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PRODUCTION AND CHARACTERISATION OF L-ASPARAGINASE FROM *Priestia megaterium* GAFA

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ABSTRACT

L-asparaginase is an enzyme used to treat acute lymphoblastic leukaemia due to its ability to break down external L-asparagine necessary for the growth of cancer cells. This work investigated the optimisation of L-asparaginase production and the effect of various factors on the activities of the produced enzyme. An L-asparaginase-producing bacterium collected from the Department of Microbiology and Biotechnology Laboratory was identified using 16S rRNA. The production of L-asparaginase was optimised using Response Surface Methodology (RSM), and the experimental design was validated. The effect of environmental factors on L-asparaginase was determined. The L-asparagine-producing bacterium was identified as *Priestia megaterium* GAFA with an accession number PP390497. Optimal production (10594.1 U/mL) was validated using glucose as a carbon source, L-asparaginase only as a nitrogen source, fermented at pH 7.76 for 73 hours with an inoculum load of 7.7%. The Ca^{2+} ion significantly increased L-asparaginase activity by 76% compared with the control at $p<0.05$. The enzyme was active over a wide pH range (4–8), with maximum activity at pH 6.0. The highest activity was observed at 60 °C after 1 hour of incubation. The production of L-asparaginase by *Priestia megaterium* GAFA was optimised, and environmental factors influenced its activity.

Keywords: Environmental Factors, Fermentation, Microbial Enzymes, Optimisation, Response Surface Methodology

INTRODUCTION

L-asparagine, a non-essential amino acid, can be synthesised via the central metabolic pathway from glutamine and aspartic acid or obtained from dietary intake (Trimpont et al., 2022). It is an amino acid that both normal and cancerous cells need for growth. Some tumour cells cannot produce L-asparagine themselves and die without it, relying instead on free L-asparagine from the body (Yuan et al., 2024). Tumour cells require an external supply of plasma-derived L-asparagine because their capacity to produce it is limited. Notably, lymphoblasts need a substantial amount of L-asparagine to support their growth, yet they have low levels of asparagine synthetase (Schmidt et al., 2021). Several researchers have highlighted the importance of L-asparaginase in medicine (Biswas et al., 2025; Yoon et al., 2025; Ikeda et al., 2025). Normal cells produce L-asparagine, making them less vulnerable to L-asparaginase. The enzyme is widely used in the treatment of acute lymphoblastic leukaemia (ALL). It works by hydrolysing L-asparagine to ammonia and L-aspartic acid, making L-asparagine unavailable for some cancer cells. The depletion of free L-Asparagine sources results in the starvation and death of some cancer cells (Kenari et al., 2011).

Microorganisms, plants, and animals all produce L-asparaginase. Microorganisms are preferred as a source of enzymes because their production can be easily controlled, takes less time, and their enzymes are easily isolated (Cunha et al., 2021; Fasiku et al., 2026). Bacteria such as E. coli, Bacillus subtilis, Pseudomonas species (Darnal et al., 2023) and fungi like Aspergillus cereus, Aspergillus oryzae (Cunha et al., 2021), and Fusarium equiseti (El-Gendy et al., 2021), Fusarium proliferatum (Yap et al., 2021) have been utilised for L-asparaginase production.

Optimisation of microbial enzyme production is vital to maximise yields. There are two approaches: one factor at a time (OFAT) and response surface methodology (RSM). The conventional OFAT method is time-consuming and does not consider the interactions between variables; it seeks the best condition based solely on the highest result for one factor, ignoring others. The RSM provides more reliable optimisation considering interactions between factors and often requires fewer experiments due to its experimental design. It can generate extensive data rapidly (Yap et al., 2021). In this study, RSM was employed to optimise the production of L-asparaginase. This work focused on optimising L-asparaginase from *Priestia megaterium* using RSM and characterising the enzyme produced.

MATERIALS AND METHODS

Collection of Organism

An L-asparaginase-producing organism was collected from the Department of Microbiology and Biotechnology, Ajayi Crowther University, Oyo Town, Nigeria, cultured on nutrient agar and preserved at 4°C for further use. The organism was molecularly identified using 16S rRNA, and its sequence was submitted to the National Centre for Biotechnology Information (NCBI) Genebank, where an accession number was assigned. The phylogenetic tree of the organism was constructed using the maximum likelihood method on MEGA X (Tamura and Nei, 1993; Kumar et al., 2018).

Production Medium

The basal production medium contains the following in g/L: L-asparagine 10, glucose 2, $MgSO_4.7H_2O$ 0.52, KCl 0.52, $Cu(NO_3)_2.7H_2O$ 0.03, KH_2PO_4 1.52, $ZnSO_4$ 0.05, and $FeSO_4$ 0.03. Glucose was the carbon source, which was substituted with other carbon sources depending on the experimental design. All experiments contain L-asparagine as nitrogen with or without another source of nitrogen (10 g/L), such as malt extract, peptone, yeast extract, and tryptone.

