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Research Article

Bacillus cereus-mediated biofermentation of Sardine offal waste: A novel approach to enhance nutritional value by Response Surface Methodology optimization

Riya Ann Samuel
Department of Life Sciences, CHRIST (Deemed to be) University, Central Campus, Hosur Road, Bangalore- 560029 (Karnataka), India
Paari K. A*
Department of Life Sciences, CHRIST (Deemed to be) University, Central Campus, Hosur Road, Bangalore- 560029 (Karnataka), India

*Corresponding Author E-mail: paari.ka@christuniversity.in

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Abstract
The rising protein demand in the aquaculture sector has significantly impacted fishmeal supply and pricing. Excessive use of fishmeal can lead to environmental issues and negatively impact marine biodiversity and human food security. Consequently, finding alternative fishmeal in aquaculture is crucial for economic and environmental sustainability. The present study aimed to determine how *Bacillus cereus* (MT355408) could enhance nutritional value of Sardine fish waste, which could replace fish meal in the market. Solid-state fermentation (SSF) represents a biotechnological method that utilizes microbes to convert discarded fish byproducts into valuable products. The bacterial ability to produce enzymes was studied and optimised for its maximum production to be used as an inoculum for the SSF technique. Different prebiotic sources were also studied for better upliftment of bacteria in the solid-state surface. A single-factor analysis was conducted to investigate the influence of varying prebiotic concentrations, inoculum quantity, and fermentation duration on protein breakdown. After studying the single-factor tests, a further response surface model was employed for better yield. The results indicated that the highest protein yield could be achieved with a fermentation time of 132.893 hours, a prebiotic quantity of 25%, and an inoculum quantity of 5.3%. The study's findings also affirmed that the model was vital in enhancing the crude protein content during fermentation. In conclusion, the model's results contribute valuable insights into fermentation processes, offering practical implications for enhancing protein content and digestibility in similar contexts.

Keywords: Bacteria, Fish waste, Prebiotics, Response surface methodology, Solid state fermentation

INTRODUCTION

Global level fish consumption has escalated over the past 60 years by 14-fold, from 0.75 million to 13.7 million, propelled by the promising health benefits associated with fish protein, such as its high nutritional content and protein quality (Shyam and Akhila, 2022). The challenge of meeting the demand of a rising population with essential fish nutrients has brought major pressure to the aquaculture industry. Fish meal has been the foundational ingredient in aquafeed formulation for ages due to its exceptional nutritional value, attributed to digestible proteins, balanced amino acid ratio, and omega-3 fatty acids. However, the excessive utilization of fish meal as the major source of protein in aquafeed triggered environmental impact, sustainability concerns and price volatility (Simat, 2021). Alternative sources of fish meal are widely studied, among which usage of agro-industrial waste resources has caught great attention as it can achieve net zero carbon emissions. Among the agro-industrial waste, one major segment of environmental pollution is caused by the accumulation of fish offal waste (FOW). Fish waste utilization has emerged as a critical area of research in sustainable resource management. The proper valorisation of discarded fish offal wastes could increase the scope for improving the availability of valuable nutrients(Essabiri *et al.,* 2021).

Among the array of techniques, fermentation offers a promising avenue for recovering and recycling essential nutrients in fish waste, which can be a promising alternative for feed ingredients. Microbial fermentation releases various enzymes such as proteases, cellulase, and amylases, that break down the raw materials (Mayta-Apaza *et al*., 2021).Among the enzymes, proteases from animal, plant, and microbial sources reign supreme in applications in diverse sectors. Versatile applications encompass a wide array of processes, including the extraction of carbohydrate gums and mucopolysaccharides, solubilization of keratin materials, and the conversion of waste materials into valuable protein concentrates (Manfredini *et al*., 2020).The proteases are efficient protein-degrading catalysts that break down proteins into smaller peptides and amino acids. Due to their remarkable specificity, they enable targeted enzymatic reactions for precise protein degradation (Araujo *et al*.,2020). Research on identifying novel strains capable of synthesising protease and optimising media components that aid in proteolysis is receiving attention.

In recent years, an increasing focus has been placed on utilizing solid waste substrates towards enzyme production. These substrates comprise a diverse range of sources, including corn steep liquor and feather meal (Do Nascimento *et al.,* 2011) shrimp shell (Mathew et al., 2021), tannery solid waste (Ravindran *et al.,* 2016). Notable bacterial strains recognised for the protease production include *Bacillus licheniformis, B. amyloliquefaciens, B. subtilis, B. lentus, B. halodurans, B. pumilus, B. circulans, B. clausii, B. alkaloophilus and B. safensis Pseudomonas fluorescens, P. aeruginosa, Geobacillus SBS-4S.* (Solanki *et al.,* 2021). Diverse microbial strains have demonstrated the capacity to secrete various types of proteases that aids them to colonize on a solid matrix and catalyse the conversion of byproducts (Briki *et al.,* 2016; Alves *et al.,* 2016). The substrate's specific microenvironment influences microbial behaviour and metabolic pathways, resulting in diverse product profiles.

Utilisation of microbes in Solid-state fermentation (SSF) is a adaptable technique in the processing of offal waste (Wang *et al.,* 2018). Offal byproducts, rich in organic matter, undergo breakdown, releasing essential nutrients and bioactive substances. Temperature, pH, types of microorganisms involved, and including other enhancers impact the bioconversion. The breakdown of fish waste generates amino acids and peptides that act as crucial animal feed components (Patsios *et al.,* 2020). With the expansion of our understanding of microbial physiology, the potential of SSF to produce new and sustainable bioproducts is expanding (Li *et al*., 2023). The present study aimed to identify a potential protease-secreting bacteria that can ferment the fish offal waste and generate a simpler biomolecule that is to be formulated as an aquafeed. *Bacillus cereus* MT355408 isolated in this study was assessed for its ability to utilise the prebiotics that fetch promising options in aqua feed development and solid waste management.

MATERIALS AND METHODS

Fish waste offal collection

Sardine offal waste, including the head, gut, tails, and viscera, was collected from fish markets in Taverkara, Bengaluru. The samples were washed with water to discard excess blood. The samples were sun-dried for 24 hours to reduce moisture. The samples were placed in the hot air oven at 80□ for 48 hours to obtain a constant weight. Samples were stored 4□ till further analysis (Hammoumi *et al.,* 1998)

Qualitative assessment of extracellular enzymes from inoculum

Extracellular enzymes detection on the starter culture

Starter culture of *Bacillus cereus* (MT355408) was previously isolated and characterized from the gut of Penaeus *indicus* (Samuel *et al.,* 2021). *Bacillus cereus* was assessed for its ability to secrete different extracellular enzyme activity such as Cellulase (Islam and Roy, 2018), Protease (Zhang X *et al*., 2021), Phytase (Lee *et al.,* 2015), Amylase (Luang-In *et al*., 2019) and Lipase (Lee *et al.,* 2015).

Quantitative analysis of protease production and its improvement

Protease production was further analyzed quantitatively using a modified assay (Cupp Enyard, 2008). To determine protease activity, the following basal medium comprising of subsequent constituents (% w/v) was used: 1% glucose, 0.5% peptone, 0.5% yeast extract, 0.02% $MgSO_4H_2O$, and 0.02% $CaCl_2$,0.1% K_2HPO_4 (pH 7.0±0.2). After incubation, the production of crude enzyme was processed by centrifugation at 10,000 rpm for 10 minutes and the resulting pellets were discarded. The activity of protease was assessed using casein as a substrate.

Enhancement of protease production and optimization in various conditions

Protease activity was optimized in different conditions like time incubation ((24 - 96 hours), Temperature 24°-50°, pH (3-13), media components (Carbon 1% and nitrogen source 0.5%), synthetic prebiotics (0.5%- 2%). (Hashmi *et al.,* 2022 and Masi *et al.,* 2021)

Selection of prebiotics

Selection of prebiotics was carried out based on the ability of the bacteria to synthesize enzyme and on the

enzyme activity. The study tested five different prebiotic sources of 1% concentration: Cane molasses, Wheat, Moringa, Noni, Nutmeg.

One Factor Analysis for Solid-state fermentation Fermentation parameters

The foundational parameters like fermentation temperature, prebiotic source, Inoculation quantity and moisture content were fixed on the aforementioned data, supporting the best protease production conditions. Throughout the single factor analysis, the fish offal waste was set up to 80% with the prebiotic source concentration to 20% and the inoculum quantity percentage to 5% with knowledge of previous literature. Subsequently, optimization was performed using varying concentration ranges. (Afreen and Ucak ,2020) (Table 1).

