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Multi-objective bayesian optimization of fermentation conditions for enhanced protease activity and microbial density in *Bacillus* spp. from traditional Vietnamese fish sauce

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Abstract The optimized fermentation for *Bacillus* strains (BM9 and HH19) isolated from traditional fish sauce mash in Nam-O village, Da Nang, Vietnam was proved to enhance protease activity and microbial community to improve food production. Fish sauce fermentation is accelerated the efficient microbial supplement to the prolonged production. Bayesian Optimization (BO in a skim milk-based medium was proved to be efficiently navigates complex biological parameter to determine the optimal conditions for nitrogen concentration, inoculum size, NaCl concentration, pH, and fermentation time. Skim milk is used to substitute peptone and yeast extract proved enhance density of bacteria and protease activity. *Bacillus* strain BM9 is achieved for protease activity of 464 U/L and a microbial density of 2.5×10^9 CFU/L, while *Bacillus* strain HH19 reached 417 U/L and 2.7×10^9 CFU/L. The potential of skim milk and Bayesian Optimization for efficient fermentation is highlighted for microbial supplement to enhance the quality of fish sauce which decreased the production time. This approach is minimized the resource use and supporting traditional food industries with sustainable agriculture and food security goals.

Keywords: *Bacillus* spp., Protease, Microbial biomass, Bayesian optimization, Fish sauce fermentation

Introduction

Fish sauce is one a traditional important fermented product of Vietnam, which originally produced from marine fish through a salt-based fermentation process. It is a common role condiment in daily meals that fish sauce is embodied the cultural and culinary identities of Vietnamese (Nguyen *et al.,* 2024).

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In Da Nang City, Nam O fish sauce craft village is located in Hai Van Ward the stands out as a representative traditional craft village to renow for uniquely rich and flavor fish sauce which is reflected the distinctive characteristics of the Quang Nam region. However, the market competition is increased which faces numerous challenges in the village for strongly competition from fish sauce industrially production which are required a shorter production time. The main limitation of the traditional method is concerned in the manual nature which prolonged fermentation period approximately 12 months, and the inconsistency in product quality and flavor that hinder economic efficiency and limit market expansion (Nguyen *et al*., 2024).

In light of these challenges, one promising approach to improving the production process is the application of microbial supplements capable of degrading fish proteins. Such bio-innovations can accelerate fermentation, shorten production time and enhance the overall quality of fish sauce (Kanjan *et al*., 2021; Lam *et al*., 2011; Zaman *et al.,* 2011). To manufacture such products, the study of fermentation conditions must be conducted thoroughly with a goal to ensure the quality of both microbial mass and protease activity during usage. It is reported that several studies have assured the positive effect upon microbial growth and protease activity of *Bacillus* spp. given the modified fermentation conditions. For instance, two assays carried out to select suitable bacterial fermentation conditions for probiotics production used in animal husbandry have shown significant improvement in biomass, of which is 26-fold the result cultivated in traditional LB medium, with both assays biomass reached over 9.64 log CFU/mL.

In 2024, a research group in Da Nang successfully performed screening of salt-tolerant bacteria from traditional Nam-O fish sauce with proteolytic ability (Nguyen *et al*., 2024). Building on this work, the present study aimed to optimize fermentation conditions influencing bacterial growth and protease activity of two selected strains using Bayesian Optimization (BO).

Bayesian Optimization (BO) was used to optimize fermentation conditions due to its efficiency in biological experiments, where trials are often time- and resource-intensive. BO builds a statistical model to predict how factors like nitrogen concentration or pH affect outcomes such as protease activity and microbial density. This model is improved with each experiment, guiding the selection of new conditions to balance exploring new possibilities with refining known high-performing settings. The traditional methods of full-factorial design or Response Surface Methodology (RSM) that BO is required for a fewer experiment to investigate the optimal conditions. This is made the idea for finding the appropriated microbial fermentation in a cost-effective and reliable

way to support a scalable and consistent fish sauce production (Gisperg *et al*., 2025).

BO is utilized to improve the fermentation process of salt-tolerant bacteria to develop more consistent, scalable, and efficient process for traditional food production. It is found to be advanced for sustainable agriculture with optimizing traditional fermentation and to support food security and biodiversity with indigenous strains. The traditional fermented fish sauce plays an important role in regional diets to optimize the production through biotechnological innovation to help a bridge the gap between heritage and innovation which enhance food quality, reduce processing time and waste, and support more resilient and sustainable local food systems. The objective was aimed to optimize fermentation using *Bacillus* strains (BM9 and HH19) isolated from traditional fish sauce mash in Nam-O village, Da Nang, Vietnam.

Materials and methods

Materials

The two strains of *Bacillus*, namely BM9 and HH19 were isolated from fish sauce produced in the Nam-O fish sauce craft village for their high protein-degrading activity and identified based on 16S rRNA gene sequencing (Nguyen *et al.,* 2024). These strains were then preserved in Luria Bertani agar (LBA*) containing 1% peptone, 0.5% yeast extract and 1% NaCl. The two strains were stored at 4^{o}C at the Danang Biotechnology Center.

