-0001-7836-0389

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