Effect of different concentrations of *Moringa oleifera* on the fermentation process by *Bacillus cereus* on sardine waste

The different concentration (10, 15, 20, 25, 30%) of *Moringa oleifera* was analyzed to determine the optimal prebiotic concentration for enhancing nutritional support in solid-state conditions. Concurrently, moisture content as assessed to identify the most favorable moisture level that promotes a higher fermentation rate (Abidin *et al.,* 2022).

Effect of different concentrations of inoculum quantity for solid-state fermentation

Bacillus cereus was administered in different concentration ranges from (4.0, 4.5, 5.0, 5.5, and 6.0 %) by keeping the basic fermentation parameters constant. The best range was selected according to the degree of hydrolysis and crude protein content (Harun *et al.,* 2019).

Effect of fermentation time on solid-state fermentation

The fermentation set up with the best parameters was analyzed for different fermentation times (1, 3, 5, 7, 9 , 11 days post-fermentation) to understand which day showed the best protein content. The crude protein content was evaluated by Bradfords method (Kamizake *et al.,* 2003).

The degree of hydrolysis was determined using the OPA method by determining the cleaved peptides using Spectrophotometric method. The OPA method was done by modifying the method of Nielsen *et al,* (2001), where serine was used as a standard and the following equations were used.

$DH = h/h_{tot} * 100\%$ Eq. 1

h= number of hydrolysed bonds and h_{tot} is the total number of peptide bonds per protein equivalent.

Optimization of Solid-state fermentation by response surface methodology

Response Surface Methodology (RSM) was employed to identify the optimal values. Three significant variables were established:(A) Fermentation time, (B) Prebiotic Concentration, (C) Inoculum Concentration.

Degree of hydrolysis and protein percentage were considered as the two responses following fermentation. The target yields for assessment were the degree of hydrolysis and crude protein. Crude protein was analysed using the Kjeldahl method using Kjeltec analyzer 2100 (Silva *et al.,* 2014). A 24 factorial Central Composite Design (CCD) was employed to analyse the response surface within the optimal range. The present research design included five coded levels (-, -1, 0, +1, +) and featured six repetitions at the central point to enable accurate error estimates. Table 2 outlines the variable ranges from the minimum to maximum values. The entire experimental plan, consisting of 20 points, was executed randomised, as described by (Molinuevo -Salces *et al.,* 2015) (Table 3). The model's validity was confirmed by comparing the point predictions with the results obtained from the design of experiments, aiming to maximize both protein yield and the degree of hydrolysis (ZhangY *et al* ., 2021)

Amino acid analysis

The bound amino acids were initially profiled through an acid/alkaline hydrolysis extraction process. The process involved hydrolyzing with 2N KOH for 6 hours at 100°C and acid hydrolysed with 2N HCl at 100°C for 8 hours. Similarly, Free amino acids were extracted by

Table 1. Fermentation conditions maintained consistently throughout the experiment.

Fermentation strain	Inoculation Quantity dilution	Fermentation Temperature	Prebiotic Source	Moisture Content
Bacillus Cereus	10^4 CFU/ml	37 Degree Celsius	Moringa oleifera	35-40%

Table 2. Response surface methodology employed ranges of 3 independent variables

Code	Independent variables	-α	-1	0	+1	+α
A	Time	66.13	90	125	160	183.863
B	Prebiotic source	18.295%	20	22.5%	25%	26.7045%
C	Inoculum quantity	4.8%	5%	5.25%	5.5%	5.6%

Table 3. Design of experiments and results of various combination Trial with yield responses.

	Factor 1	Factor 2	Factor 3	Response 1	Response 2
Run	A: Time Hour	B: Prebiotic %	C: Probiotic %	DH %	Protein %
1	125	26.7045	5.25	21.8	42.2
2	66.1373	22.5	5.25	17.9	37.4
3	125	22.5	5.25	21.3	42.1
4	125	22.5	5.25	21.2	42.24
5	90	25	5.5	19.1	38.5
6	183.863	22.5	5.25	16.4	33.7
7	125	22.5	5.25	21.5	42
8	125	22.5	5.25	21.4	42.3
9	90	25	5	19.5	38.9
10	160	20	5	20.5	41.6
11	90	20	5	19.3	38.5
12	125	22.5	4.82955	20.8	41.67
13	160	25	5.5	20.7	40.8
14	125	18.2955	5.25	21.27	41.9
15	160	25	5	20.34	40.9
16	160	20	5.5	20.4	40
17	125	22.5	5.67045	21	41.6
18	125	22.5	5.25	21.29	42
19	125	22.5	5.25	21.31	41.98
20	90	20	5.5	19.3	38

homogenizing dry samples of fish waste in 0.1% formic acid n 20% v/v methanol. After centrifugation, homogenate aliquots were collected, redissolved in formic acid, and filtered through a 0.2 µm nylon membrane. A 5µl of the sample aliquot was injected into the UPLC-MS/MS system for amino acid analysis. The Acquity UPLC-H class system with TQD-MS/MS instruments was employed under optimized Multiple Reaction Monitoring (MRM) mode conditions, ensuring the highest detection sensitivity. Operational settings for the MS/MS system were individually optimized for each amino acid in positive ionization mode (ES+) to obtain mass spectra revealing the most abundant protonated forms of amino acid molecules.

Analysis of nutritional content before and after fermentation

Proximate analysis of Non-fermented Fish waste (NFW) and Fermented Fish waste (FFW) using the standard procedures according to the AOAC guidelines. Assessment of moisture, pH, crude protein to understand the quality of fermented products. The secondary nutrients like phosphorus, Potassium, calcium, sulfur and magnesium were analyzed using AAS (McBride, 2020).

Statistical analysis

The analysis of variance (ANOVA) of the Central composite model was done using the RSM Package of Design Expert 1 software3. All experiments were replicated three times and Data is represented as mean values and standard deviation.

RESULTS AND DISCUSSION

Quantitative analysis of protease production by *Bacillus cereus*

Effect of different time incubation

The study investigated the protease activity over a 72-hour incubation period. The highest protease activity was observed between the 36th and 48th hours, with enzyme activity levels measuring 223.9 ± 4.4 U/ml and 121.14 ± 8.3 U/ml, respectively. However, starting from the 72nd hour of incubation, there was a noticeable decrease in enzyme production. Specific activity measurements were conducted to assess the efficiency of the protease at different time points. At the 36th hour, the specific activity was determined to be 1.065 U/µg, while at the 40th hour, specific activity increased to 1.171 U/µg. The production of extracellular proteases is known to be influenced by various physical factors, including pH, temperature, inoculum density, and incubation time, as well as the composition of the growth medium, particularly the carbon and nitrogen sources (Alahmad, Aljammas *et al.,* 2022).In industrial fermentation processes, substrates represent a substantial portion of the production cost, accounting for around 30 -40% of industrial enzyme production expenses. Consequently, selecting cost-effective growth media is cru-

cial in reducing the overall cost of enzyme production (Tennalli *et al.*, 2022). By optimizing the growth medium, significant cost savings can be achieved, making enzyme production processes more economically viable and efficient. These values were calculated based on the enzyme activity obtained from the protease assay using β-casein as the substrate and the total protein content of the protease solution. Overall, the study reveals that the optimum protease activity was achieved during the 36th hour of incubation, with a subsequent decline in enzyme production beyond this time point (Fig. 1)

Effect of different temperatures

The optimum temperature range for protease production was between 37°C and 40°C, resulting in enzyme activity levels of 198.8525 U/ml and 344.4959 U/ml, respectively. However, as the temperature exceeded 40°C and reached 47°C, the protease activity decreased. At 40°C, the protease activity reached its maximum, and the specific activity of the enzyme was calculated to be 1.19 U/µg. Interestingly proteases from different *Bacillus* genera have been reported to exhibit maximum activity at varying temperature ranges. *Bacillus cereus* AUST-7 locally isolated from soil showed peak activity at temperatures between 44 to 55 °C (Ullah *et al.,* 2022). In another study where protease isolated from *Bacillus cereus* HMRSC30, derived from Terasi exhibited a better stability within the temperature from 30- 50°C (Cahyaningtyas *et al.,* 2021). The present study highlights the temperature-dependent nature of protease production, with the highest activity observed at 40°C in this experiment. These findings are consistent with previous reports showing variations in protease activity among different genera and under different temperature conditions (Fig. 2)

Effect of pH

During the fermentation process, the pH of the surrounding medium often fluctuates due to the metabolites produced by the microorganisms supplemented (López-Trujillo *et al.,* 2023); there are chances of varying pH exerted to the surrounding medium it is important to check the ability of Bacillus cereus to withstand various pH conditions. Here, the exposure of protease medium to different pH levels showed that *Bacillus cereus* could withstand various ranges of pH from 1 - 13. But, the optimum was at pH 7 and 9 with 358.9 ± 14.45 U/ml and 320.4 ± 15.23 U/ml. (Fig. 3)

Effect of carbon sources

Carbon sources play an important role in growth media, as they are essential. The study supplemented five different carbon sources, including lactose, fructose, xylose, starch and arabinose. Among these sources,1% of lactose has shown the highest protease activity. The average protein activity of lactose is 345.33.4 U/ml. Followed by arabinose and xylose, which also showed significant protease activity, reaching a value of 258.273.7 U/ml and 232.8U/ ml, respectively. In order to analyze the effectiveness of lactose as a carbon source further, the total protein content was assessed, and the specific activity of lactose was determined at 1.18U/g. The results show that lactose, one of the carbon sources tested, is the most effective for promoting protease activity and has the highest average protease and specific activity. The study conducted by Asha *et al.* (2018) suggested that the reported *B. cereus* FT1 showed enhanced production by utilizing 2% lactose compared to other types of carbon sources (Fig. 4).

