Original Article

FUTURE JOURNAL OF PHARMACEUTICALS AND HEALTH SCIENCES

Published by Pharma Springs Publication Journal Home Page: https://pharmasprings.com/fjphs

Production of bioactive nutraceuticals from autoclave and surface sterilized fish skin waste using microbial fermentation

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Article History:

Received on: 07 Jan 205
Revised on: 10 Feb 2025
Accepted on: 11 Feb 2025

Keywords:

Fermented fish skin, Bioactive compounds, Anticancer activity, Antimicrobial activity, Antioxidant activity.

Abstract

The fish processing industry generates substantial by-products, including viscera, heads, scales, and bones, which pose environmental challenges if not managed properly. This study explores the potential of fish skin waste from Carangoides malabaricus (Malabar Kingfish/Trevally/Parai) for bioactive compound production through microbial fermentation using Aspergillus oryzae, Bacillus subtilis, and Saccharomyces cerevisiae. Fish skin was pretreated via autoclaving and surface sterilization before controlled fermentation. The resulting extracts were analyzed for phytochemical constituents and evaluated for antioxidant, anti-inflammatory, antimicrobial, and anticancer properties. The extracts contained bioactive compounds such as alkaloids, phenols, terpenoids, carbohydrates, and proteins. Among surface-sterilized samples, Saccharomyces cerevisiae fermentation produced the highest protein content, while Aspergillus oryzae extracts from autoclaved samples showed the strongest antioxidant activity. The Aspergillus oryzae fermented autoclaved sample also exhibited excellent anti-inflammatory effects. Bacillus subtilis extracts demonstrated antibacterial activity against E. coli, B. cereus, Staphylococcus, and Klebsiella species, along with moderate antifungal activity. Additionally, both Saccharomyces cerevisiae and Bacillus subtilis extracts displayed strong anticancer activity. This study highlights fish skin waste as a sustainable source of bioactive compounds, contributing to waste valorization, environmental sustainability, and potential pharmaceutical applications.

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eISSN: 2583-116X
DOI: https://doi.org/10.26452/fjphs.v5i1.711

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INTRODUCTION

Fishery resources are vital to our economy, producing over 170 million metric tons of fish, shellfish, and other seafood annually (FAO, 2018). The fish processing industry generates substantial byproducts, including viscera, heads, scales, and bones. These byproducts can account for 30 to 70% of processed fish, creating a significant environmental challenge due to improper disposal, which can lead to pollution and greenhouse gas emissions [1]. Instead of discarding these byproducts, there is a push towards converting them into valuable products. Protein recovery

from fish waste is a key focus, as it helps manage waste, reduces environmental impact, and provides low-cost protein.

Methods like enzymatic hydrolysis and fermentation extract proteins, lipids, and peptides from fish waste. These compounds have potential applications in food, nutraceuticals, pharmaceuticals, and cosmetics. Enzymatic hydrolysis, though effective, can be complex and environmentally taxing, while microbial fermentation offers a more sustainable and cost-effective alternative.Fish consumption is significant globally, providing around 17% of animal protein and 6.7% of total protein intake [2]. Fish is rich in high-quality protein, omega-3 fatty acids, vitamins, and minerals, contributing to a balanced diet and offering health benefits [3]. Fish byproducts, similar to fish fillets, have high nutritional value and are rich in proteins, fats, and minerals.Fermentation, a traditional method used for centuries, enhances food's nutritional value and flavor by releasing bioactive peptides through microorganisms and enzymes. Various fermented fish products, such as Thai shrimp paste and squid miso, have demonstrated bioactivity. Fermentation can convert fish waste into valuable products, including bioactive peptides, antioxidants, and preservatives, making it a sustainable solution for reusing fish byproducts [4].

METHODOLOGY

Sample Collection:

Fresh Malabar Kingfish (Trevally / Parai) were collected from Kasimedu fishing harbor. The fish were thoroughly washed to remove dust and debris. [5] The skin was peeled off after descaling and collected in a sterile conical flask for further analysis.