Methods

Determination of antagonism between two bacterial strains by cross-strike method

Evaluation of potential antagonistic interactions between the two *Bacillus* strains is a crucial step to ensure their compatibility prior to co-formulation in microbial preparation. Antagonism between two bacterial strains was assessed by cross-impact method on agar plates containing meat peptone medium. Each strain was inoculated in a straight line on the agar surface, arranged perpendicular to each other. The plates were incubated at 37°C for 24 h. After incubation, growth at the intersection was observed. Overlapping or overlapping growth at the intersection indicated the presence or absence of antagonistic activity (Nguyen and Le, 2021).

Determination of bacterial density using colony counting method

Preparation of serial dilutions: 1 mL of the original solution was transferred into a test tube containing 9 mL of distilled water to obtain a 10^{-1} dilution. This process is continued to obtain serial dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} (Ben-David and Davidson, 2014).

Inoculation diluted samples: a micropipette was used as 0.1 mL of the suspension from each dilution, then transferred to Plate Count Agar (PCA). The inoculum was spread across the surface of the agar by a sterile spreader to ensure well-isolated colonies. (Ben-David and Davidson, 2014).

The inoculated Petri dishes were sealed and incubated in an incubator. After 24 to 48 hours of incubation, the number of colonies on each plate were counted. The colony counts for dilution levels recorded yields between 25 and 250 colonies per plate (JECFA, 2006).

$$\text{A (CFU/g or CFU/mL)} = \frac{N}{n1.V.f1+\cdots+n2.V.f2}$$

Where A: Number of viable microorganisms in 1 g or 1 mL of the sample.
N: Total number of colonies counted.
n_i: Number of plates at the i-th dilution level.
V: Volume of sample inoculated onto each plate (mL).
f_i: Dilution factor at the i-th level.

Determination of bacterial density using optical density (OD) measurement

A microbial suspension was serially diluted at levels of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. Each dilution was plated on PCA (Plate Count Agar) to determine viable cell counts.

The colony-forming unit (CFU/mL) of each suspension was quantified using the plate count method. Corresponding optical density at 600 nanometers wavelength (OD_{600} nm) values were measured for each dilution. A calibration curve was constructed by plotting cell density (CFU/mL) on the y-axis against OD_{600} nm on the x-axis to establish a linear correlation between bacterial density and turbidity (Nguyen *et al.,* 2024).

To estimate cell density based on turbidity, the OD_{600} nm value of a bacterial suspension was measured. Using the established standard curve, the corresponding CFU/mL value was then calculated from the OD_{600} nm reading.

Determination of protease activity using modified Anson method

Protease activity was determined following a modified Anson method and was calculated using the following equation (Cupp-Enyard and Aldrich, 2008).

$$\text{Protease activity } \left(\frac{\text{U}}{\text{mL}}\right) = \frac{\mu\text{mol Tyrosine} \times 11}{15 \times 1 \times 1}$$

Where

11: Total volume of the enzyme reaction mixture (mL) = 1 mL enzyme + 5 mL casein solution + 5 mL TCA

15: Reaction time (minutes)

1: Volume of enzyme used in the reaction (mL)

1: Volume used for absorbance measurement after reaction with Folin reagent (mL)

Preparation of culture

Nitrogen-substituted medium: The initial nitrogen source in Luria Bertani was replaced by skim milk at a concentration of 1.5% (w/v). Skim milk was chosen as high protein content and cost-effectiveness which boost the protease production in *Bacillus* spp. (Abbas *et al.,* 2020). The media were sterilized using autoclave at 121°C for 20 minutes before inoculation.

Optimal multi-objective fermentation condition design using Bayesian Optimization (BO)

Multi-objective for Bayesian Optimization approach with PyCharm Community Edition 2024.2.4 software was designed. Data were collected with input variables as Inoculum (%), Nitrogen (%), NaCl (%), pH, and Time (h). The output responses were enzyme activity and microbial density. The experiment was assessed for variability, and pair exhibited over 15% deviation in target values which was filtered by removing the replicates with the higher index to ensure data consistency. A multi-objective optimization problem was formulated using the PyMOO v0.6.1 library, incorporating defined input bounds and objective functions. To enhance the performance of the Gaussian Process (GP) models, enzyme activity was linearly scaled to the [0, 1] range, microbial density log-transformed then scaled to [0, 1]. Two separate GP regression models were trained for each response variable using a Matern kernel ($\nu = 2.5$) combined with a WhiteKernel to account for noise. An exploration strategy was implemented by weighing the predictive standard deviation of the GP models, with an exploration

weight of 1.0 applied during the first 50 generations and reduced to 0.5 thereafter to balance exploration and exploitation during optimization (Cosenza *et al.,* 2022; Narayanan *et al*., 2025; Siedentop *et al*., 2023).