Effect of different nitrogen sources

Nitrogenous materials are vital as they serve as a source for amino acid production, which, in turn, plays a crucial role in protein production, essential for the development and functioning of all organisms. In this study, various nitrogen sources were tested at a concentration of 0.5 %, including yeast extract, sodium nitrate, ammonium chloride, glycine, and ammonium sulfate. The results revealed that glycine and ammonium chloride were the optimum nitrogen sources for promoting protease activity. The protease activity with glycine was measured at 262.98 ± 2.4 U/ml, while ammonium chloride displayed an activity of 179.6 ± 1.4 U/

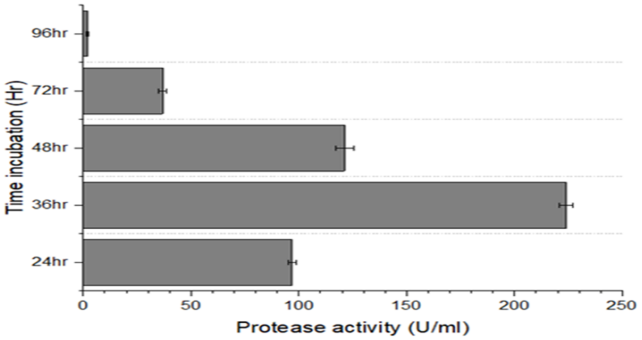

Fig. 1. *Effect of time incubation on the protease production by Bacillus cereus*

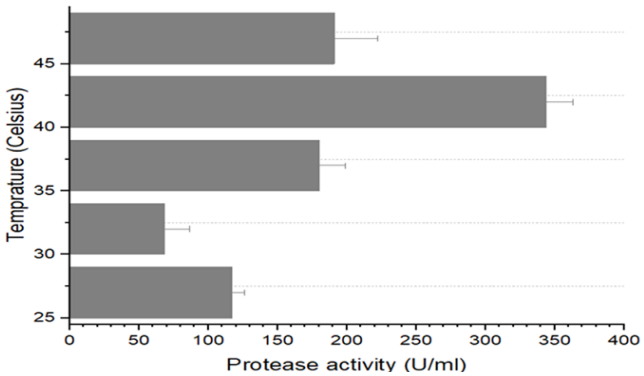

Fig. 2. *Effect of different temperatures on the protease production by Bacillus cereus*

ml. In a more detailed analysis of the efficiency of glycine as a nitrogen source, specific activity is calculated, and the value is 1.213U/g. The study conducted by Gaddad *et al.* (2019) has shown that peptone and yeast extract are the most effective sources of nitrogen to improve the activity of proteins. In particular, in this study, glycine showed the highest protease activity and specific activity among the tested nitrogen sources, highlighting its importance as a key component of the growth medium for efficient protease production (Fig. 5).

Effect of different synthetic prebiotics

Different synthetic prebiotics, including inositol, sorbitol, and mannitol, were used to investigate their effect on protease activity. The prebiotics were tested at various percentages, including 0.5%, 1.5%, 1.0%, and 2.0%. Among the prebiotics tested, inositol at 0.5% showed the highest protease activity, with a measurement of 390.71±19.34 U/ml. Following this, 1.5% inositol exhibited a substantial protease activity of 363.03± 18.14 U/ml. Mannitol also showed better protease activity ranges when compared to sorbitol. The findings highlight the significant impact of different synthetic prebiotics on protease activity, with inositol and mannitol demonstrating notable effects at specific concentrations. Further investigations into the mechanisms behind these effects could contribute to a better understanding of how prebiotics can modulate protease production, with potential implications in various biotechnological applications and industrial processes (Hadjidj *et al.,* 2018) ((Fig. 6).

Effect of natural prebiotics

The research focused on solid fermentation; choosing a source of nutrients that can ensure the survival and spread of Bacillus cereus in fish waste is important. Here, the study selected five different sources of natural prebiotics which are Cane molasses (*Saccharum officinarum L*), Wheat (*Triticum aestivum), Moringa oleifera*, *Myristica fragrans, M. citrifolia.* The results suggested that *M. oleifera* when supplemented with 1% showed the highest protease activity with 395± 15.6 U/ml compared to other sources added (Fig. 7).

The present study observed that pH, temperature and time incubation influence the proliferation of Bacillus cereus and the enzyme activity. Given the use of Solid-State Fermentation (SSF) in this study, the choice of an appropriate fermentation temperature was 40ºC as it is associated with maximum production of protease enzyme. The optimal fermentation time for incubation for *Bacillus cereus* was set as 24 hour and subsequently, the time incubation was progressively elevated to 160 hours. *M. oleifera*, a prebiotic supplement to enhance protease production, showed prominent results. Hence *M. oleifera* was considered a nutrient source in solid-state fermentation for elevating bacterial survival.

One factor analysis of solid-state fermentation

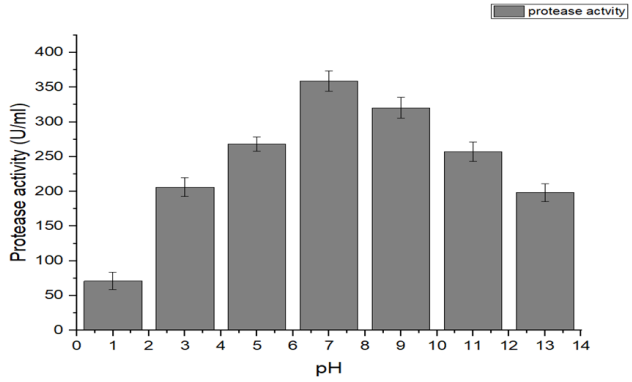

Fig. 3. *Effect of pH on protease production by Bacillus cereus*

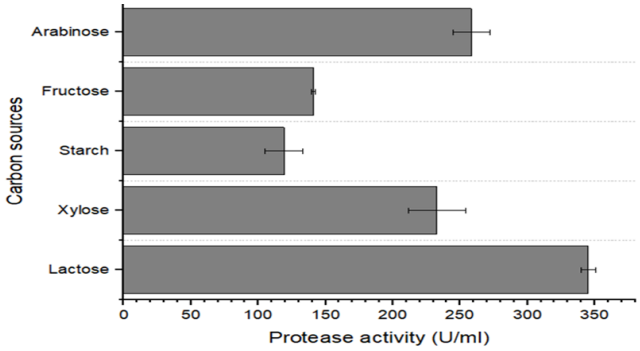

Fig. 4. *Effect of Carbon sources on protease production by Bacillus cereus*

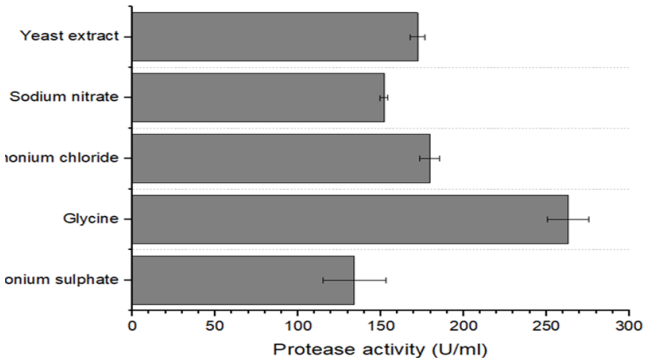

Fig. 5. *Impact of different Nitrogen sources on the protease production by Bacillus cereus*

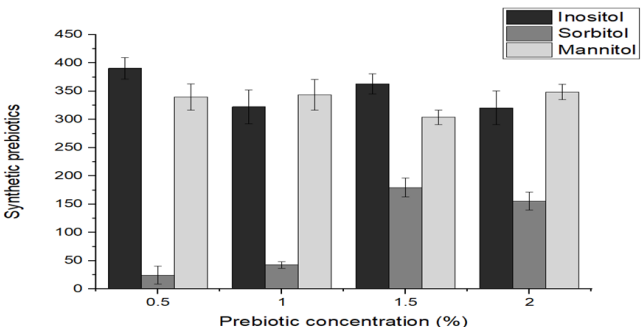

Fig. 6. *Effect of different synthetic prebiotics on the maximum production of protease by Bacillus cereus*

Table 4. Analysis of variance for regression equation of degree of hydrolysis of fermented fish waste.