Optimisation of L-asparaginase production using response surface methodology

Experimental Design

The experimental design employed was a D-optimal design, the study type was response surface methodology (RSM), and the algorithm was point exchange in Design Expert software (Stat-Ease Inc.) version 11. The design model was a quadratic, and the total number of experimental runs was 65, comprising 5 groups. The independent factors included pH (A), fermentation period (B), inoculum load (C), carbon source (D), and nitrogen source (E), as shown in Table 1. The pH, fermentation period, and inoculum size were numeric, while the carbon and nitrogen sources were categorical. The minimum and maximum values for pH, fermentation period, and inoculum size were 4 and 8, 24 and 120 hours, and 1 and 10%, respectively. The carbon sources used were glucose, sucrose, fructose, maltose, and galactose, while the nitrogen source was L-asparagine only and L-asparagine with other nitrogen sources such as yeast extract, peptone, malt extract, and tryptone. The response was L-asparaginase yield (U/mL), and the experimental design is displayed in Table 2.

Model generation and Optimisation

The 65 points experimental data collected were subjected to statistical analysis. The significance of each experimental variable, reliability, and the derivation of mathematical models for the process were assessed using the R^2, F test, and model coefficients. Using Design Expert 11 software (Stat-Ease Inc.), a mathematical model that depicts the link between L-asparaginase yield and the process parameters was developed, along with a statistical analysis of the model. The modelled equation was fitted using regression analysis, and then the software underwent numerical optimisation (Oladunni et al., 2025). To maximise L-asparaginase yield, the optimisation constraints for pH (A), fermentation time (B), inoculum load (C), carbon source (D), and nitrogen source (E) were all "in range". Experiments were then carried out in triplicate in the laboratory to validate the predicted optimal point.

Table 1: Independent factors and coding

Factor	Name	Units	Change	Type	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev.
A	pH		Hard	Numeric	4.00	8.00	-1 ↔ 4.00	+1 ↔ 8.00	6.00	1.80
B	Fermentation Period	Hr	Easy	Numeric	24.00	120.00	-1 ↔ 24.00	+1 ↔ 120.00	69.78	46.03
C	Inoculum Load	%	Easy	Numeric	1.0000	10.00	-1 ↔ 1.00	+1 ↔ 10.00	5.57	4.32
D	Carbon Source	g/L	Easy	Categoric	Glucose	Sucrose			Levels:	5
E	Nitrogen Source	%	Easy	Categoric	Yeast Extract	Asparagine only			Levels:	5

Table 2: Design expert-selected variables with the experimental runs

Group	Run	Factor 1 A: pH	Factor 2 B: Fermentation Period	Factor 3 C: Inoculum Load	Factor 4 D: Carbon Source	Factor 5 E: Nitrogen Source
1	1	8	72	1	Galactose	Yeast Extract
1	2	8	120	1	Fructose	Peptone
1	3	8	120	10	Fructose	Malt Extract
1	4	8	120	10	Maltose	Asparagine only
1	5	8	24	1	Sucrose	Malt Extract
1	6	8	24	10	Fructose	Yeast Extract
1	7	8	120	5.5	Sucrose	Tryptone
1	8	8	120	10	Glucose	Yeast Extract
1	9	8	120	1	Glucose	Asparagine only
1	10	8	24	10	Maltose	Tryptone
1	11	8	24	1	Glucose	Malt Extract
1	12	8	24	1	Galactose	Asparagine only
1	13	8	24	1	Fructose	Asparagine only
2	14	4	72	5.5	Galactose	Asparagine only
2	15	4	120	1	Galactose	Yeast Extract
2	16	4	24	1	Sucrose	Tryptone
2	17	4	72	10	Sucrose	Peptone
2	18	4	24	5.5	Fructose	Peptone
2	19	4	24	10	Maltose	Asparagine only
2	20	4	120	10	Maltose	Peptone
2	21	4	24	10	Fructose	Malt Extract
2	22	4	24	1	Glucose	Asparagine only
2	23	4	72	10	Maltose	Yeast Extract
2	24	4	24	1	Glucose	Yeast Extract
2	25	4	120	1	Fructose	Tryptone
2	26	4	120	10	Fructose	Asparagine only

