A comprehensive study of tuna dry fish protein hydrolysates (DFPH) via papain-mediated hydrolysis and fermentation with *Lactobacillus delbrueckii*

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Abstract

Tuna dry fish demonstrates remarkable potential in nutraceutical applications due to its high content of essential proteins and bioactive compounds. This research examines the bioactive characteristics of fermented tuna dry fish protein hydrolysate (DFPH) which was produced through enzymatic papain hydrolysis followed by Lactobacillus delbrueckii fermentation. The bioactivity assessment of both fermented and non-fermented DFPH involved antioxidant testing using DPPH radical scavenging evaluation and inflammatory response testing via albumin denaturation inhibition analysis alongside MTT testing against HCT 116 colorectal cancer cells to determine anticancer activity.

This research demonstrates that fermenting DFPH led to enhanced bioactive properties resulting in improved radical scavenging ability at IC_{50} = 107.91 µg/mL and enhanced anti-inflammatory capacity at IC_{50} = 198.06 µg/mL along with cytotoxicity against cancer cells. The bioactive compounds identified by GC-MS analysis within fermented DFPH showed antioxidant, anticancer and anti-inflammatory properties. This study highlights that fermentation processes improve bioactive features of tuna dry fish hydrolysates making them suitable for food production and pharmaceutical applications.

Keywords: Tuna dry fish protein hydrolysate, Fermentation, Antioxidant activity, Anti-inflammatory activity, Anticancer activity.

Introduction

Dried fish serves as a significant provider of essential nutrients such as crude protein, amino acids, water, lipids, fatty acids and minerals. The proximate compositions including moisture, protein, lipid and ash content, play a crucial role in evaluating the quality of fishery products, influencing their nutritional value, quality, functional properties and sensory properties[17].

The composition of such nutrients of a specific species often seems to differ across habitats and seasons, primarily influenced by variations in the quantity and quality of the fish's diet as well as their level of movement[1,6,26]. When compared with fresh fish, the drying process leads to a notable reduction in moisture content and an increase in other nutritional components, including protein, lipid and ash percentage[24].

In this context, it was observed that the nutritional value of 1 kg of dried fish is the same as approximately 5 kg of fresh fish[28]. Dried fish is a cheaper and exceptional source of protein and necessary amino acids with fewer calories than beef. It contains methionine and lysine, which are absent in terrestrial meat proteins. Fish fats are recognized for their abundant supply of important dietary components such as essential fatty acids and n-3 polyunsaturated fatty acids (PUFA) which maintains healthy blood circulation, lowering blood pressure and triglyceride levels, enhancing the immune system, supporting optimal brain function and combating diabetes and obesity.

Tuna is an economically significant seafood, harvested throughout the world and is heavily traded due to its high nutritional quality and consumer acceptance. Tuna meat is rich in essential nutrients such as amino acids, polyunsaturated fatty acids (PUFA) and trace minerals. Taurine, found abundantly in various fish species like tuna, plays a role in mammalian development. Taurine shows promise in reducing blood pressure, enhancing cardiac function and lowering blood cholesterol levels. It exhibits cytoprotective properties through mechanisms such as antioxidation, modulation of energy metabolism, gene expression and regulation of calcium and osmotic balance, among others[4]. Though, Tuna fish has high nutritional values, it is expensive and only occasionally available especially in its fresh state.

Alternatively, Tuna dry fish is affordable and easily available, which serves as a potential dietary supplement. Fish protein hydrolysate (FPH) is a product derived from fish proteins obtained from fish meat or by-products of fish processing using either enzymatic or chemical methods[7]. Tuna fish protein hydrolysate can inhibit the hydroxyl radical induced damage in DNA, which demonstrates the antioxidant activity. DNA damage would bring about an onset of various grave diseases including coronary heart disease, diabetes and cancer[4]. Although, the nutritional values of FPH are known, the bioactive properties of fermented tuna dry fish hydrolysates (DFPH) have not been well studied.

Previous studies had addressed the nutritional benefits, antioxidant potential and anti-inflammatory ability of fresh fish protein hydrolysates. Nonetheless, the impact of the

process of fermentation using *Lactobacillus delbrueckii* on the bioactive properties of DFPH is not fully understood especially its antioxidant, anti-inflammatory and anticancer potential. Fermentation is a metabolic process that derives energy from organic compounds without the need for an external oxidizing agent. It enhances the levels of bioactive compounds in food and reduces antinutrients through hydrolysis.

The consumption of fermented foods can have positive effects attributed to either the live microorganisms present in them, some of which exhibit probiotic properties, or compounds synthesized during fermentation because of bacterial metabolism[32]. Fermentation is a secure method that is both eco-friendly and economically beneficial. Furthermore, fermentation leads to the production of more easily digestible proteins because of their hydrolysis and breakdown into shorter peptides and amino acids, making it a highly valuable technique for enhancing the nutritional value of fish products used as a protein source in feed.