The optimization process automatically stops when the hypervolume over the last 20 generations < 0.02 and the current hypervolume value $>10^{-6}$. This ensures that the algorithm terminates only when further improvements are negligible, indicating convergence.

Table 1. Summary of the parameters and their corresponding boundaries of the factors used in Bayesian optimization

Factor	Unit	Range
Nitrogen concentration	%	0.5 – 2
Inoculation rate	%	1 – 7
NaCl concentration	%	1 – 5
pH	–	5 – 8
Time	hours	16 – 22

Data processing using software

All experiments were performed in triplicate, and the mean values were calculated. Statistical analysis was conducted using Microsoft Excel, PyCharm Community Edition 2024.2.4, Design Expert 11 and IBM SPSS Statistics 27.

Results

Determination of antagonism between two bacterial strains by cross-strike method

The results showed that all three experimental plates did not show any inhibition zones at the intersection of the two bacterial strains. The bacterial colonies grew continuously, without being dispersed or interrupted when encountering the other strain. This proved that the two strains BM9 and HH19 did not have antagonism under the current culture conditions, and they could be applied simultaneously in the same fermentation system or in mixed microbial preparations (Figure 1).

Figure 1. Antagonism test results of two strains BM9 and HH19

Design of optimal multi-objective fermentation conditions using Bayesian Optimization (BO)

Figure 2. Glass fermentation bottles of BM9 and HH19 strains

Strain BM9

Significant variation in microbial densities across the experimental conditions highlighted the multifactorial effects of environmental parameters on *Bacillus* growth (Figure 2). Treatments showed high inoculum levels (4%–7%) and mildly alkaline pH values (7.8–8.0), such as condition 14 (Table 2) and condition 18 (Table 2). It showed the microbial densities of 1.5×10^9 CFU/mL which found that mild alkaline in combination with an appropriate inoculum ratio was appropriated for a favorable condition of microbial proliferation. Morevover, low to moderate concentrations (1% – 4.5%) of NaCl found to be supported the growth of microbial and maintain stable cell densities. However, as 5% in treatment 10 was higher NaCl concentrations was declined in microbial density to $7.7–8.2 \times 10^8$ CFU/mL. It indicated that the elevated salt concentrations forced osmotic stress in the cells (Table 2).

The protease activity showed a wide range from low activity (20 – 50 U/L) to high levels (300–464 U/L). The conditions were characterized by alkaline pH

(7.8 – 8.0), low to moderate nitrogen concentration (0.5%–1.5%), and high inoculum percentages that higher yield of protease activity.

Treatment 18 was the highest protease activity at 464 U/L. The fermentation time was prolonged to enhance enzyme activity within 22-hour incubation period (Table 2). Conversely, acidic pH conditions and elevated salt concentrations suppressed enzyme activity due to biological stress which is induced by acidic environments and osmotic pressure with adversely affected in extracellular protease activity (Table 2 and Figure 3).

Table 2. Summary of the results from three rounds of Bayesian optimization on microbial density and protease activity of the BM9 strain

Round	Treatment	Nitrogen (%)	Inoculum (%)	NaCl (%)	pH	Time (h)	Microbial density (CFU/mL)	Protease activity (U/L)
1	1	0.85	3.21	2.45	6.2	18.5	$8.2^{hi} \times 10^8$	$256^{c} \pm 19$
	2	1.73	5.89	4.12	7.1	20	$2^{l} \times 10^8$	$100^{g} \pm 12$
	3	1.12	1.45	1.67	5.8	16.5	$1.1^{f} \times 10^9$	$225^{d} \pm 8$
	4	0.62	6.75	3.89	6.7	21	$3.5^{l} \times 10^8$	$162^{ef} \pm 14$
	5	1.95	2.33	1.05	5.1	17.5	$1^{fg} \times 10^9$	$135^{f} \pm 54$
	6	1.28	4.56	2.78	7.5	20.5	$1.9^{d} \times 10^9$	$235^{cd} \pm 9$
	7	0.53	1.98	4.95	5.5	17.0	$5.4^{k} \times 10^8$	$19^{l} \pm 5$
	8	1.41	7.00	3.21	8.0	22.0	$1.4^{e} \times 10^9$	$220^{d} \pm 25$
	9	1.67	3.75	1.33	6.0	18.0	$2.3^{l} \times 10^8$	$173^{e} \pm 1$
	10	0.75	2.11	5.00	7.8	19.5	$7.2^{ik} \times 10^8$	$48^{kl} \pm 13$
	11	1.50	4.00	2.00	6.5	20.0	$9.5^{gh} \times 10^8$	$308^{b} \pm 6$
	12	0.95	5.25	4.50	5.3	17.0	$2.8^{b} \times 10^9$	$29^{lm} \pm 5$
2	13	1.90	1.45	3.63	8.0	22.0	$8.9^{h} \times 10^8$	$57^{hi} \pm 3$
	14	1.95	6.54	5.00	5.7	16.5	$3.3^{a} \times 10^9$	$74^{h} \pm 3$
	15	1.95	4.48	4.48	6.9	16.5	$2^{l} \times 10^8$	$119^{g} \pm 11$
	16	1.94	1.60	4.49	8.0	18.0	$5.8^{k} \times 10^8$	$30^{lm} \pm 1$
	Repeated 11	1.50	4.00	2.00	6.5	20.0	$9.4^{gh} \times 10^8$	$350^{gh} \pm 17$
3	17	0.50	7.00	1.00	8.0	16.8	$1.5^{e} \times 10^9$	$322^{b} \pm 17$
	18	0.84	4.12	1.00	8.0	22.0	$2.5^{c} \times 10^9$	$464^{a} \pm 20$
	19	0.50	7.00	2.20	5.0	16.0	$1.5^{e} \times 10^9$	$227^{d} \pm 6$
	Repeated 10	0.75	2.11	5.00	7.8	19.5	$7.8^{ik} \times 10^8$	$46^{ik} \pm 3$
P-value							<0.001	<0.001