ANOVA for Reduced Cubic model						
Response 1: DH						
Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	35.53	12	2.96	173.82	< 0.0001	Significant
A-Time	1.12	1	1.12	66.05	< 0.0001	
B-Prebiotic	0.0099	1	0.0099	0.5827	0.4702	
C-Probiotic	0.0028	1	0.0028	0.1658	0.6961	
AB	0.0024	1	0.0024	0.1438	0.7157	
AC	0.0544	1	0.0544	3.20	0.1169	
BC	0.0005	1	0.0005	0.0264	0.8755	
A^2	30.45	1	30.45	1787.95	< 0.0001	
B^2	0.1346	1	0.1346	7.90	0.0261	
C^2	0.2356	1	0.2356	13.83	0.0075	
ABC	0.0924	1	0.0924	5.43	0.0526	
AB^2	3.57	1	3.57	209.80	< 0.0001	
B^3	0.0650	1	0.0650	3.82	0.0917	
Residual	0.1192	7	0.0170			
Lack of Fit	0.0657	2	0.0328	3.07	0.1351	not significant
Pure Error	0.0535	5	0.0107			
Cor Total	35.65	19				

Single factor analysis suggested the influence of varying prebiotic concentrations for maximum protein yield during the fermentation process. The prebiotic content of *M. oleifera* was administered at a concentration ranging from 10 % to 30 %. The results suggested a maximum protein percentage of 37.6 % and 37.9% that was obtained at 20 and 25% of prebiotic concentration. During solid-state fermentation, including carbohydrate or nutrient sources is crucial in ensuring bacterial growth and metabolism. The concentration of *M. oleifera* in samples above 30 % showed fungal contaminations, suggesting the optimal moisture content to remain better at the 40-45% range. The results also indicated that the bacterial content maintained at a constant range between 20- 25% produced better proteolytic enzymes to break down the complex bound protein content into simpler proteins (Fig. 8)

Effect of inoculum quantity on solid state fermentation

The best inoculum quantity was observed to be in the

Table 5. Analysis of variance for regression analysis of protein content in fermented fish waste

ANOVA FOR REDUCED CUBIC MODEL						
RESPONSE 2: PROTEIN						
SOURCE	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
MODEL	97.07	12	8.09	536.40	< 0.0001	significant
A-TIME	6.84	1	6.84	453.92	< 0.0001	
B-PREBIOTIC	0.1658	1	0.1658	10.99	0.0128	
C-PROBIOTIC	0.0025	1	0.0025	0.1625	0.6989	
AB	0.0800	1	0.0800	5.31	0.0547	
AC	0.0800	1	0.0800	5.31	0.0547	
BC	0.3200	1	0.3200	21.22	0.0025	
A^2	76.69	1	76.69	5085.49	< 0.0001	
B^2	0.0011	1	0.0011	0.0726	0.7954	
C^2	0.3482	1	0.3482	23.09	0.0020	
ABC	0.2450	1	0.2450	16.25	0.0050	
A^2C	0.3066	1	0.3066	20.33	0.0028	
AB^2	17.15	1	17.15	1137.34	< 0.0001	
RESIDUAL	0.1056	7	0.0151			
LACK OF FIT	0.0116	2	0.0058	0.3094	0.7470	not significant
PURE ERROR	0.0939	5	0.0188			
COR TOTAL	97.17	19				

range of 5.0% - 5.5%. The inoculum provided at 4.0 and 4.5% showed less protein content, revealing insufficient bacterial culture to sustain fermentation kinetics. On the other hand, when the inoculum percentage was increased to 5.0%, a significant improvement in protein content was observed, reaching 37.8%. A similar pattern was noted when the inoculum was raised to 5.5% (Fig. 9).

Effect of time incubation at fermentation analysis

There was a positive correlation between the duration of fermentation, measured in hours, and the extent of protein breakdown. Tropea *et al.* (2021) studied the combination of fish waste and lemon peel fermented with *Lactobacillus reuteri* and *Saccharomyces cerevisiae* and the study found that fermentation could potentially increase the protein content from 11.68 ± 0.48 to 48.55 ± 0.72 from 0 to 120 hours. The fermentation setup for this study was kept for 1- 7 days (24h – 168 h) (Fig. 10). Fermentation byproducts increased with the extension in the fermentation time. From day 1 to day 5, there was an increase in protein, and from day 5 to day 7, a similar increase in protein percentage was noted. From the 7th day, the protein content decreased mostly because of the deprived nutrient sources inhibiting the fermentation kinetics. Nevertheless, beyond 160 hours, the protein content demonstrated a tendency to decline. This could potentially be attributed to the fact that the fermentation time surpassed the optimum range for *B. cereus*, resulting in a deceleration of fermentation kinetics. Utilizing a center composite design, a time incubation hour from 90 ºC to 168 hours was chosen as the baseline for the fermentation time in the Response Surface Methodology (RSM) approach.

Fermentation optimization of parameters using central composite design.

After doing a preliminary analysis of single factors, further optimizations were done using central composite design utilizing the software programme of design of experiments version 16. For solid-state fermentation involving sardine fish wastes, three parameters were considered: fermentation time (hours), prebiotic quantity (%), and inoculum quantity (%). Central composite design was selected to find efficient and balanced factorial points that can promise optimal conditions for the desired response variable. Even the small deviations from optimal conditions can be determined in CCD, providing precise estimates of model factors. Two response yields were fixed for the model: crude protein and degree of hydrolysis. A second-order polynomial equation is used in CCD to model the relationship between the factors and responses. The fermentation time was set in the range from 90 hours to 160 hours as the range showed a dip in protein content and increase in degree of hydrolysis when analyzed during One factor at a time, which possibly suggests initial breakdown of protein to peptides and then amino acids. The second factor was prebiotic quantity, which significantly enhanced the nutrient nourishment for *Bacillus*

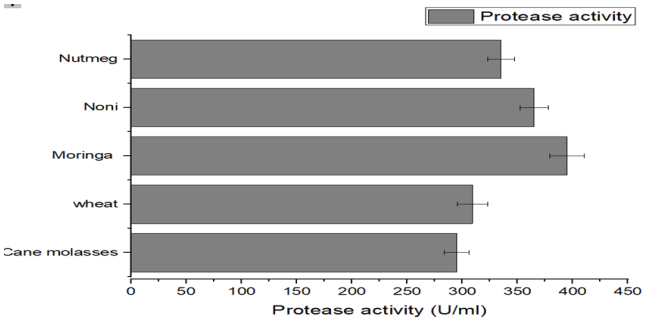

Fig. 7. *Effect of Natural prebiotics on the production of Protease by Bacillus cereus*

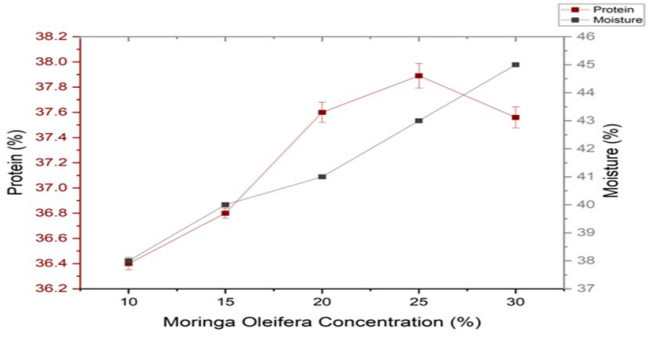

Fig. 8. *Effect of different concentrations of Moringa Oleifera on the fermentation process by Bacillus cereus on Fish waste*