Preparation and Pretreatment:

The fish skin samples were washed with distilled water and collected in a conical flask. Sterilization was done using two methods: autoclave and surface sterilization. The sterile fish skin samples were then used in fermentation to produce bioactive nutraceutical compounds [6].

Autoclave Method:

To prepare the seed culture, 100 mL of medium at a skin/water ratio of 2:3 (w/v) was placed in a 250 mL Erlenmeyer flask. The mixture was then homogenized and autoclaved at 121°C for 20 minutes before fermentation [7].

Surface Sterilization Method:

1. Collection of Fish Skin: Obtain the fish skin.

2. Wash with Distilled Water: Rinse the skin thoroughly to remove surface contaminants.

3. Bleaching Solution Treatment: Treat the skin with a bleaching solution for further disinfection.

4. Rinse with Distilled Water: Rinse again to remove any bleaching solution residue.

5. Wash with Ethanol: To kill any remaining microorganisms, submerge the skin in 100% ethanol.

6. Final Rinse with Distilled Water: Rinse once to remove any ethanol, ensuring the skin is clean and sterilized.

These steps effectively sterilized the fish skin, preparing it for further use [8].

Collection and Maintenance of Standard Strains:

Standard strains of *Aspergillus oryzae* MTCC 1846, Bacillus *subtilis* MTCC 1133, and *Saccharomyces cerevisiae* MTCC 181 were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC) for use in the fermentation process [9].

These strains were maintained on Sabouraud Dextrose Agar (SDA) slants. The strains were inoculated for fermentation into Sabouraud Dextrose Broth (SD Broth), which contained 20 g/L of glucose and 10 g/L of special peptone, with a pH of 5.8. The pure cultures of *Saccharomyces cerevisiae* and *Aspergillus oryzae* were inoculated into SD Broth flasks and incubated at 28°C for 2 to 3 days. *Bacillus subtilis* was maintained on nutrient agar slants, inoculated into nutrient broth with a pH of 7 to 7.5, and incubated at 37°C for 72 hours [10].

Extract preparation from fermented medium:

Dimethyl sulfoxide (DMSO) was added to the filtrate in a separating flask and shaken well. The extract was then collected and condensed [11].

Qualitative analysis of phytochemical:

The extract was tested for qualitative analysis of phytochemicals for two Sample they are Autoclave

and surface sterilized samples; each Sample, Aspergillus oryzae, Bacillus subtilis, and Saccharomyces cerevisiae tested against Alkaloids, phenols, protein, terpenoids, carbohydrates, Flavonoids, tannins, saponins, steroids, anthraquinones were tested for all organism [12].

Quantitative Analysis :

Protein Estimation by Lowry's Method

Reagent 1: 48 ml of Solution A + 1 ml of Solution B + 1 ml of Solution C

Reagent 2:1:1 Folin phenol reagent to distilled water

Procedure:

1. Pipette 0.2 ml of the Sample into a test tube and adjust the volume to 1 ml with distilled water.

2. Add 4.5 ml of Reagent 1 and incubate for 10 minutes.

3. Add 0.5 ml of Reagent 2 and incubate for 30 minutes at room temperature.

4. Measure the absorbance at 660 nm.

Antioxidant activity[13]

DPPH radical scavenging activity [14]:

To assess antioxidant activity using the DPPH radical scavenging method (Molyneux, 2004), 3.7 ml of absolute methanol was added to each test tube, while 3.8 ml was added to the blank. Then, 100 µl of BHT was added to the standard tube and 100 µl of Sample to the test tubes. Next, 200 µl of DPPH reagent was added to all tubes, including the blank. The tubes were incubated in the dark at room temperature for 30 minutes, and absorbance was measured at 517 nm [14].

Calculation:

$$\% \text{ antioxidant activity } = \frac{(\text{absorbance at blank}) - (\text{absorbance at test})}{(absorbance\ at\ blank)} \text{ x100}$$

Antimicrobial assay:

The antibiotic activity of the fermented broth samples was analyzed for antibacterial activity against Klebsiella, Escherichia coli, Staphylococcus, Bacillus cereus, Salmonella, and antifungal activity Candida, Malassezia furfur using the clinical isolates collected from Life Teck Research Centre [15].