Various natural compounds contribute to the antioxidative defence mechanism of fish. These include enzymes such as catalase, peroxidase, glutathione and superoxide dismutase as well as carotenoids, peptides, amino acids and phenolic compounds like tocopherols and ubiquinone. These substances are present in the plasma and mitochondria of cell membranes. Moreover, antioxidative protein hydrolysates derived from fish can be used in various industries including food, pharmaceuticals, cosmetics and nutritional supplements. They can substitute synthetic antioxidants like butylated hydroxytoluene, butylated hydroxyanisole, tertbutyl hydroquinone and propyl gallate in the production of diverse products[5].

Protein products with anti-inflammatory properties often contain abundant radical-scavenging Aas. Glutamate and aspartate, which serve as electron donors, demonstrate antioxidant capabilities. Furthermore, amino acids with radical-scavenging side chains, such as hydrophobic and aromatic amino acids, may alleviate oxidative stress and provide defence against inflammation induced by reactive oxygen species (ROS)[9].

Bioactive peptides, particularly those derived from fish hydrolysates, can mitigate oxidative stress by reducing reactive oxygen species (ROS), consequently impeding genetic changes like mutations and chromosomal abnormalities, which play a significant role in carcinogenesis[15]. However, bioactive compounds of fermented Tuna dry fish hydrolysates were not explored much. In the present study we performed GC-MS analysis to identify the presence of bioactive compounds.

The present study explores the fermentation of tuna dry fish hydrolysates and investigates the changes in their antioxidant, anti-inflammatory, activities pre- and post-fermentation followed by analysing the anticancer activity of fermented DFPH. Through comparative analysis, the study aims to elucidate the influence of fermentation on the bioactive properties of hydrolysates.

Material and Methods

Tuna dry fish was purchased from local fish market and stored in the refrigerator until use. Bovine serum albumin was used as a protein standard for the denaturation assay. Milky Mist yoghurt was selected as the primary source of the yoghurt sample for the isolation of *Lactobacillus delbruecki*. DPPH solution, phosphate-buffer saline, methanol, DMS0, streptomycin, penicillin, helium and n-hexane were used. All chemicals and reagents used in the experiments were of high purity and analytical grade. The tuna dry fish was processed by grinding it with an electric blender until it reached a specific size, approximately 0.5 to 1cm. Subsequently, the ground dry fish was effectively homogenized using a mortar and pestle, resulting in a uniform mixture.

Preparation of Dry Fish protein hydrolysates: Fish protein hydrolysate was prepared by using 1:4 of dry fish and distilled water. Therefore 25 grams of minced tuna dry fish were appropriately weighed using weighing balance with 25 ml of distilled water using measuring cylinder and completely homogenised with the help of mortar and pestle. A crude mixture of Papain was purchased from TM media. To the homogenized mixture, 25 grams of papain enzyme were added and mixed thoroughly. The resulting mixture was then suspended in 75 ml of distilled water making up to 100ml.

The mixture was evenly distributed in two clean conical flasks: one designated for fermentation and other flask serving as a control where fermentation was not conducted. Both the tubes were sealed tightly. For enzymatic hydrolysis, both flasks were placed in a water bath set to 70°C and maintained at a pH of 6 for a duration of 6 hours[23]. Following the incubation period, the hydrolysate from the control flask was initially strained using a sterile strainer and subsequently filtered using Whatmann filter paper to remove the insoluble substances from the hydrolysate. The filtrate (supernatant) is then stored in the deep freezer until further use.

Isolation of *Lactobacillus delbrueckii*: MRS agar and MRS broth media were used for isolation and growth of LAB and to inhibit the growth of unwanted bacteria. MRS agar and broth were also used for enrichment of LAB culture. 1 ml of the yoghurt sample was taken in 9 ml of MRS Broth (Hi Media®, India) and incubated at 37° C for 36-48 hrs. One loopful broth culture was streaked on MRS agar plates and was incubated for 36-48 hrs. The isolates were screened by performing Gram staining and followed microscopic examination to confirm the morphology of isolated colonies as Gram-positive rod. Pure culture was obtained by multiple sub-culturing and was stored in MRS broth at refrigerator temperature.

Fermentation of DFPH: Fermentation was carried out using a sterile conical flask containing the hydrolysate, 50 ml of dry fish protein hydrolysate was measured and taken into the conical flask. 500 µl of the Isolated *Lactobacillus delbrueckii* was inoculated and closed tightly using a cotton plug to avoid contaminants. The flask was kept for incubation for 48 hrs at 37°C in an incubator. The fermentation process was frequently monitored. After 48hrs of fermentation, the fermented hydrolysate was taken out and centrifuged and filtered to remove the bacteria thus stopping the fermentation. The fermented hydrolysate was initially strained using sterile strainer and subsequently filtered using Whatmann filter paper. The filtrate(supernatant) was transferred to a sterile conical flask and stored in the deep freezer for further analysis.