* Data were shown as Means ± SD of triplicate

* Values with different letters in the same column are significantly different at $p < 0.05$ according to Dunican's test

The optimization process of the five factors for the BM9 strain was terminated for the following reasons (Table 2).

Hypervolume convergence showed the change in hypervolume which was 0.846% (<1%), and the hypervolume value reached 0.2813 ($>10^{-6}$). This indicated that the Pareto front had stabilized, and subsequent generations were unlikely to yield significant improvements.

Significant improvement in objective functions showed that the experiments proposed in the third optimization round (Treatment 18 in Table 2) achieved an enzyme activity of 464 U/L, representing a substantial improvement compared to 350 U/L in experiment 11 (an increase of 32.57%). Although the bacterial density in treatment 18 was 2.508 × 10^9 CFU/mL, did not surpass that of treatment 12 was 2.888 × 10^9 CFU/mL (Table 2). The notable increase in enzyme activity indicated that the optimization process successfully identified a region within the Pareto front synthesis.

Experimental variability showed the variation observed in the replicated treatments (treatments 10 and 11, Table 2) were 13.64% and 4.17% for enzyme activity, and 0.779% and 9.11% for bacterial density, respectively. This suggested the presence of experimental interference. However, these variations remained within the acceptable threshold (<15%) synthesis (Table 2 and Figure 3).

According to the Pareto front plot (Figure 3), treatment 18 (Table 2) lay on the Pareto-optimal front and was considered suitable for the objective functions.

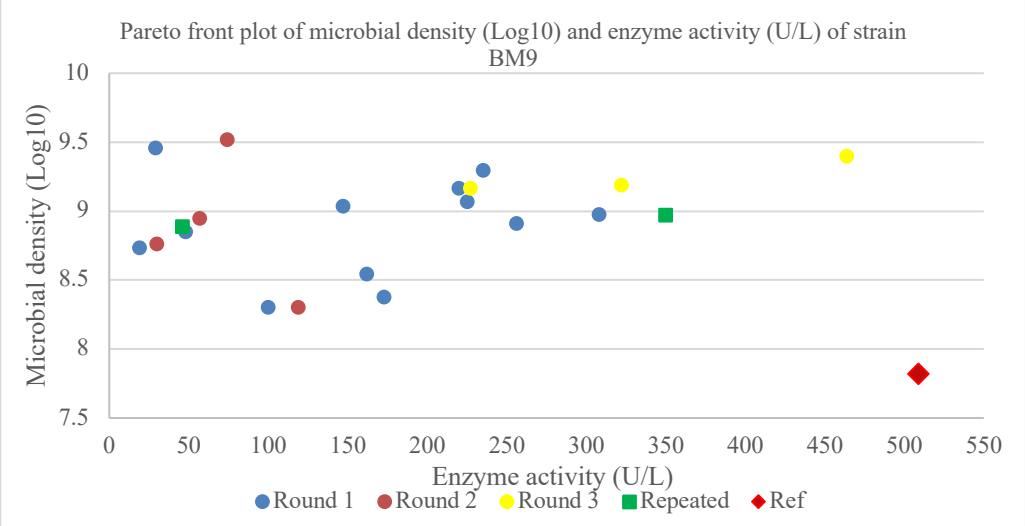

Figure 3. Pareto front plot of microbial density (Log10) and enzyme activity (U/L) of strain BM9

Strain HH19

The relationship between microbial density (CFU/mL) and protease enzyme activity (U/L) in the *Bacillus* strain HH19 did not follow a simple linear trend. Treatments 5 and 20 showed very high (2.7×10^9 CFU/mL) microbial densities which the protease activities were not follow up. Moreover, treatment

8, showed a moderate microbial density (6.0 $\times 10^8$ CFU/mL) which expressed a high enzyme activity (398 U/L) (Table 3 and Figure 4).