Anti-Inflammatory Assay:

Inhibition of protein denaturation:

Procedure:

100µl of test was added with 500µl of 1% BSA. The mixture was incubated for 10 minutes at 37ºC. The contents were then heated in a water bath at 51ºC for 20 minutes. Afterward, the mixture was cooled to room temperature, and the absorbance at 660nm was checked against the blank. Acetyl Salicylic acid was used as the positive control, and water was used as the product control [16].

Calculation:

$$\% \text{ Inhibition } = \frac{\text{Control OD} - \text{ Sample OD)}}{\text{Control O. D} * 100}$$

Anticancer Activity

The BETA TC-6 cell line, derived from a mouse pancreatic tumor (insulinoma), was used for the anticancer activity study. These cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a humidified atmosphere with 5% CO2 [17]. For the in vitro MTT assay, cells were plated at 1×10^5 cells/well in 24-well plates and incubated until they reached confluence. Various concentrations of the samples were then added and incubated for 24 hours. After incubation, the samples were removed, and the wells were washed with phosphate-buffered saline or DMEM without serum. Then, 100 µl/well of 0.5% MTT (5 mg/ml) was added and incubated for 4 hours. Subsequently, 1 ml of DMSO was added to dissolve the formazan crystals. Absorbance was measured at 570 nm using DMSO as a blank. The percentage of cell viability was calculated, and the IC50 value was determined graphically [18].

$$\% \text{ Cell viability } = \frac{\text{A570 of treated cells}}{A570\ of\ control\ cells} \text{ x100}$$

RESULT AND DISCUSSION

Sample collection:

Fresh Malabar King fishes (Trevally / Parai) were collected from the Kasimedu fishing harbour and washed thoroughly for fermentation.

Preparation and pretreatment:

Fish skin samples were washed thoroughly with distilled water and collected in the conical flask.

Samples were sterilized in two ways - the autoclave method and the surface sterilization method. The sterile fish skin samples were used for fermentation to produce bioactive nutraceutical compounds.

Collection and maintenance of standard strains:

The sub-cultured standard strains of *Aspergillus oryzae* MTCC 1846, *Bacillussubtilis* MTCC 1133and *Saccharomyces cerevisiae* MTCC 181 were used for fermentation.

Fermentation of the fish skin:

Fermentation is carried out in two samples. They are

Autoclave Sample and

Surface sterilized Sample.

Fermentation by Autoclave sample:

The resulting autoclaved fermented Sample was done in *Aspergillus oryzae, Bacillussubtilis,* and *Saccharomyces cerevisiae. Bacillussubtilis* fermentation was conducted at 37 °C in an incubator, whereas fermentation by *Aspergillus oryzae* and *Saccharomyces cerevisiae* was carried out at ambient temperature (25 °C) for 5 days. After fermentation, the cultures were filtered.

Figure 1 Autoclave Sample

Fermentation by Surface sterilization sample:

The resulting surface sterilization of fermented samples was done in *Aspergillus oryzae, Bacillussubtilis,* and *Saccharomyces cerevisiae. Bacillussubtilis* fermentation was conducted at 37 °C in an incubator, whereas fermentation by *Aspergillus oryzae* and *Saccharomyces cerevisiae* was carried out at ambient temperature (25 °C) for 5 days. After fermentation, the cultures were filtered.

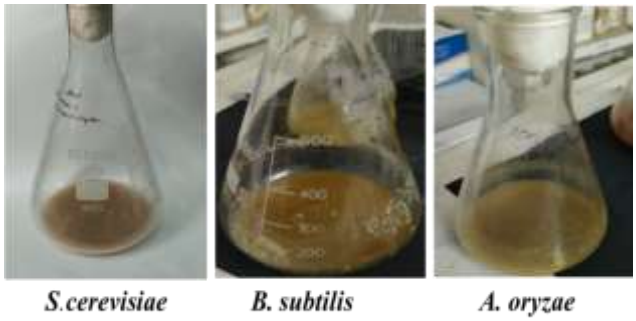

Figure 2 Surface sterilization sample

Extract preparation from fermented medium:

Dimethyl sulfoxide (DMSO) was added to the filtrate in a separating flask and shaken well. The extract was then collected and condensed.