Antioxidant Activity – DPPH Assay: DPPH assay was done on the fermented DFPH and unfermented DFPH (control) to find out their antioxidant activity. The radical scavenging capacity of both DFPH was measured by 1,1-diphenyl-2picrylhydrazyl (DPPH) method[20]. 0.4 mM solution of DPPH in methanol was prepared and 2mL of this solution was added to concentrations of 50,100,150, 200, 250mg in both the samples and was allowed to stand at room temperature for 15mins and then absorbance was read at 517 nm against the blank sample which consists of only 2mL of 0.4mM of DPPH solution.

Anti-inflammatory activity - Inhibition of albumin denaturation assay: The anti-inflammatory activities of both the fermented and unfermented DFPHs were evaluated according to Anoop and Bindu[3]. The reaction mixtures consisted of 0.5 ml of bovine albumin (1% aqueous solution), 1 ml of phosphate buffered saline (PBS, pH 7.2) and different concentrations of both the fermented and unfermented tuna dry fish sample (50, 100, 150, 200, 250 mcg/ml). Similar volume of PBS served as control. The mixtures were incubated at 37 ºC for 15 mins and heated at 70ºC for 10 mins. After cooling, absorbance was measured at 660 nm by using PBS as blank.

Anti-Cancer Activity

Cell Culture Maintenance: HCT 116 (Human colorectal carcinoma epithelial cell line) was obtained from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained in the logarithmic phase of growth in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin. They were maintained at 37°C with 5% CO_2 in 95% air humidified incubator.

Anticancer activity: The anticancer activity of the fermented tuna dry fish hydrolysate was tested against HCT 116 cell line by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The cells were seeded in 96-well microplates (1 x 106 cells/well) and incubated at 37°C for 48 h in 5% CO_2 incubator and allowed to grow 70-80% confluence. Then the medium was replaced and the cells were treated with different concentrations of fermented DFPH and incubated for 24 h. The morphological changes of untreated (control) and the treated cells were observed under digital inverted microscope (20X magnification) after 24 h and photographed. The cells were then washed with phosphate-buffer saline (PBS, pH-7.4) and 20 µL of (MTT) solution (5 mg/mL in PBS) was added to each well.

The plates were then stand at 37ºC in the dark for 2 h. The formazan crystals were dissolved in 100 µL DMSO and the absorbance was read spectrometrically at 570 nm. Percentage of cell viability was calculated using the formula:

Cell viability (%) = (Absorbance of sample/Absorbance of control) X 100

GC-MS analysis: GC-MS analysis of the fermented and non-fermented DFPH was conducted using an Agilent GC 7890A 240MS system, featuring an Ion Trap gas chromatograph coupled with an HP5 capillary column (30 meters long, 0.32mm inner diameter, 0.25µm coating thickness), interfaced with an Agilent 240 MS Ion Trap mass detector. The analytical conditions were set as follows: injector and transfer line temperatures were maintained at 220°C and 240°C respectively; oven temperature was programmed to increase from 80°C to 300°C at a rate of 40°C/min; helium was employed as the carrier gas at a flow rate of 1 ml/min; and 0.2 µl of n-hexane was injected. Component identification was achieved by comparing their retention times with those of authentic samples, utilizing linear retention indices and by computer matching against commercial mass spectra libraries such as NIST and MS literature data[8].

Results and Discussion

Tuna fish holds significant biological applications due to its rich nutritional profile and bioactive compounds. Its high protein content makes it valuable for muscle growth and repair while omega-3 fatty acids support cardiovascular health and brain function. Additionally, tuna's vitamins and minerals contribute to overall well-being. Its anti-inflammatory properties aid in reducing inflammation. Tuna contains significant amounts of selenoneine.

In fact, it is the predominant chemical form of organic selenium in the blood and other tissues of these fish. The selenium levels in tuna are closely related to their radical scavenging activity against red blood cells[33]. Hence, tuna dry fish was selected in the current study.

Isolation of *Lactobacillus delbrueckii*: Milky mist yoghurt sample was collected from the supermarket. The sample was cultured on MRS agar plate for 36-48 hrs and these strains were identified as *Lactobacillus spp*. after observation of their colony morphology. The result showed that the bacterial isolates were Gram-positive, rod shaped, creamish in color, dull appearance, smooth texture, non-motile based

on the morphological characteristics and identified as *Lactobacillus delbrueckii* (Fig. 1).