Nitrogen concentration plays a crucial role in regulating the protease biosynthesis. Result found that the optimal nitrogen was ranged between 1.4% and 1.7%. Treatments 8 (1.41%), 9 (1.67%), and 20 (1.46%) which expressed protease activities of 280 U/L. Low (0.5–0.75%) nitrogen levels revealed that bacteria lacked sufficient resources for enzyme activity. The excessive nitrogen concentrations of ≥ 1.9% showed in treatments 2 and 5 were decreased activities which indicated secondary metabolic repression.

Critical factor of inoculum size influences microbial density and enzyme secretion. The finding showed that the optimal inoculum ranged between 3.5% to 5.0% which found to be favorable for other culture conditions. Treatments 11 was 4% and treatment 9 was 3.75% which gave high enzyme activities of ~270 to 290 U/L (Table 3). Treatment 8 was 7% which a peak activity of 398 U/L. It was interacted of inoculum size, pH, incubation time, and NaCl concentrations. low inoculum was prolonged the lag phase, while an excessively high inoculum resulted to be competition of nutrients or accumulated toxic by-products. The under optimum conditions showed high activity of enzyme.

The finding revealed that the optimum NaCl ranged from 2.0% to 3.5%, which showed high enzyme activity without osmotic stress. It revealed that treatment 8 was 3.21%, treatment 11 was 2.0% and treatment 9 waa1.33% (Table 3). Moreover, treatments with NaCl over 4% was significantly decreased activity of ~34–37 U/L which known as a physiological characteristic of *Bacillus* spp.

Activity of protease is normally highly sensitive to pH conditions. Enzyme activities were highest at pH ranged from 6.0 to 8.0 which pH 8.0 in treatment 8, pH 6.0 in treatment 9, and pH 6.5 in treatment 11. The mild alkaline to neutral showed that that pH was strongly affected. The optimal range of pH was denatured enzyme and biosynthesis repressed. For example, at lower pH values (5.1–5.5) in treatments 5 and enzyme activities dropped in treatment 13 (≤ 50 U/L).

Incubation hour is a decisive factor in allowing microorganisms to reach the stationary phase, during which extracellular enzymes are typically produced in large quantities. Treatments with incubation times between 18 and 22 hours such as treatment 8 (22 hours), treatment 9 (18 hours), treatment 18 (20.8 hours), and treatment 20 (18.7 hours) showed the highest enzyme activities. If incubation is too short (≤16 hours), the system may not reach the phase necessary for maximal enzyme secretion; on the other hand, overly long durations (>24 hours) may result in enzyme degradation due to reabsorption or hydrolysis by endogenous proteases. Therefore, a range of 18–22 hours is considered optimal to balance biomass accumulation with enzyme production (Table 3 and Figure 4).

Table 3. Summary of the results from three rounds of Bayesian optimization on microbial density and protease activity of the HH19 strain

Round	Treatment	Nitrogen (%)	Inoculum (%)	NaCl (%)	pH	Time (h)	Microbial density (CFU/mL)	Protease activity (U/L)
	1	0.85	3.21	2.45	6.2	18.5	$1.5^{kl} \times 10^8$	$71^{klmn} \pm 14$
	2	1.73	5.89	4.12	7.1	20.0	$3.3^{m} \times 10^7$	$35^{p} \pm 1$
	3	1.12	1.45	1.67	5.8	16.5	$3.6^{h} \times 10^8$	$54^{nop} \pm 6$
	4	0.62	6.75	3.89	6.7	21.0	$2.8^{hi} \times 10^8$	$57^{mno} \pm 2$
	5	1.95	2.33	1.05	5.1	17.5	$3.4^{a} \times 10^9$	$48^{op} \pm 3$
	6	1.28	4.56	2.78	7.5	20.5	$3.2^{hi} \times 10^8$	$75^{klm} \pm 8$
1	7	0.53	1.98	4.95	5.5	17.0	$3.2^{hi} \times 10^8$	$78^{kl} \pm 11$
	8	1.41	7.00	3.21	8.0	22.0	$6.1^{g} \times 10^8$	$398^{b} \pm 4$
	9	1.67	3.75	1.33	6.0	18.0	$5.5^{g} \times 10^8$	$289^{cd} \pm 31$
	10	0.75	2.11	5.00	7.8	19.5	$2.3^{ik} \times 10^8$	$64^{klmno} \pm 11$
	11	1.50	4.00	2.00	6.5	20.0	$1.1^{lm} \times 10^8$	$273^{d} \pm 15$
	12	0.95	5.25	4.5	5.3	17.0	$1.3^{c} \times 10^9$	$122^{h} \pm 5$
	13	0.53	1.45	4.50	7.8	16.5	$2.6^{hi} \times 10^8$	$34^{p} \pm 8$
	14	0.53	1.45	5.00	5.1	16.6	$3.3^{hi} \times 10^8$	$74^{klm} \pm 1$
2	15	0.53	2.27	4.50	8.0	21.4	$3.5^{h} \times 10^8$	$82^{k} \pm 1$
	16	0.53	1.46	4.50	6.0	16.5	$3.4^{hi} \times 10^8$	$61^{lmno} \pm 3$
	Repeated 11	1.50	4.00	2.00	6.5	20.0	$1.1^{lm} \times 10^8$	$280^{d} \pm 6$
	17	0.50	7.00	1.05	8.0	17.7	$8.1^{ef} \times 10^8$	$200^{e} \pm 21$
3	18	0.90	5.15	1.05	7.2	20.8	$1.3^{c} \times 10^9$	$303^{c} \pm 19$
	19	0.53	7.00	2.25	5.1	16.5	$8.4^{e} \times 10^8$	$157^{g} \pm 13$
	20	1.46	4.04	3.00	7.4	19.5	$2.8^{b} \times 10^9$	$417^{a} \pm 8$
	Repeated 2	1.73	5.89	4.12	7.1	20.0	$3.8^{m} \times 10^7$	$34^{p} \pm 1$
	21	1.59	4.76	5.00	7.3	19.3	$1.0^{d} \times 10^9$	$176^{f} \pm 15$
	22	0.98	1.79	1.95	7.0	16.5	$5.9^{g} \times 10^8$	$102^{i} \pm 1$
4	23	1.10	2.35	1.79	7.2	16.5	$7.2^{f} \times 10^8$	$101^{i} \pm 1$
	Repeated 4	0.62	6.75	3.89	6.7	21.0	$2.9^{hi} \times 10^8$	$52^{mno} \pm 1$
	Repeated 8	1.41	7.00	3.21	8.0	22	$6.1^{g} \times 10^8$	$386^{b} \pm 22$
	24	1.70	5.80	4.80	6.6	21.7	$3.8^{m} \times 10^7$	$35^{p} \pm 5$
	25	1.60	5.70	4.40	6.6	21.8	$3.9^{m} \times 10^7$	$34^{p} \pm 1$
5	26	1.70	5.90	5.00	6.6	21.7	$3.9^{m} \times 10^7$	$32^{p} \pm 8$
	27	1.60	5.70	4.30	6.6	22.0	$3.6^{m} \times 10^7$	$37^{p} \pm 1$
	Repeated 17	0.50	7.00	1.05	8.0	17.7	$8.3^{ef} \times 10^8$	$221^{e} \pm 1$
P-value							<0.001	<0.001