Figure 3 Extract preparation from fermented medium

Qualitative analysis of phytochemical:

Phytochemical analysis- autoclave samples and surface sterilized samples:

The six fermented extracts were tested for qualitative analysis of phytochemicals for *Aspergillus oryzae, Bacillus subtilis, and Saccharomyces cerevisiae.* Alkaloids, phenols, protein, terpenoids, and carbohydrates tested positive for all organisms. Flavonoids, tannins, saponins, steroids, and anthraquinones were tested negative for all organisms.

Quantitative analysis:

Protein estimation by Lowry's method:

After the incubation, the OD value was taken at 660nm for two samples. Autoclaved samples and surface sterilized samples are used.

High protein concentration was present in the *Saccharomyces cerevisiae* fermented surface

Reading estimation of fermented autoclave sample:

Table 1 Reading estimation of fermented surface sterilized Sample

Sample	OD Value	Protein mg/ml	OD Value	Protein mg/ml
Aspergillus Oryzae	0.355	3.7	0.561	5.5
Bacillus Subtilis	0.254	2.6	0.543	5.4
Saccharomyces Cerevisiae	0.349	3.6	0.581	5.7

Table 2 Antibacterial activity of Autoclave sample

Organisms	Standard Ampicillin (mm)	Zone of Inhibition		
		Aspergillus Oryzae	*Bacillus Subtilis*	*Saccharom Yces Cerevisiae*
E. Coli	27	-	21	-
Bacillus Cereus	26	-	-	-
Salmonella	9	-	-	-
Staphylococcus	20	13	18	11
Klebsiella	32	9	11	9

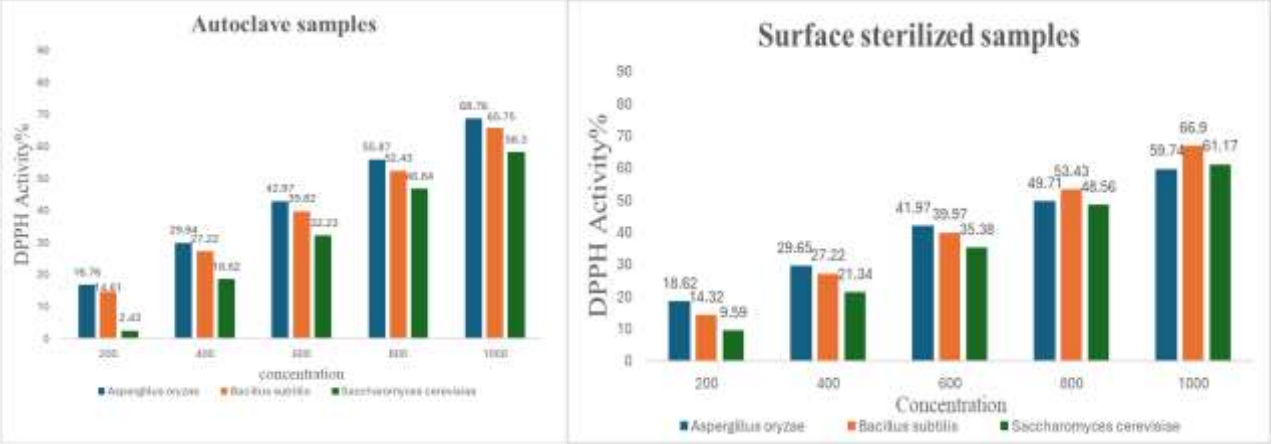

Figure 4 Antimicrobial activity of the extract

Table 3 Antibacterial activity of Surface sterilized Sample

Organisms	Standard Ampicillin (mm)	Zone of Inhibition		
		Aspergillus Oryzae	*Bacillus Subtilis*	*Saccharom Yces Cerevisiae*
E. Coli	26	9	9	-
Bacillus Cereus	27	10	-	-
Salmonella	20	-	8	-
Staphylococcus	18	8	11	8
Klebsiella	32	9	14	-

sterilized fish skin sample extract compared to the other extracts of this study.