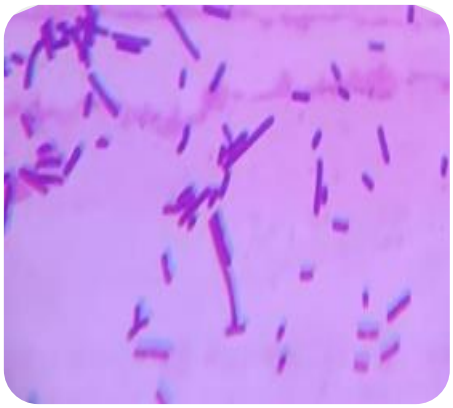

Figure 1: Gram's Staining and morphological characteristics of *Lactobacillus delbrueckii* isolated from Milky Mist yoghurt.

Fermentation of DFPH: Fermentation of the protein hydrolysate with *Lactobacillus delbrueckii* was done and noticeable colour change from brown to orange was observed in the hydrolysate flask along with an alteration in odour. The change in odour intensity was evident, with the fermented sample exhibiting a stronger and more pungent aroma compared to the unfermented sample, suggesting microbial activity during the fermentation process.

Fermentation with *Lactobacillus delbrueckii* induces significant changes in the odour profile of the dry fish protein hydrolysate, suggesting metabolic activity and microbial transformation of the substrate. Microbial fermentation represents a cost-effective technology and is more eco-friendly than the enzymatic hydrolysis process in transforming by-product materials into value-added products[21].

In general, microorganisms involved in FBPs fermentation are responsible for dietary enrichment through nutritional and mineral improvement[31], production of lactic acid and acetic acid[1,29] and transformation of FBPs protein to FPHs[25,27]. To maximize the potential of FBPs, a microbial fermentation approach can be used to convert FBPs into bioactive fish protein hydrolysate (FPH).

Antioxidant Activity - DPPH assay: The DPPH radical-scavenging test is commonly employed to examine the scavenging capabilities of antioxidant substances. DPPH, a stable free radical, exhibits its highest absorbance levels at 517 nm when dissolved in ethanol. Upon encountering an antioxidant that donates protons, the DPPH radical is scavenged, resulting in a decrease in absorbance. Both the unfermented and fermented DFPH were assayed for its antioxidant activity. The absorbance of the different concentration was measured at 571nm and it was found that DPPH radical scavenging assay of both the fermented and unfermented dry fish protein hydrolysates increases with increasing their concentration.

The IC50 value of the fermented and non-fermented sample, was calculated using Log dose-inhibition curve. From the obtained graph (Fig. 2), the IC50 values of both the samples were calculated using linear regression: y = mx + c

For fermented DFPH,
y = 50
m = 0.2842
c = 25.473
x = IC50 = 107.91µg/mL

For non-fermented DFPH,
y = 50
m = 0.2388
c = 24.307
x = IC50 = 86.36µg/mL

Fish protein hydrolysates from various fishes were studied for antioxidant activity by performing radical scavenging assays for DPPH, superoxide and nitric oxide free radicals *in vitro*. In addition, the ability of the extracts to chelate ferrous ions (Fe^{2+}) was also determined. The antioxidant FPH exhibits metal chelation or hydrogen/electron donating activity, enabling them to interact with free radicals and stop the radical chain reaction or prevent their formation. In the current study the fermented fish protein hydrolysates showed enhanced antioxidant activity compared to the non-fermented fish protein hydrolysates. Srikanya et al[30] reported that the fish protein hydrolysate prepared from tilapia wastes has exhibited varied antioxidant and antimicrobial properties. Their results suggested that FPH showed the highest of 77.40% at 100 mg/L with decrease in antioxidant activity with increase in concentration.

In the current study, the fermented DFPH showed the highest of 84.5% at 250mg/m when compared with non-fermented DFPH with antioxidant activity increase with increase in the concentration of DFPHs. Anti-inflammatory bioactive peptides (BAPs), derived from sources like seafood processing by-products, offer a promising alternative to NSAIDs[18].

Anti-inflammatory Activity - Inhibition of albumin denaturation assay: Fish protein hydrolysates have been shown to possess anti-inflammatory effects, which could be beneficial for various health conditions. These effects are attributed to the presence of bioactive peptides within the hydrolysates. Studies have demonstrated that fish protein hydrolysates can reduce inflammation in different experimental models, including *in vitro* cell culture studies and *in vivo* animal models. For example, they may alleviate inflammation associated with conditions like arthritis, cardiovascular disease and gastrointestinal disorders.

In the present study, the fermented DFPH showed the anti-inflammatory activity at the highest of a 63.23% at 250mg compared with non-fermented DFPH with the inhibition of 45.58% at 250mg.