* Data were shown as Means ± SD of triplicate

* Values with different letters in the same column are significantly different at $p < 0.05$ according to Dunican's test

The optimization process for the five factors affecting strain HH19 was concluded. Hypervolume (HV) convergence: The HV indicator stabilized at a value of 1.3145 ($>10^{-6}$) with minimal variation (0.09% < 0.2%), indicating that the algorithm converged appropriately when further improvements became negligible. This is suggested that a stable Pareto front was reached. Significant improvement of the objective function: The experiment proposed in the fourth

iteration (treatment 20, Table 3) achieved a maximum enzyme activity of 417 U/L, representing a 4.56% increased as compared to 398 U/L in treatment 8. Although bacterial density in treatment 20 (2.776×10^9 CFU/mL) (Table 3) did not exceed that of treatment 5 (3.436×10^9 CFU/mL) (Table 3), the enhancement in enzyme activity confirmed that the optimization process successfully identified a promising region of the Pareto front. Experimental variability showed the variation among replicate treatments (treatments 11, 8, 4, and 20) were 3.02% for enzyme activity and 2.54% for bacterial density which indicated the presence of experimental noise. These variations remained in an acceptable range.

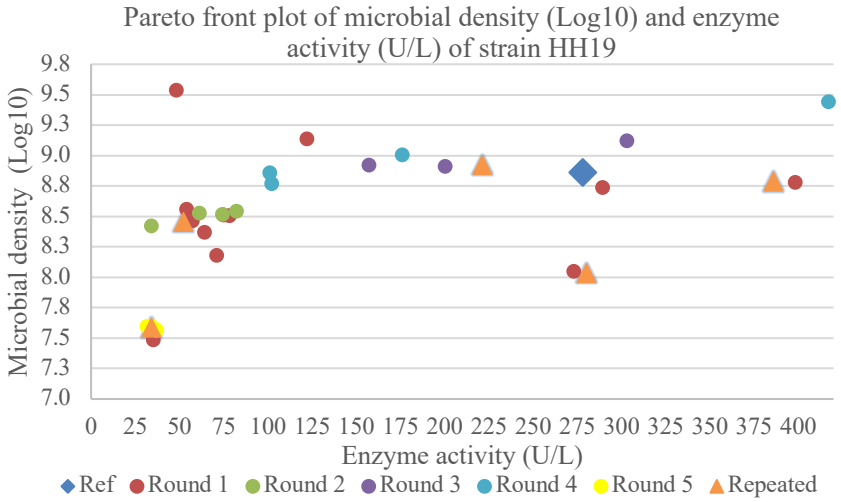

Figure 4. Pareto front plot of microbial density (Log10) and enzyme activity (U/L) of strain HH19

The optimization was hypothesized using RSM which constructed with various levels of factor for individual and interactive effects with 43 runs was shown in Table 4. The five investigated factors showed distributed to comprehensive coverage of the experiment. Nitrogen concentration (%) was evaluated for three levels of 0.50, 1.25 and 2.00 resulted 1.25% was the most frequently served as the central point. Inoculum size (%) was evaluated for three levels of 1, 4 and 7 which resulted of 4% was the most activity. NaCl concentration at levels of 1, 3 and 5 were distributed with 3%. The pH values were tested at 5.0, 6.5, and 8.0 which resulted pH at 6.5 showed the central level. The incubation time was ranged as 16 hours, 19 hours and 22 hours which resulted 19 hours the was most frequently as the central condition.