Antioxidant Activity of the Extract:

The OD value was taken for autoclaved sample and surface sterilized sample. Both samples have three different fermentation microorganisms (*Aspergillus oryzae, Bacillus subtilis, Saccharomyces cerevisiae*). The best activity was recorded for Aspergillus oryzae fermented autoclaved sample extract (68.76), followed by Bacillus subtilis fermented surface sterilized sample extract (66.90).

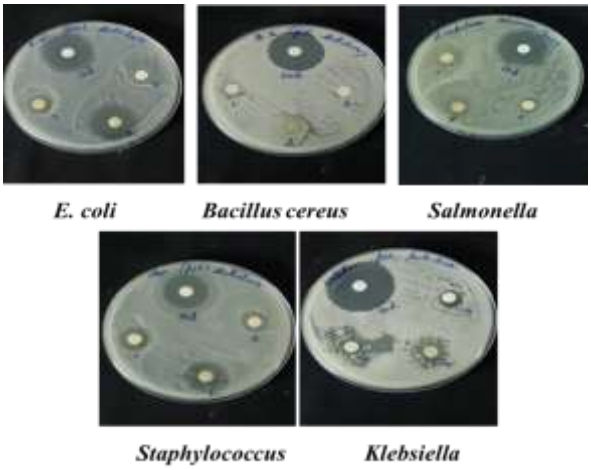

Figure 5 Antibacterial activity of Autoclave sample

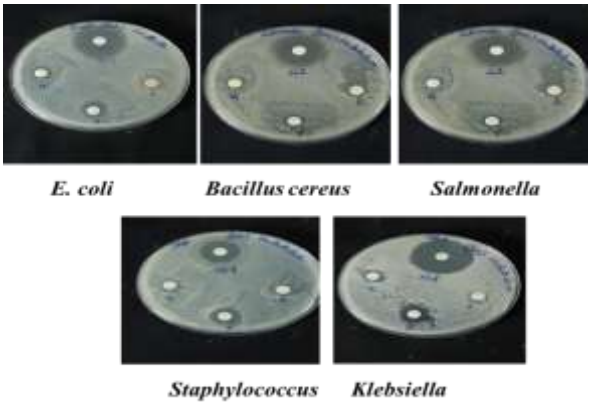

Figure 6 Antibacterial activity of Surface sterilized Sample

Antifungal activity of the extract:

Antifungal activity of Autoclave samples against *Candida albicans and Malassezia furfurs.*

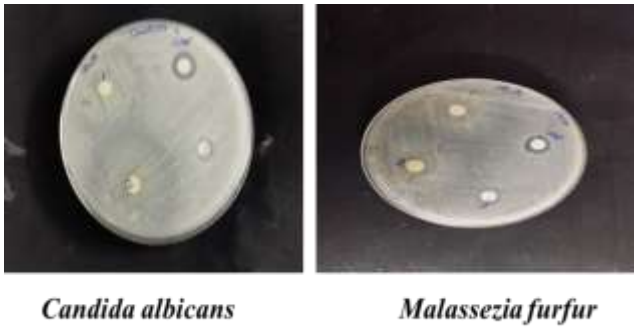

Figure 7 Antifungal activity of the extract

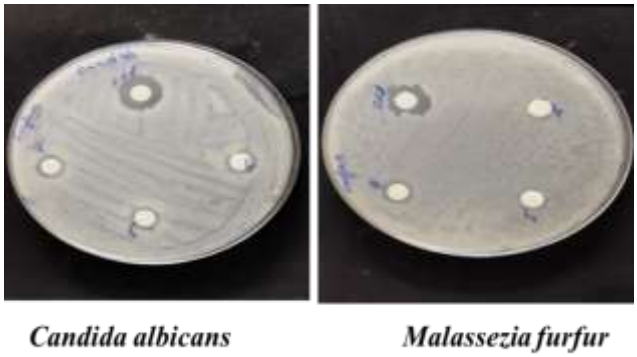

Figure 8 Antifungal activity of Surface sterilized Sample

Anti-Inflammatory Activity of the Extract:

An anti-inflammatory assay was conducted using the protein denaturation method for all six extracts. All the extracts were found to possess anti-inflammatory activity. Excellent results were obtained from the *Aspergillus oryzae* fermented autoclaved sample extract, and it had 64.97% activity compared to other surface sterilized samples.