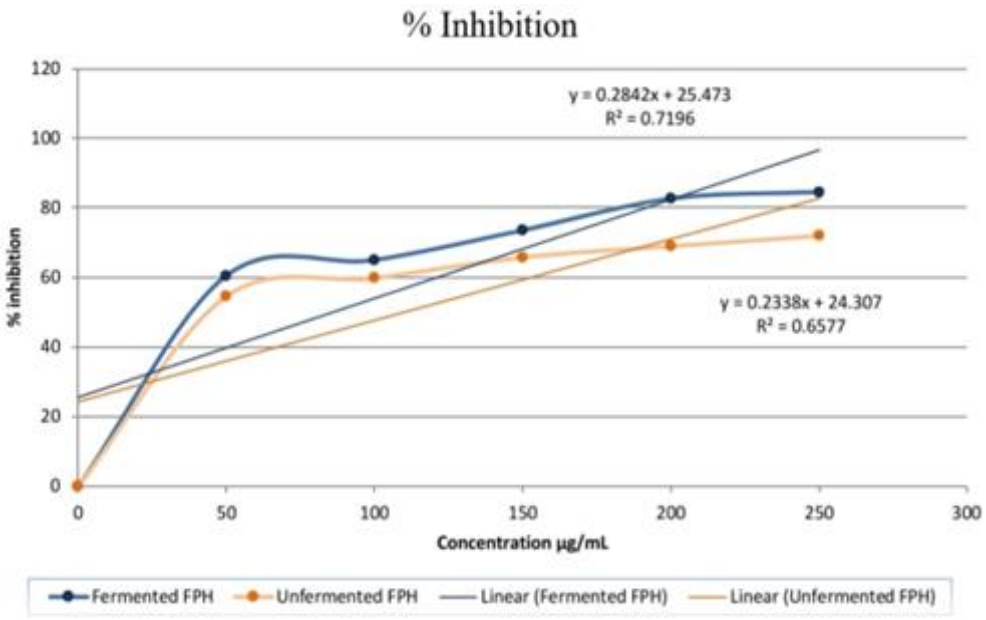

Figure 2: Percentage of inhibition of the Fermented and Non-fermented DFPH samples at different concentrations.

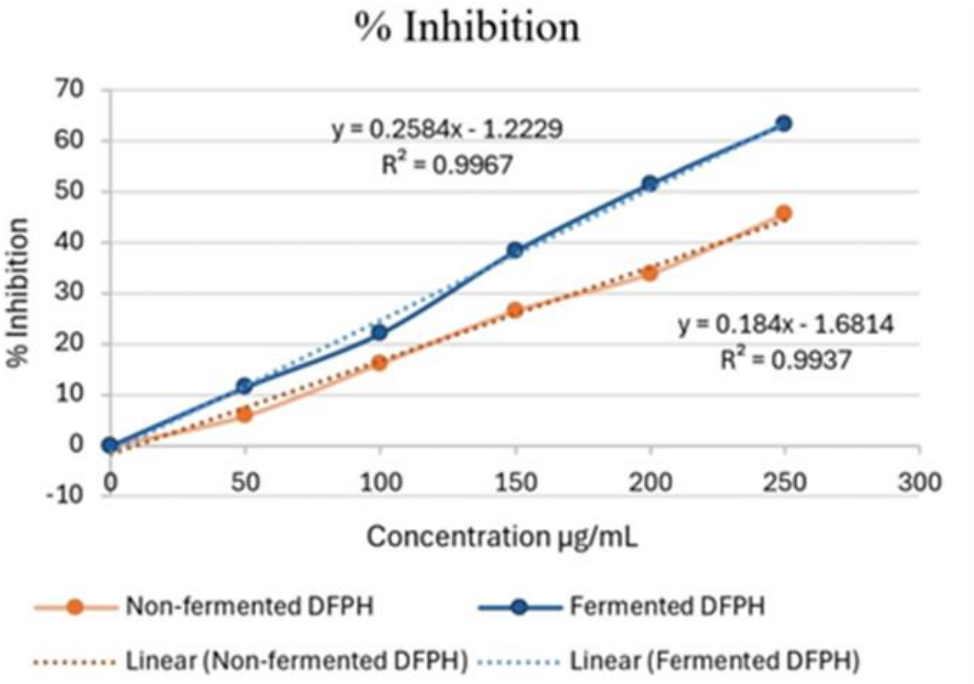

Figure 3: Percentage of inhibition of the Fermented and Non-fermented DFPH samples at different concentrations.