Table 4. Total treatments designed using RSM-BBD on microbial density and protease activity of BM9 and HH19, with 43 treatments in each strain

Treatment	Nitrogen (%)	Inoculum (%)	NaCl (%)	pH	Time (h)
1	1.25	7.00	3.00	6.5	16
2	1.25	7.00	5.00	6.5	19
3	1.25	7.00	3.00	8.0	19
4	1.25	4.00	3.00	8.0	16
5	1.25	7.00	3.00	6.5	22
6	1.25	4.00	3.00	5.0	16
7	0.50	4.00	3.00	6.5	22
8	1.25	7.00	1.00	6.5	19
9	1.25	1.00	3.00	6.5	16
10	0.50	4.00	1.00	6.5	19
11	1.25	4.00	1.00	5.0	19
12	0.50	4.00	5.00	6.5	19
13	1.25	1.00	5.00	6.5	19
14	2.00	4.00	3.00	8.0	19
15	2.00	4.00	3.00	5.0	19
16	1.25	1.00	1.00	6.5	19
17	0.50	7.00	3.00	6.5	19
18	1.25	4.00	1.00	6.5	16
19	1.25	4.00	3.00	6.5	19
20	1.25	4.00	3.00	8.0	22
21	0.50	4.00	3.00	8.0	19
22	1.25	4.00	5.00	6.5	22
23	1.25	4.00	1.00	8.0	19
24	2.00	4.00	5.00	6.5	19
25	1.25	4.00	3.00	5.0	22
26	2.00	4.00	3.00	6.5	16
27	2.00	1.00	3.00	6.5	19
28	0.50	4.00	3.00	5.0	19
29	0.50	4.00	3.00	6.5	16
30	1.25	7.00	3.00	5.0	19
31	0.50	1.00	3.00	6.5	19
32	1.25	4.00	5.00	5.0	19
33	2.00	4.00	1.00	6.5	19
34	1.25	4.00	3.00	6.5	19
35	2.00	7.00	3.00	6.5	19
36	1.25	1.00	3.00	6.5	22
37	1.25	4.00	5.00	8.0	19
38	1.25	4.00	5.00	6.5	16
39	1.25	1.00	3.00	8.0	19
40	1.25	1.00	3.00	5.0	19
41	1.25	4.00	3.00	6.5	19
42	2.00	4.00	3.00	6.5	22
43	1.25	4.00	1.00	6.5	22

Discussion

It is reported that *B. subtilis* showed highly selective ability for kin discrimination which characterized by the inhibition of most non-kin strains win the same lineage to compete for resources (Lyons and Kolter, 2017). This mechanism promotes cooperation of intraspecies and found to limit the intrusion of competing strains (Lyons and Kolter, 2017).

The strains BM9 and HH19 were found to be closely relationship which isolated from Nam-O fish sauce. These strains are not exhibited any antagonistic action to each other that reflected as a symbiosis to maintain the microbial population efficiency. A low inoculum level may result in insufficient bacterial biomass, thereby extending the fermentation time and reducing product yield. In contrast, an excessively high inoculum can lead to oxygen depletion, negatively impacting target product synthesis (Zhang *et al.,* 2023). An inoculum size of 4% was found to be optimal for both BM9 and HH19 strains. A study on *B. pumilus* NJM4 reported similar results (Yao *et al*., 2011) and 5% inoculum yielded the highest protease activity for *B. licheniformis (*Zhang *et al.,* 2023).

The optimal NaCl concentrations for the two strains BM9 and HH19 are 1% and 3%, respectively. The results of this study agreed with the study on thermophilic *Geobacillus thermoglucosidasius* SKF4 isolated from Hot Spring Park, Malaysia, a NaCl concentration of 1% supported optimal growth and protease production (182000 U/L). When the NaCl concentration increased to 4–5%, both biomass and protease activity decreased significantly (Suleiman *et al*., 2020). The optimal growth of *B. subtilis* T9-05 was observed at a salt concentration of 0 – 1%, higher salt concentrations negatively impacted its growth, which indicated a decrease in the number of colonies to a salt concentration of 12% (Sanjaya *et al*., 2023). NaCl concentration influences osmotic pressure and cell surface structure, thereby indirectly affecting enzyme secretion. The investigation is aligned with the physiological characteristics of *Bacillus* spp., which is tolerated only the moderate salinity levels and susceptible to osmotic stress when is exceeded and inhibited the secretion of extracellular enzyme (Hoper *et al*., 2006). The findings found that high salinity limited accumulation of biomass which should consider when designing for culture conditions to optimize enzyme activity and microbial density.