Table 4 Antifungal activity of Autoclave sample

Organisms	Standard Ampicillin (mm)	Zone of Inhibition		
		Aspergillus Oryzae	*Bacillus Subtilis*	*Saccharom Yces Cerevisiae*
Candida	14	-	-	7
Malassezia furfur	11	-	-	7

Antifungal activity of Surface sterilized sample against *Candida albicans and Malassezia furfur.*

Table 5 Antifungal activity of Surface sterilized Sample

Organisms	Standard Ampicillin (mm)	Zone of Inhibition		
		Aspergillus Oryzae	*Bacillus Subtilis*	*Saccharom Yces Cerevisiae*
Candida albicans	15	10	9	7
Malassezia furfur	13	9	7	7

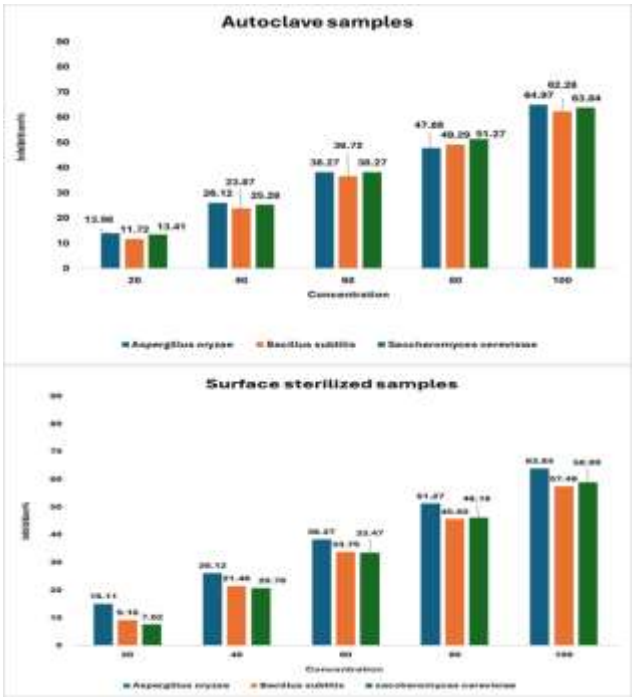

Figure 9 Autoclave and Surface sterilized samples of Anti-Inflammatory Activity

Anticancer Activity of the Extract:

The Autoclave and Surface sterilized Samples are tested for anticancer properties against the BETA TC-6 cell line by MTT assay, and the results are given below.

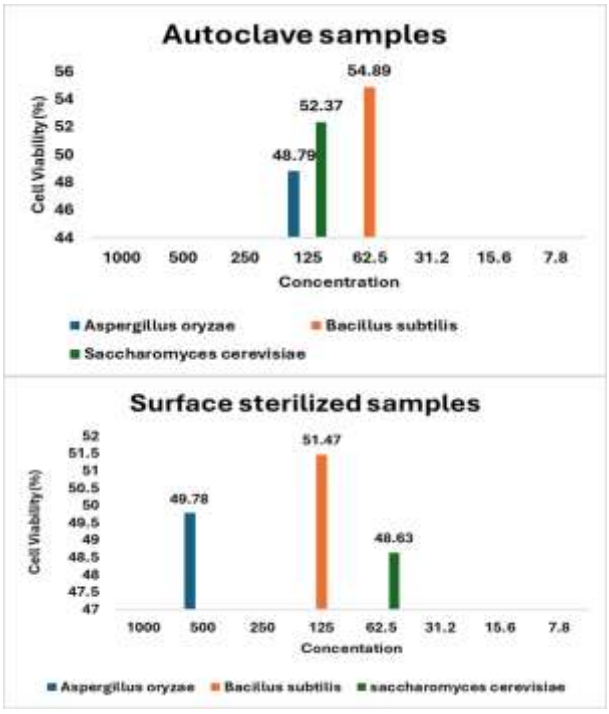

Figure 10 Autoclave and Surface sterilized samples of Anticancer Activity

Aspergillus oryzae demonstrated an IC_{50} 49.78(Con.500)