The IC50 value of the fermented and non-fermented DFPH sample, which is the concentration of sample required to inhibit 50% of the protein denaturation induced by an inflammatory stimulus, was calculated using Log dose-inhibition curve. From the obtained graph (Fig. 3), the IC50 values of both the samples were calculated using linear regression: y = mx + c

For fermented DFPH,
y = 50
m = 0.2584
c = -1.2229
x = IC50 = 198.065µg/mL

For non-fermented DFPH,
y = 50
m = 0.184
c = -1.6894
x = IC50 = 280.436µg/mL

Anticancer Activity: The anticancer activity of the fermented tuna dry fish hydrolysate was tested against HCT 116 cell line by MTT assay. Morphological alterations were visually assessed and documented. These observations provide qualitative insights into the effects of the fermented dry fish protein hydrolysate sample on cell morphology which is shown in fig. 4. The absorbance values obtained at

570 nm were used to quantify cell viability (Fig. 5). Percentage cell viability was calculated relative to the untreated control group. A dose-response curve was generated to evaluate the concentration-dependent effects of the sample on HCT 116 cell viability. The results of the MTT assay provide insights into the anticancer potential of the sample against HCT 116 cells. Morphological observations, coupled with quantitative analysis of cell viability, contribute to understanding the cytotoxic effects of the sample and its potential as an anticancer agent. Anticancer activity of fermented fish protein hydrolysate is given in Supplementary. The anticancer effects of fish hydrolysates have garnered increasing interest in recent years due to their potential therapeutic benefits. These hydrolysates, derived from various fish sources, contain bioactive peptides with properties that may inhibit cancer cell growth, induce apoptosis (programmed cell death) and inhibit angiogenesis (the formation of new blood vessels that supply tumors). While the concept of isolating anticancer peptides from various parts of fish is relatively recent in research, significant progress has been made in this area over the past decade. Hsu et al[11] utilized papain and protease enzymes to isolate two peptides from the muscle tissue of Tuna fish (*Thunnus tonggol*), demonstrating their effective anticancer activity against the Human breast cancer cell line (MCF-7) in a dose-dependent manner. Similarly, Kannan et al[16] identified anticancer properties in peptides extracted from Shrimp shell hydrolysates using cryotin and pepsin enzymes for digestion. The purified shrimp peptides exhibited antiproliferative activity against both human colon cancer and human liver cancer (HepG2) cell lines.

In a parallel study, You et al[34] isolated peptides from the muscle tissues of *Misgurnus anguillicaudatus* (*Loach*) and evaluated their effects on HepG2 (liver), MCF-7 (breast) and Caco-2 (colon) cancer cell lines. They observed a significant reduction in cancer cell proliferation rates upon treatment with these peptides.