As result, pH 8 and 7.4 for strains BM9 and HH19 showed the highest growth and protease production. Similarly, Suleiman *et al*. (2020) stated that *G. thermoglucosidasius* SKF4 reached the growth and protease production peak at pH 7 and 8. *Bacillus subtilis* T9-05 isolated from fish sauce was achieved at pH 7-8 showed the highest growth (Sanjaya *et al*., 2023). The optimal pH for the production of extracellular protease using *Bacillus* spp. was 8.0 (Akhavan

Sepahy and Jabalameli, 2011). It affected the mild alkaline to neutral of the enzyme and strongly influenced by pH condition.

The research finding found time duration of 16 to 22 hours affected protease activity and biomass. Strain BM9, HH19 were the highest reaction at 22 and 18.5 hours respectively. A peak protease activity was found at 18 hours, which corresponded to late stationary phase (Kebabcı and Cihangir, 2011).

Kumara *et al*. (2012) stated that time is required a critical factor that influenced the microorganism transition to the stationary phase during production of extracellular enzyme. If cultivation period is too short (≤16 hours), the microbial biomass may not reach the stationary phase and not sufficient to stimulate enzyme secretion. However, incubation time is extended to >24 hours that leaded to degradation of enzyme which affected reabsorption or hydrolysis by endogenous proteases. Finally, an appropriated duration is approximately 18–22 hours to optimize enzyme production and biomass growth.

Strain BM9 showed optimization rounds from 21 experimental runs which included two repeated treatments. The hypervolume was changed to -0,846% <1% showing stabilization. The treatments were significantly expressed enzyme production from 350 U/L to 464 U/L. The bacterial density was not surpassing the initial level of 2,888 × 10^9 CFU/mL and trade-off was considered as variation across runs which remained within reasonable limits (<15%).

The other optimization rounds were significantly improving the yield under the stabilized hypervolume and the near-optimal performance.

All treatments were run to get 18 yielded highest enzyme production at 464 U/L with a bacterial density of 2.508 × 10^9 CFU/mL which was selected as optimal condition to enhance the enzyme production which maintained a high bacterial density. Bayesian Optimization process for strain BM9 was found with experimental run 18 as the optimum treatment.

Strain HH19 was done five optimization rounds of 32 experimental runs, with four repeated treatments. The hypervolume change was 0.09% <1%) for convergence confirmation. The treatments proved enzyme production from 398 U/L to 417 U/L. The bacterial density was not exceeded to initial level of 3.436 × 10^9 CFU/mL which the trade-off was acceptable, and variability remained within acceptable limits. The hypervolume is stabilized to function which reached near-optimal values, and other rounds resulted for improvement. Experimental run 20 yielded the highest enzyme activity was at 417 U/L, and a bacterial density was 2.776 × 10^9 CFU/mL to improve enzyme production and maintained a high microbial density. Bayesian Optimization process for strain HH19 resulted with experimental run 20 selected as the optimum treatment.

Response Surface Methodology (RSM) has traditionally been employed for optimizing fermentation conditions. Traditional RSM focuses on accurately

modelling response variation across the entire experimental domain. In contrast, Bayesian optimization emphasizes modelling the objective function more precisely in regions near the optimum. Toward the latter stages of the experimental sequence, Bayesian optimization progressively concentrated its sampling around these optimal regions, selecting experiments that refined the search for the best conditions (Rummukainen *et al*., 2024). RSM becomes increasingly impractical as the number of variables rises, since the required experiments grow exponentially. Moreover, with limited data, the fitted model may lack accuracy. In contrast, Bayesian optimization is better suited to capture complex variable interactions under experimental conditions, overcoming the dimensional constraints of conventional RSM (Tachibana *et al.,* 2023). In this study, RSM required 43 fixed treatments, whereas Bayesian Optimization delivered comparable or superior performance in significantly fewer runs—requiring just 21 treatments across 3 rounds for strain BM9, and 32 treatments across 5 rounds for strain HH19. These findings demonstrate that multi-objective Bayesian optimization provides a more efficient and effective approach than RSM for enhancing protease activity and microbial density in *Bacillus spp*. isolated from traditional Vietnamese fish sauce, making it well-suited for complex fermentation research.

Based on the findings of this study, several suggestions can be made to increase improvement. Although strains BM9 and HH19 demonstrated high protease production, high salt concentrations restricted their enzyme activity, highlighting the need to optimize salt levels or introduce these strains during controlled, lower-salinity fermentation phases. Future research findings should enhance the accuracy and industrial relevance of the optimization model which include temperature and dissolved oxygen for more understanding of environmental interactions and industrial conditions.

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Conflicts of interest

The authors declare no conflict of interest.

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