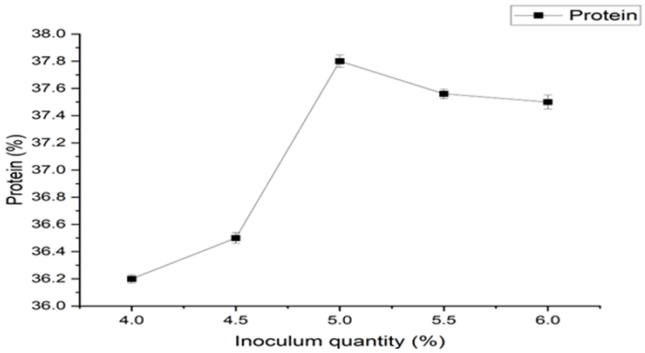

Fig. 9. *Effect of Inoculum quantity on protein content on fermentation process of fish waste*

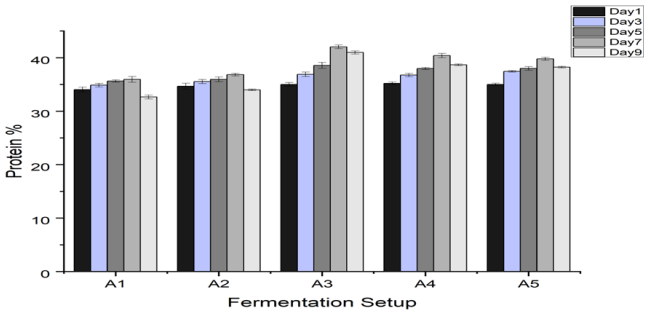

Fig. 10. *Fermentation Time Incubation: Day-wise Analysis from Day 1 to Day 7*

Table 6. The amino acid composition of non-fermented fish waste and fermented fish waste

Amino acid composition	Non-fermented Fish waste (Mg/g)	Fermented Fish waste (Mg/g)
Glycine	0.4925±0.017	0.486±0.0155
Alanine	13.235±0.205	12.416±0.033
Serine	13.8985±0.219	14.641±0.07
Proline	13.373±0.055	22.412±0.115
Valine	3.6765±0.009	4.704±0.06
Threonine	3.1335±0.068	6.1405±0.105
Cysteine	0.089±0.009	0.4335±0.03182
Leucine	12.7515±0.1124	25.398± 0.192
Asparagine	1.7755±0.0544	1.1775±0.03
Aspartic acid	9.7485±0.095	10.4985±0.0997
Lysine	4.364±0.033	20.972±0.123
Glutamic acid	4.5375±0.00919	9.73±0.02
Methionine	6.987±0.079	12.398±0.101
Histidine	0.7895±0.012	5.3135±0.05586
Ethionine	0.0125±0.0000748	0.0085±0.00071
Phenylalanine	9.2515±0.048	28.5175±0.0502
Arginine	3.4765±0.075	9.158±0.0155
Citrulline	0.0475±0.00353	0.0755±0.000212
Tyrosine	5.404±0.080	6.309±0.10041
Beta 3-4 dihydroxy phenyl alanine	0.0165±0.00212	0.011±0.000141
Tryptophan	0.382±0.00028	0.926±0.00424

Table 7. Proximate analysis of fermented and non-fermented fish waste.

Parameters	Non-fermented Fish waste	Fermented Fish waste
pH	6.10	5.12
EC	1.73	1.75
Organic Carbon (TOC) %	42.92	40.02
N(nitrogen) %	6.05	6.75
P (phosphorous)%	2.40	2.71
K(potash) %	0.40	0.42
Calcium %	5.91	6.75
Magnesium %	0.19	0.20
Sulphur%	1.78	2.01
C:N ratio	32.10	42.62

cereus. The range was fixed from 20- 25%. The third factor was the inoculum quantity and was set in range of 5-6%. The experimental setup was carried out using 1 gram of fish waste in sterile plastic boxes. A combination run of a total 20 runs was executed and the response was updated in Table 4.

Regression analysis was performed and the protein and degree of hydrolysis results were evaluated. Based on the yield analysis of degree of hydrolysis the regression analysis suggested that the Model F-value of 173.82 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P value suggested that the model is significant as the p value of A, A^2, $B^{2,}$ $C^{2,}$ and AB^2 are significant. The Lack of Fit F-value of 3.07 suggested that the Lack of Fit was not significant compared to the pure error. There was a 13.51% probability that such a Lack of Fit F-value could happen randomly. A lack of fit being non-significant is desirable, which indicated a good fit of the model. The predicted R^2 of 0.8703 was in reasonable agreement with the adjusted R^2 of 0.9909 which ideally has a difference less than 0.2, with an adequate precision of 51.321 which indicates an adequate signal that can be used to navigate the design space. The final equation in terms of coded factors for degree of hydrolysis based on the CCXD model is given below.

Fish waste degree of hydrolysis= 21.3295 + -0.445953 * A + -0.0591068 * B + 0.014378 * C + 0.0175 * AB + 0.0825 * AC + 0.0075 * BC + -1.45369 * A^2 + 0.096645 * B^2 + -0.127861 * C^2 + 0.1075 * ABC + 1.03845 * AB^2 + 0.0766068 * B^3.

Where the A, B, C, AB, AC, BC, A^2, B^2, C^2, ABC, AB^2 and B^3 are the coded values of (A) Fermentation time, (B) Prebiotic quantity, (C) Probiotic quantity

Amongst the coded factors A, B, BC, A^2, C^2, ABC, A^2, AB^2 showed significant p value. To understand the optimal value ranges 3D response surface graphs were plotted. From the graph, the coded colours observed evident interactions and optimal values (Fig. 11). In this fermentation time factor vs Probiotic quantity showed a

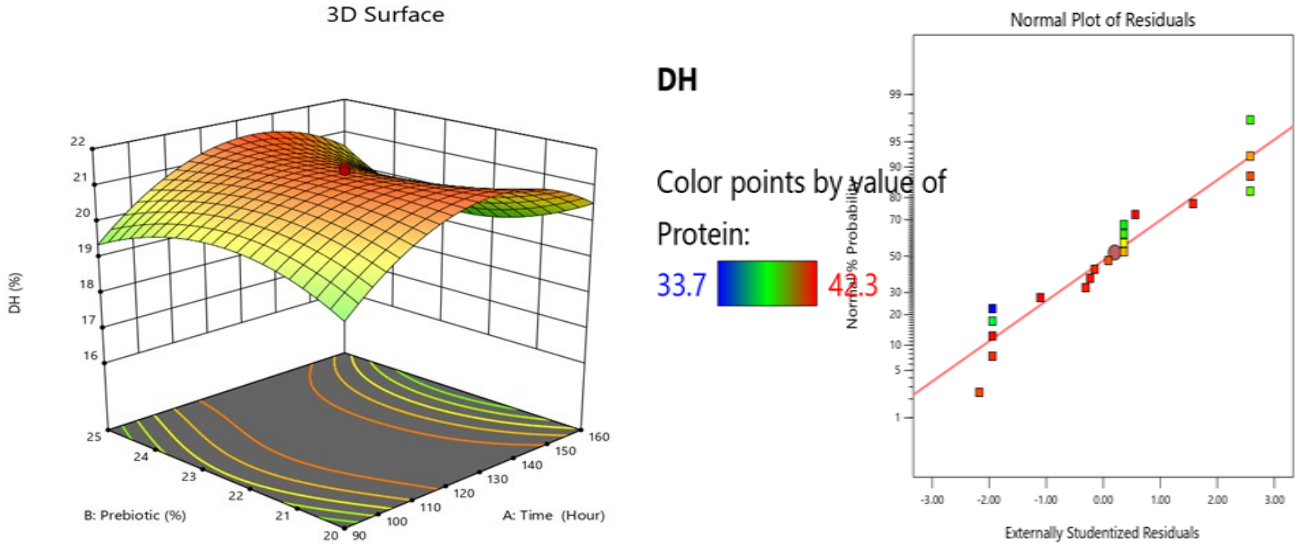

Fig. 11. *Design of Experiments Response surface plots for degree of hydrolysis. a) Effect of prebiotics vs Time incubation b) Plot for predicted vs actual values*

significant increase in the degree of hydrolysis compared to the other factors.

The second targeted yield was to see the combination of fermentation factors that increase the crude protein content of fish waste, which can be directly correlated to the amino acid nitrogen content in the fermented raw material (Table 5). After running regression analysis for protein content, it was observed that model F- value was 536.40 which determined the model's significance. Model terms are significant if their p-values are less than 0.0500. In this scenario, terms A, B, BC, A^2, C^2, ABC, A^2C, and AB^2 are significant. Conversely, values exceeding 0.1000 indicate insignificance. Reducing the model might enhance its effectiveness if numerous insignificant terms (excluding those essential for hierarchy) exist.