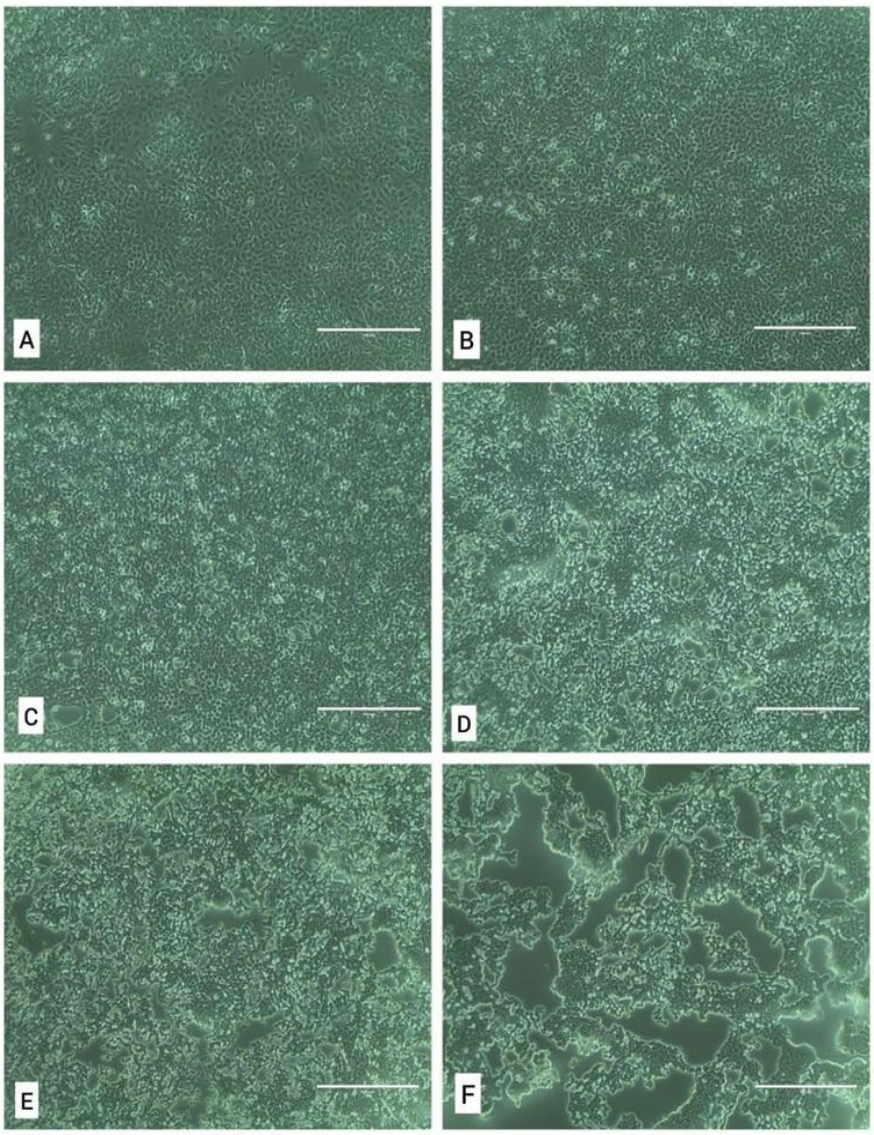

Figure 4: Anticancer activity of fermented fish protein hydrolysates against human colorectal carcinoma cell line (HCT 116) by MTT assay. Representative microscopy images of cell viability at different concentrations are shown (A) Control; (B) 20µl; (C) 40µl (D) 60µl (E) 80µl (F) 100µl

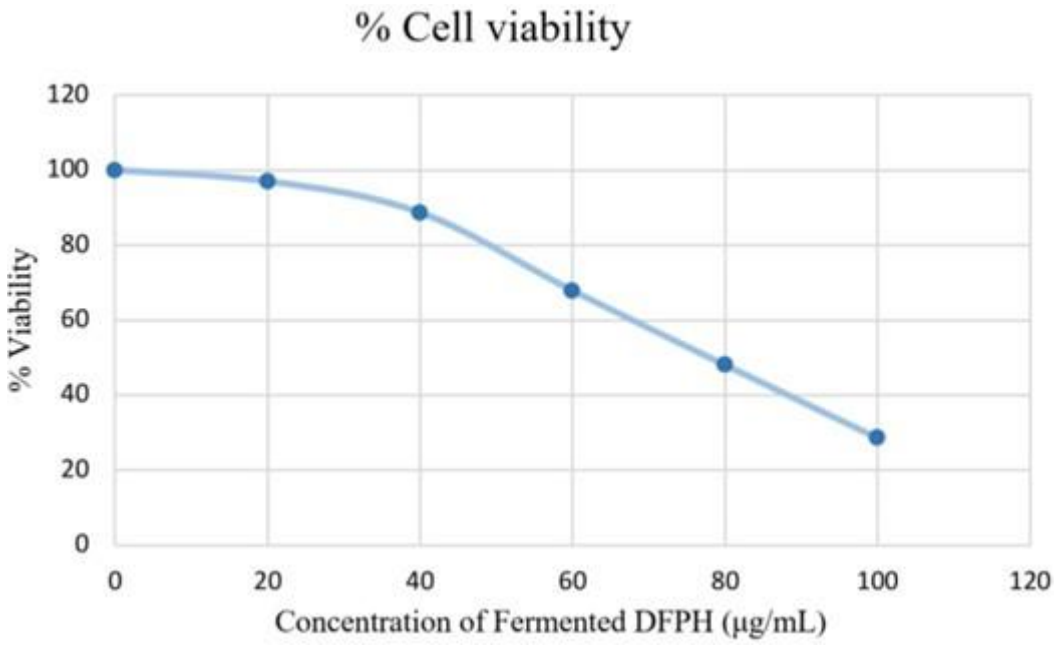

Figure 5: Percentage of cell viability of the Fermented FPH samples at different concentrations.