Bacillus subtilis demonstrated an IC_{50} 51.47(Con.125)

Saccharomyces cerevisiae demonstrated an IC_{50} 48.63 (Con.62.5)

DISCUSSION

Fermented fish skin waste produces bioactive nutraceutical compounds. The fermentation process used the standard strain *Aspergillus oryzae* MTCC 1846, *Bacillus subtilis* MTCC 1133, and *Saccharomyces cerevisiae* MTCC181. Fermentation experiments were conducted using two samples: one sterilized in an autoclave and the other surface sterilized. The fermentation procedures were carried out using the organisms mentioned above. They only used Aspergillus oryzae for fermentation. All six sample extracts contained the phytochemicals – Alkaloids, phenols, protein, terpenoids, and carbohydrates. High protein concentration was present in the Saccharomyces cerevisiae fermented surface sterilized fish skin sample extract. It reported a protein concentration of 4.58 µg/ml, while our fermentation process yielded a better 5.7 µg/ml concentration.

Antioxidant activity was assessed using the DPPH assay for both samples extracted. The best activity was recorded for *Aspergillus oryzae* fermented autoclaved sample extract (68.76). This report correlates with DDPH activity studies. The *Aspergillus oryzae* fermented autoclaved sample extract demonstrated excellent anti-inflammatory activity, recording a percentage activity 64.97. The anti-inflammatory activity using the fish skin ferment and the reports complement the current findings. So far, no anticancer activity studies have been delineated using fermented fish skin samples. Hence, an attempt was made to determine the anticancer activity in the present studies. Anticancer activity was evaluated using the MTT assay for all extracts against the pancreatic cancer cell lines isolated from mice (BETA TC-6 cell line). *Saccharomyces cerevisiae* fermented surface sterilized sample extract and *Bacillus subtilis* fermented autoclaved sample extract were revealed to possess Strong anticancer activity with 48.63 and 54.89 IC50 62.5 µg/ml concentration.

CONCLUSION

Fermentation of fish skin waste by microorganisms such as *Aspergillus oryzae, Bacillus subtilis,* And *Saccharomyces cerevisiae* yields bioactive nutraceutical compounds. Both autoclaved, and Surface-sterilized fish skin samples exhibit consistent production of bioactive compounds, including phytochemicals and proteins, manifesting antioxidant, anti-inflammatory, antimicrobial,

And anticancer properties. *Aspergillus oryzae* possesses the highest antioxidant and anti-inflammatory activities, while Bacillus subtilis exhibits superior anticancer and antibacterial Effects. Saccharomyces cerevisiae excels in anticancer activity and protein estimation. Notably, *Aspergillus oryzae* displays broad-spectrum activity across all tested parameters, suggesting its Potential as a versatile source of bioactive compounds.

Therefore, this study concludes that *Aspergillus Oryzae* has a commendable ability to yield numerous nutraceutical compounds with diverse.

ACKNOWLEDGEMENT:

The Authors are thankful to the principal and management of Valliammal College for Women, E9 Anna Nagar East, Chennai, for their constant support in completing this research work.

Author Contribution

All authors made substantial contributions to the conception, design, acquisition, analysis, or interpretation of data for the work. They were involved in drafting the manuscript or revising it critically for important intellectual content. All authors gave final approval of the version to be published and agreed to be accountable for all aspects of the work, ensuring its accuracy and integrity.

Funding Support:

The Author declares that there is no funding.

Conflict of Interest:

The Author declares that there is no conflict of interest.

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