The Lack of Fit F-value of 0.31 suggested that the Lack of Fit was not significant compared to the pure error. There iss a 74.70% probability that such a Lack of Fit F -value could occur due to noise. A lack of fit being non-significant is favorable; it indicates a good fit of the model. The protein analysis also suggested that predicted R^2 (0.9906) is in reasonable agreement with the adjusted R^2 (0.9971) with an adequate precision of 86.2; hence the model can be suggested for navigating the design space. The final equation in terms of coded factors for the protein yield is built on the above model.

Fish waste protein =42.1018 + -1.10002 * A + 0.110167 * B + -0.0208111 * C + -0.1 * AB + -0.1 * AC + 0.2 * BC + -2.30681 * A^2 + -0.00871502 * B^2 + -0.15544 * C^2 + 0.175 * ABC + -0.304189 * A^2C + 2.27502 * AB^2.

Where A, B, C, AB, AC, BC, A^2, B^2 C^2, ABC, A^2C, and AB^2 are the combinations coded for the above-mentioned fermentation factors which are fixed for the CCD. A graphic representation in 3D (Fig. 12) was analyzed for optimal regions. The equation using coded factors allows for the prediction of the response based on specific levels of each factor. In this representation, high levels are coded as +1, and low levels are coded as -1 by default. This coded equation is valuable for assessing the factors' relative impact by comparing their coefficients. The fermentation time and prebiotic quantity showed better increase in the yield of protein.

Based on the above interactions and point prediction, the range of fermentation time and the prebiotic quantity was maximum. Probiotic quantity was also set in the range to identify the maximum degree of hydrolysis and protein content (Fig. 13). The model suggests that maximum yield can be procured at fermentation time at 132.893 with an addition of prebiotic quantity of 25% and inoculum quantity of 5.3%. The desirability of the model was seen to be 0.982. The model combinations to obtain the maximum protein and degree of hydrolysis were confirmed in triplicates, and the results showed value near the predicted range. The degree of hydrolysis of the above fermentation parameter combination was seen to be 19.8± 0.103 % and the crude protein content was 42.09%. Both the results verified that the model has significantly helped in increasing the crude protein content obtained during OFAT from 38.75 to 42.09%.

Amino acid analysis in non-fermented and fermented fish sample

Fermentation parameters suggested by design of experiments was extended for lab scale production in 300 ml glass jars. Amino acid analysis from the non-fermented and fermented fish waste sample suggested that the amino acid composition after fermentation has increased considerably (Table 6). This indicated that the fermentation has helped the complex and simpler proteins to break down into peptides and later amino

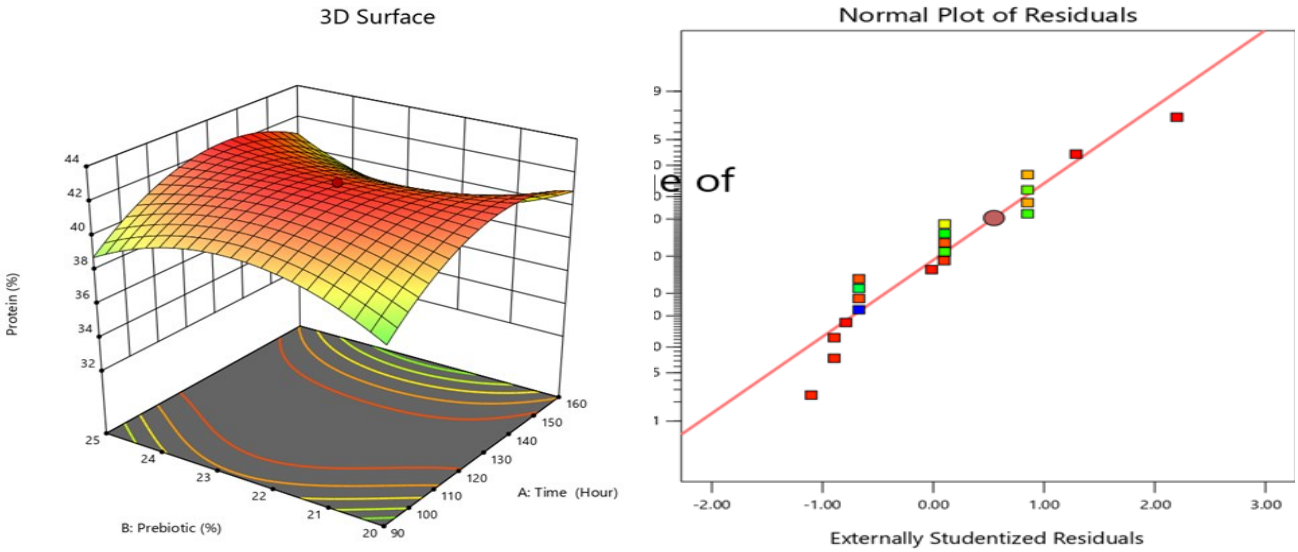

Fig. 12. *Design of Experiments Response surface plots for production of protein. a) Effect of prebiotics vs Time incubation in hour b) Plot for predicted vs actual values*

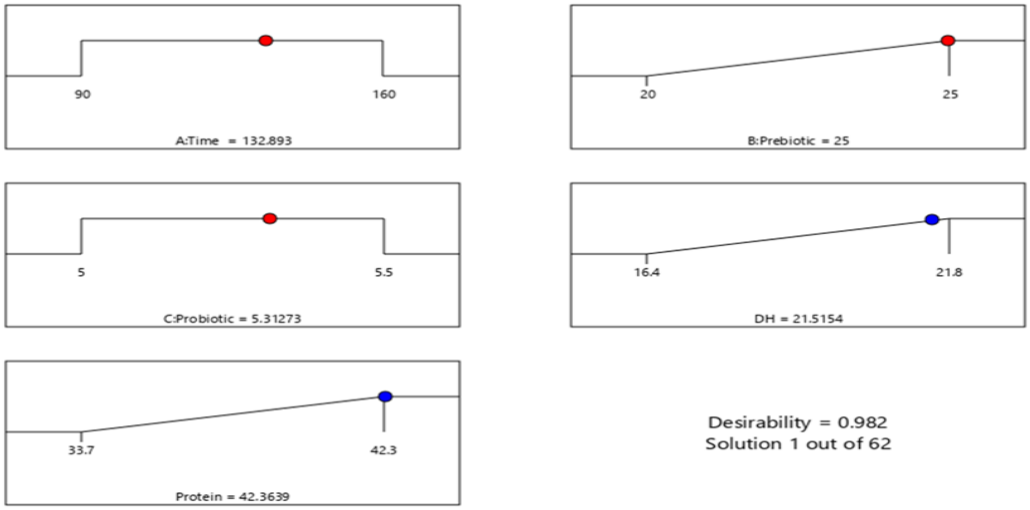

Fig. 13. *The optimal response of the desired factor limit and the possibility of solid-state fermentation of fish waste in prebiotic reaction mode*

acids. The protease enzyme released by *Bacillus cereus*, could have hydrolysed and separated proteins at specific targeted peptide site, which lead to the digestion of amino acids (Kieliszek *et al*., 2021). Leucine, proline, lysine, methionine and arginine showed notable increase compared to the other amino acids. This increase can be attributed to the transformation of complex storage proteins into simpler and more soluble forms due to microorganisms' enzymatic activity, making them easier to digest. This process not only improves the digestion of proteins, but also improves the overall nutritional quality and bioavailability of fermented foods (Nkhata *et al*., 2018). Imran and Wei (2019) in their study demonstrated the role of solid-state fermentation in the enhancement of five essential amino acids such as tryptophan, lysine, methionine, isoleucine and valine, about 43.1± 1.62 gl $^{-1}$ by utilising *Bacillus cereus* (MH027625). The result promises that the fermented fish waste can be further used to replace fish meal in aquafeed diets. Zhou *et al.* (2020) reported that optimum dietary leucine, arginine and methionine-fed diets could promote growth, immunity, gut health and antioxidant activity levels in various fishes.