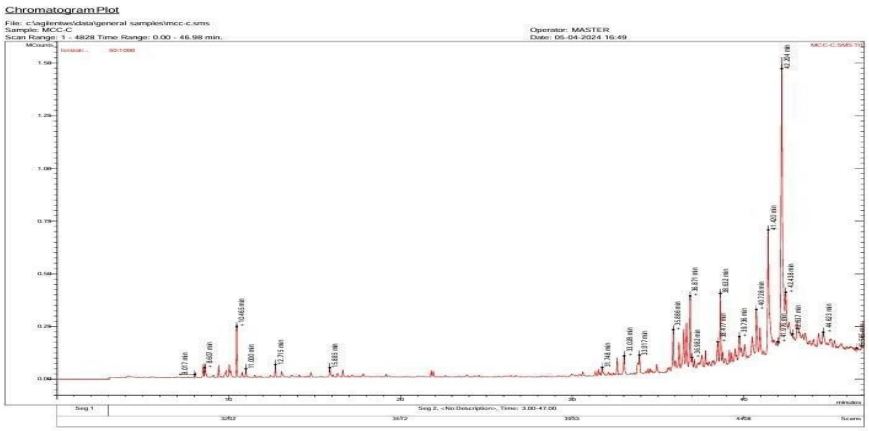

Figure 6: Chromatogram (GC-MS) of non-fermented dry fish protein hydrolysate

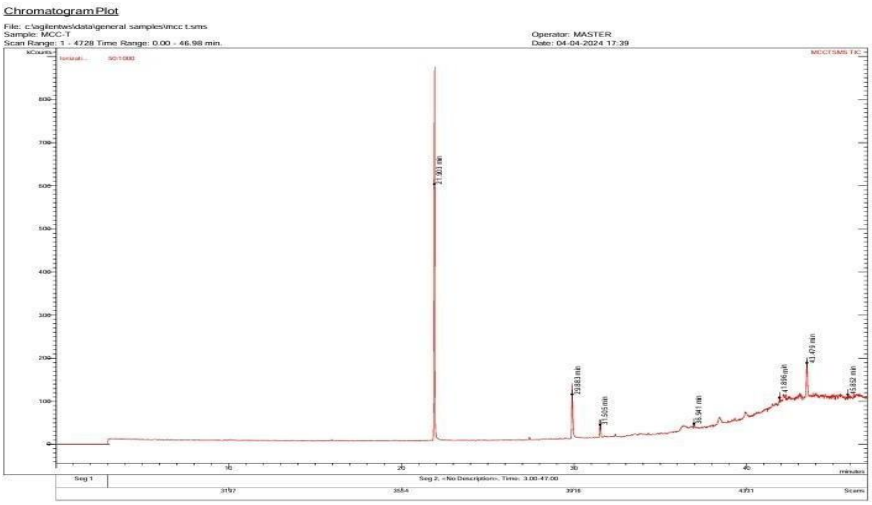

Figure 7: Chromatogram (GC-MS) of fermented dry fish protein hydrolysate

GC-MS analysis: The samples of dry fish protein hydrolysate, both fermented and non-fermented, underwent analysis using GC-MS to detect a variety of bioactive components and metabolites. GC-MS is a technique utilized for identifying and quantifying chemical compounds by their distinct fragmentation patterns at specific retention times. Chromatogram (GC-MS) of non-fermented and fermented fish protein hydrolysate is shown in figures 6 and 7. The

results of this analysis revealed numerous compounds, showing the molecular weights and biological activities of the fermented and non-fermented DFPH. In the non-fermented sample, a total of 55 compounds were identified. Most of these compounds have been studied for their properties such as antioxidant, anti-inflammatory, antitumor, antiviral, antibacterial, antipyretic and wound healing effects.

However, some of the other compounds have biological characteristics that have not been reported. Conversely, the fermented DFPH sample yielded eight identified compounds, demonstrating various effects including antibacterial, antiviral, antioxidant, anti-inflammatory, antidiabetic, anticancer, reno protective, neuroprotective, cardioprotective and hepatoprotective properties. The differences in compound abundance between fermented and non-fermented DFPH samples observed in GC-MS analysis could be attributed to a combination of factors related to microbial metabolism, biodegradation, formation of new compounds, fermentation conditions and sample preparation techniques etc.

The GC-MS analysis of both the fermented fish protein hydrolysate (FPH) and the non-fermented FPH revealed the presence of bioactive compounds with diverse potential applications including antioxidant, anti-inflammatory and anticancer, antidiabetic, antimalarial and wound healing properties. Few compounds from non-fermented DFPH like Trans-2-Carene-4-ol, 1-Phenanthrenecarbaldehyde[19], (6E,9E,12E)-6,9,12-Hexadecatrienoic acid[2], isopimaric acid[12,13], andrographolide[10] etc. have shown anti-inflammatory activity.

The compound 1,2-Diphenyl-1,2-di(morpholin-4-yl) ethane (threo) shows a wide range of bioactivities like antimicrobial, antioxidant, anti-inflammatory, antidiabetic, anticancer, reno protective, neuroprotective, cardioprotective and hepatoprotective effects[22]. Other compounds from fermented DFPH like phthalic acid, pentyl tridec-2-yn-1-yl ester[12,13] etc. have shown antioxidant, anti-inflammatory and antimicrobial activities.

Therefore, based on the comprehensive analysis of GC-MS results and bioactivity assays, it can be inferred that the observed positive outcomes from the antioxidant, anti-inflammatory and anticancer activity analysis, are likely attributed to the presence of bioactive compounds identified in the fermented and non-fermented DFPH.

Conclusion

This study shows that fermentation of tuna dry fish protein hydrolysate enhances the bioactive properties which resulted in impressive antioxidant, anticancer and anti-inflammatory activities. The bioactive properties of protein hydrolysates substantially increase after the fermentation process demonstrating the transformative power of microbial fermentation methods. The GC-MS analysis specifically confirmed the presence of major bioactive compounds that mediate therapeutic effects.

The finding opens new possibilities for the implementation of fermented fish protein hydrolysates as a sustainable and cost-effective natural solution in pharmaceutical and nutraceutical markets. Further studies are required to explore both molecular and *in vivo* processes to have a better understanding of their mechanisms.

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