Proximate analysis of fermented and non-fermented fish waste

Proximate analysis of the fermented and non-fermented samples showed a decrease in pH, which suggested bacteria might have released organic acids during the fermentation process, which increased the acidic content of the setup and eventually caused a dip in pH. The moisture content of non-fermented sample was observed to be 7% which was constantly maintained at 40- 45% of moisture by adding prebiotic and inoculum. Organic carbon content showed a slight decrease from 42.92 %to 40. 02% could be due to the

bacteria's utilization of carbon sources from the fermentation medium. The increase in nitrogen content correlated with the breakdown of protein into amino acids. There was only a slight difference in secondary nutrients compared to non-fermented fish waste (Table 7).

Conclusion

The study optimized protease production to be at 40ºC with 48-hour incubation, pH range of 7-9, lactose as carbon source at 1% and 0.5% of glycine as nitrogen source. Among the various prebiotic sources examined, *M. oleifera* stood out for its superior protease production, making it the preferred nutrient source for supplementation as prebiotics in solid-state fermentation. Optimal protein yields during fermentation were obtained at 20% and 25% prebiotic concentrations, while excess prebiotics led to unfavourable conditions and reduced protein content. Similarly, 5- 5.5 % of bacterial inoculum and 1-5 days of fermentation time showed synthesis of maximum protein content during single factor analysis. The insights obtained from single-factor analysis provided the rationale for implementing Response surface methodology to create a statistical model for optimization. In the study, the model indicated that the highest yield could be achieved with a fermentation time of 132.893 hours, a prebiotic quantity of 25%, and an inoculum quantity of 5.3%. The model's desirability was high at 0.982. The suggested combinations were tested three times to confirm these findings, and the results closely matched the predicted values. For the specified parameters, the degree of hydrolysis was measured at 19.8% ± 0.103, and the crude protein content reached 42.09%. These results significantly improved from the initial value of 38.75% obtained through One Factor at a Time (OFAT) experiments. The study's findings affirm that the model played a vital role in enhancing crude protein content during fermentation. The proximate analysis showed that the fermented fish waste had a better nutritional quality than the non-fermented fish waste. Further the amino acid analysis demonstrated improved protein degradation into amino acids post-fermentation. Therefore, the study concludes that utilizing a central composite design of Response surface methodology to optimize fermentation parameters with *B. cereus* can result in formulating a diet with improved protein and nutritional content.

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Conflict of interest

The author declare that they have no conflict of interest.

REFERENCES

1. Alves, M. P., Salgado, R. L., Eller, M. R., Vidigal, P. M. P. & Fernandes de Carvalho, A. (2016). Characterization of a heat-resistant extracellular protease from *Pseudomonas fluorescens* 07A shows that low temperature treatments are more effective in deactivating its proteolytic activity. *Journal of Dairy Science,* 99(10), 7842–7851.
2. Abidin, Z., Huang, H.T., Hu, Y.F., Chang, J.J., Huang, C.Y., Wu, Y.S. & Nan, F.H. (2022). Effect of dietary supplementation with Moringa oleifera leaf extract and Lactobacillus acidophilus on growth performance, intestinal microbiota, immune response, and disease resistance in whiteleg shrimp (*Penaeus vannamei). Fish & Shellfish Immunology, 127*, 876-890. https://doi.org/10.1016/j.fsi.2022.07.007.
3. Afreen, M. & Ucak, I. (2020). Fish processing wastes used as feed ingredient for animal feed and aquaculture feed. *Journal of Survey in Fisheries Sciences*, 55-64. https://doi.org/10.17762/sfs.v6i2.198
4. Alahmad, Aljammas, H., Yazji, S. & Azizieh, A. (2022). Optimization of protease production from *Rhizomucor miehei* Rm4 isolate under solid-state fermentation. *Journal of Genetic Engineering and Biotechnology,* 20(1),1-3. https://doi.org/10.1186/s43141-022-00358-9.
5. Araujo, J., Sica, P., Costa, C. & Márquez, M.C. (2021). Enzymatic hydrolysis of fish waste as an alternative to produce high value-added products. *Waste and Biomass Valorization*, 12, 847-55. https://doi.org/10.1007/s12649-020-01029-x.
6. Asha, B. & Palaniswamy, M. (2018). Optimization of alkaline protease production by Bacillus cereus FT 1isolated from soil. *Journal of Applied Pharmaceutical Science*, 8 (2), 119-27. 10.7324/JAPS.2018.8219.
7. Briki, S., Hamdi, O. & Landoulsi, A. (2016). Enzymatic dehairing of goat skins using alkaline protease from Bacillus sp. SB12. *Protein Expression and Purification*, 121, 9–16. 10.1016/j.pep.2015.12.021.
8. Cahyaningtyas, H. A., Suyotha, W., Cheirsilp, B. & Yano, S. (2021). Statistical optimization of halophilic chitosanase and protease production by Bacillus cereus HMRSC30 isolated from Terasi simultaneous with chitin extraction from shrimp shell waste. *Biocatalysis and Agricultural Biotechnology*, 1, 31:101918. https://doi.org/10.1016/j.bcab.2021.101918.
9. Cupp -enyard, C. (2008). Sigma's Non-specific Protease Activity Assay - Casein as a Substrate. *Journal of visualized experiments*, 10,3791/899.
10. Do Nascimento, R. P., Junior, N. A. & Coelho, R. R. R. (2011). Brewer's spent grain and corn steep liquor as alternative culture medium substrates for proteinase production by Streptomyces malaysiensis AMT-3. *Brazilian Journal of Microbiology,* 42(4), 1384–1389. 10.1590/S1517-838220110004000020.
11. Essabiri, H., Damrani, R., Boumalkha, O., Hachi, T., Laiboud, C. & Abba, E.H. (2022). Fish waste: Valorisation methods on a local scale. *InIOP Conference Series: Earth*

and Environmental Science , 1090(1)- 012015. 10.1088/1755-1315/1090/1/012015.
12. Gaddad, S.M. (2019). Enhanced production of extracellular alkaline protease by Bacillus cereus GVK21 by optimized formulations. *International Journal of Pharmacy and Biological Sciences, Transactions B: Applications,* 9 (1),1103-13.
13. Hadjidj, R., Badis, A., Mechri, S., Eddouaouda, K., Khelouia, L., Annane, R., El, Hattab. M. & Jaouadi, B. (2018). Purification, biochemical, and molecular characterization of novel protease from Bacillus licheniformis strain K7A. *International Journal of Biological Macromolecules*,114,1033-48. https://doi.org/10.1016/j.ijbiomac.2018.03.167.
14. Hammoumi, A., Faid, M. & Amarouch, H. (1998). Characterization of fermented fish waste used in feeding trials with broilers. *Process Biochemistry*, *33*(4), 423-427. https://doi.org/10.1016/S0032-9592(97)00092-7.
15. Harun, A.A.C., Mohammad, N.A.H., Ikhwanuddin, M., Jauhari, I., Sohaili, J. & Kasan, N.A. (2019). Effect of different aeration units, nitrogen types and inoculum on biofloc formation for improvement of Pacific Whiteleg shrimp production. *The Egyptian Journal of Aquatic Research*, *45* (3), 287-292. https://doi.org/10.1016/j.ejar.2019.07.001
16. Hashmi, S., Iqbal, S., Ahmed, I. & Janjua, H.A., (2022). Production, optimization, and partial purification of alkali-thermotolerant proteases from newly isolated *Bacillus subtilis S1* and *Bacillus amyloliquefaciens* KSM12. *Processes*, *10*(6),1050. https://doi.org/10.3390/pr10061050.
17. Islam, F. & Roy, N. (2018). Screening, purification and characterization of cellulase from cellulase producing bacteria in molasses. *BMC Research Notes*, 11(1), 445. https://doi.org/10.1186/s13104-018-3558-4.
18. Imran, S.Z. & Wei, L.S. (2019). Selected Essential Amino Acid Enhancement by Bacillus cereus from Solid State Fermentation of Soy Pulp Through Carbon Concentration and Fermentation Period. *Journal of Aquaculture Research & Development*, *10*(566), p.2. 10.4172/2155-9546.1000566.
19. Kieliszek, M., Pobiega, K., Piwowarek, K. & Kot, A. M. (2021). Characteristics of the Proteolytic Enzymes Produced by Lactic Acid Bacteria. *Molecules*, *26*(7). https://doi.org/10.3390/molecules26071858.
20. Kamizake, N.K., Gonçalves, M.M., Zaia, C.T. & Zaia, D.A. (2003). Determination of total proteins in cow milk powder samples: a comparative study between the Kjeldahl method and spectrophotometric methods. *Journal of Food composition and analysis*, *16*(4),507-516. https://doi.org/10.1016/S0889-1575(03)00004-8.
21. Lee, L. P., Karbul, H. M., Citartan, M., Gopinath, S. C. B., Lakshmipriya, T. & Tang, T. H. (2015). Lipase-Secreting Bacillus Species in an Oil-Contaminated Habitat: Promising Strains to Alleviate Oil Pollution. *BioMed Research International*, 820575. https://doi.org/10.1155/2015/820575.
22. Luang-In, V., Yotchaisarn, M., Saengha, W., Udomwong, P., Deeseenthum, S. M. & Maneewan, K. (2019). Isolation and Identification of Amylase-producing Bacteria from Soil in Nasinuan Community Forest, Maha Sarakham, Thailand. *Biomedical and Pharmacology Journal,* 12(3), 1061–1068. https://dx.doi.org/10.13005/bpj/1735.
23. Li, C., Ma, L., Wang, L., Zhang, Z., Chen, Y., Chen, J., Jiang, Q., Song, Z., He, X., Tan, B., Xiao, D. & Ma, X. (2023). Optimization of Solid-State Fermentation Conditions of *Quercus liaotungensis* by *Bacillus subtilis. Fermentation*, *9*(1), 75. https://doi.org/10.3390/fermentation9010075.
24. López-Trujillo, J., Mellado-Bosque, M., Ascacio-Valdés, J. A., Prado-Barragán, L. A., Hernández-Herrera, J. A. & Aguilera-Carbó, A. F. (2023). Temperature and pH Optimization for Protease Production Fermented by *Yarrowia lipolytica* from Agro-Industrial Waste. *Fermentation*, *9*(9), 819. https://doi.org/10.3390/fermentation9090819.
25. Manfredini, P.G., Cavanhi, V.A., Costa, J.A. & Colla, L. M. (2021). Bioactive peptides and proteases: characteristics, applications and the simultaneous production in solid-state fermentation. *Biocatalysis and Biotransformation,* 39 (5),360-77. https://doi.org/10.1080/ 10242422. 2020. 1849 151.
26. Masi, C., Gemechu, G. & Tafesse, M. (2021). Isolation, screening, characterization, and identification of alkaline protease-producing bacteria from leather industry effluent. *Annals of Microbiology*, 71(1), 1–11. https://doi.org/10.1186/s13213-021-01631-x.
27. Mathew, G. M., Huang, C. C., Sindhu, R., Binod, P., Sirohi, R., Awsathi, M. K., Pillai, S. & Pandey, A. (2021). Enzymatic approaches in the bioprocessing of shellfish wastes*. Biotech*, 11(8), 367.
28. Mayta-Apaza, A.C., García-Cano, I., Dabrowski, K. & Jiménez-Flores, R. (2021). Bacterial diversity analysis and evaluation proteins hydrolysis during the acid whey and fish waste fermentation. *Microorganisms,* 9(1),100. https://doi.org/10.3390/microorganisms9010100.
29. Mcbride, C. H. (2020). Determination of Secondary and Minor Plant Nutrients in Fertilizers by Atomic Absorption Spectrophotometry: Third Collaborative Study. *Journal of Association of Official Analytical Chemists*, 50(2), 401–407. https://doi.org/10.1093/jaoac/50.2.401.
30. Molinuevo-Salces, B., Ahring, B.K. &Uellendahl, H. (2015). Optimization of the co-digestion of catch crops with manure using a central composite design and reactor operation. *Applied biochemistry and biotechnology*, *175*,1710-1723. 10.1007/s12010-014-1391-3.
31. Nkhata, S. G., Ayua, E., Kamau, E. H. & Shingiro, J. B. (2018). Fermentation and germination improve nutritional value of cereals and legumes through activation of endogenous enzymes. *Food Science & Nutrition*, *6*(8), 2446–2458. https://doi.org/10.1002/fsn3.846.
32. Nielsen, P. M., Petersen, D. & Dambmann, C. (2001). Improved Method for Determining Food Protein Degree of Hydrolysis. *Food chemistry and toxicology* 66:5. https://doi.org/10.1111/j.1365-2621.2001.tb04614.x
33. Oreopoulou, V. & Russ, W. (2006). Utilization of Byproducts and Treatment of Waste in the Food Industry. Springer Science & Business Media.
34. Patsios, S. I., Dedousi, A., Sossidou, E. N. & Zdragas, A. (2020). Sustainable Animal Feed Protein through the Cultivation of *YARROWIA* Lipolytica on Agro-Industrial Wastes and byproducts. *Sustainability: Science Practice and Policy*, 12(4), 1398. https://doi.org/10.3390/su12041398.
35. Ravindran, B., Wong, J. W. C., Selvam, A., Thirunavukarasu, K. & Sekaran, G. (2016). Microbial biodegradation of proteinaceous tannery solid waste and production of a

novel value-added product - Metalloprotease. *Bioresource Technology*, 217, 150–156. 10.1016/j.biortech.2016.03.033.

36. Samuel, R.A., Dash, S.S., Ali, L. Riyas. & Paari, K.A. (2021). Formulation and characterization of plant, animal, and probiotic based fish meals and evaluating their efficacy on growth and performance in zebrafish (danio rerio). *Advances in Animal and Veterinary Sciences*, 9(8)
37. Silva, J.F.X., Ribeiro, K., Silva, J.F., Cahú, T.B. & Bezerra, R.S. (2014). Utilization of tilapia processing waste for the production of fish protein hydrolysate. *Animal feed science and technology*, *196*, 96-106. https://doi.org/10.1016/j.anifeedsci.2014.06.010
38. Simat, V. (2021). Valorization of seafood processing by-products. In Valorization of agri-food wastes and byproducts. *Agricultural and biological sciences*, 515-536.
39. Shyam, S.S. & Akhila, K. (2022). Domestic fish consumption in India: Challenges and opportunities, *Shrimp farming – made better for the Future* ,10-25.
40. Solanki, P., Putatunda, C., Kumar, A., Bhatia, R. & Walia, A. (2021). Microbial proteases: ubiquitous enzymes with innumerable uses. *3 Biotech*, 11(10), 428. https://doi.org/10.1007/s13205-021-02928-z.
41. Tropea, A., Potortì, A. G., Lo Turco, V., Russo, E., Vadalà, R., Rando, R. & Di Bella, G. (2021). Aquafeed Production from Fermented Fish Waste and Lemon Peel. *Fermentation,* 7(4), 272. https://doi.org/10.3390/fermentation7040272.
42. Tennalli, G.B., Garawadmath, S., Sequeira, L., Murudi, S., Patil, V., Divate, M.N.& Hungund, B.S. (2022). Media optimization for the production of alkaline protease by Bacillus cereus PW3A using response surface methodology. *Journal of Applied Biology and Biotechnology*, 1,10(4)-17-26.
43. Ullah, N., Rehman, M.U., Sarwar, A., Nadeem, M., Nelofer, R., Shakir, H.A., Irfan, M., Idrees, M., Naz, S., Nabi, G.& Shah, S. (2022). Purification, characterization, and application of alkaline protease enzyme from a locally isolated Bacillus cereus strain. *Fermentation*, *8*(11), 628. https://doi.org/10.3390/fermentation8110628.
44. Wang, Lan., Liu Yang. & Chen, Hong- Zhang. (2018). Advances in Porous Characteristics of the Solid Matrix in Solid-State Fermentation. *In Current Developments in Biotechnology and Bioengineering*, 19-29). https://doi.org/10.1016/B978-0-444-63990-5.00002-5.
45. Zhang, Y., Ishikawa, M., Koshio, S., Yokoyama, S., Dossou, S., Wang, W., Zhang, X., Shadrack, R.S., Mzengereza, K., Zhu, K. & Seo S. (2021). Optimization of Soybean Meal Fermentation for Aqua-Feed with Bacillus subtilis natto Using the Response Surface Methodology. *Fermentation*, 7:306. https://doi.org/10.3390/fermentation7040306.
46. Zhang, X., Shuai, Y., Tao, H., Li, C. & He, L. (2021). Novel Method for the Quantitative Analysis of Protease Activity: The Casein Plate Method and Its Applications. *American Chemical Society Omega*, *6*(5), 3675–3680. https://doi.org/10.1021/acsomega.0c05192.
47. Zhou, C., Lin, H., Huang, Z., Wang, J., Wang, Y. & Yu, W. (2020). Effects of dietary leucine levels on intestinal antioxidant status and immune response for juvenile golden pompano (*Trachinotus ovatus*) involved in Nrf2 and NF-κB signaling pathway. *Fish & Shellfish Immunology*, *107*, pp.336-345. https://doi.org/10.1016/j.fsi.2020.10.012.