Group	Run	Factor 1 A: pH	Factor 2 B: Fermentation Period	Factor 3 C: Inoculum Load	Factor 4 D: Carbon Source	Factor 5 E: Nitrogen Source
3	27	4	24	10	Glucose	Tryptone
3	28	4	120	10	Galactose	Malt Extract
3	29	4	120	1	Maltose	Asparagine only
3	30	4	24	1	Galactose	Peptone
3	31	4	24	1	Fructose	Yeast Extract
3	32	4	120	1	Sucrose	Yeast Extract
3	33	4	24	1	Maltose	Malt Extract
3	34	4	24	5.5	Sucrose	Malt Extract
3	35	4	120	1	Glucose	Peptone
3	36	4	120	10	Glucose	Asparagine only
3	37	4	24	10	Galactose	Yeast Extract
3	38	4	120	10	Maltose	Tryptone
3	39	4	120	5.5	Glucose	Malt Extract
4	40	8	120	10	Glucose	Tryptone
4	41	8	120	1	Maltose	Malt Extract
4	42	8	120	1	Galactose	Tryptone
4	43	8	24	10	Sucrose	Yeast Extract
4	44	8	24	10	Glucose	Asparagine only
4	45	8	120	10	Galactose	Asparagine only
4	46	8	24	10	Fructose	Tryptone
4	47	8	24	1	Maltose	Peptone
4	48	8	24	1	Glucose	Tryptone
4	49	8	24	10	Maltose	Malt Extract
4	50	8	24	10	Galactose	Peptone
4	51	8	120	10	Sucrose	Peptone
4	52	8	24	1	Maltose	Yeast Extract
5	53	6	120	1	Maltose	Yeast Extract
5	54	6	72	10	Fructose	Peptone
5	55	6	72	1	Maltose	Tryptone
5	56	6	120	1	Fructose	Malt Extract
5	57	6	24	10	Glucose	Peptone
5	58	6	24	1	Galactose	Malt Extract
5	59	6	120	5.5	Galactose	Peptone
5	60	6	24	10	Sucrose	Asparagine only
5	61	6	24	10	Galactose	Tryptone
5	62	6	120	10	Sucrose	Malt Extract
5	63	6	120	10	Fructose	Yeast Extract
5	64	6	24	1	Sucrose	Peptone
5	65	6	120	1	Sucrose	Asparagine only

All experimental runs contain either L-asparagine only or L-asparagine with another source of nitrogen

L-asparaginase assay

After fermentation, each production medium was centrifuged at 1790 g for 5 minutes, and the supernatant was collected and used as crude enzyme. L-asparaginase activity was evaluated by measuring the rate at which L-asparagine is hydrolysed through the liberation of ammonia via Nesslerisation (Imada et al., 1973). Crude enzyme (0.5 mL) was mixed with 0.5 mL of Tris-HCl buffer (0.1 M, pH 8) and 0.5 mL of L-asparagine (0.04 M). The mixture was incubated at 37 ℃ for 30 minutes. To terminate the reaction, 0.5 mL of trichloroacetic acid (1.5 M) was added to the mixture after incubation. A portion of the terminated-reaction mixture (0.1 mL) was combined with 3.75 mL of distilled water, followed by the addition of 0.2 mL of Nessler reagent. This new mixture was incubated at 25 ℃ for 10 minutes. The optical density was measured using a spectrophotometer (500 spectrophotometers, Buck Scientific Inc.) at 450 nm. The optical density of various L-asparaginase concentrations was determined and used to extrapolate the ammonia released. One unit (U) of L-asparaginase is the quantity of enzyme that releases one micromole of ammonia in one minute.

Characterisation of L-asparaginase

Effect of Temperature and Time on L-asparaginase Activity

The crude L-asparaginase (0.5 mL) was added to 0.5 mL of L-asparagine (0.04 M) and Tris HCl buffer (0.05 M, pH 8.0). The mixture was incubated at different temperatures (25, 37, 45, and 60 ℃) for one hour at intervals of 15 minutes. The reaction was stopped using 0.5 mL of 1.5 M trichloroacetic acid after incubation. The enzyme activity was determined through Nesslerisation as explained in the L-asparaginase assay.

Effect of pH on L-asparaginase activity

The crude enzyme (0.5 mL) was added to 0.5 mL of 0.04 M L-asparaginase and 0.5 mL of different buffers of varying pH (sodium acetate – 0.1 M, pH 4.0 and 5.0; potassium phosphate – 0.1 M, pH 6.0 and 7.0; and Tris HCl – 0.1 M, pH 8.0). Each mixture was incubated at 37 °C for 30 minutes. The reaction was terminated with 0.5 mL of 1.5 M trichloroacetic acid after incubation. The L-asparaginase activity was evaluated as described in the L-asparaginase assay.

Effect of metal ions on L-asparaginase activity

The effects of different metal ions were determined by incubating crude enzymes with metal ions (Mg^{2+}, Mn^{2+}, Fe^{2+}, Na^{+}, K^{+}, and Ca^{2+}) and L-asparagine at 37 °C for 30 minutes at pH 8.0. The reaction was stopped by adding 0.5 mL of trichloroacetic acid (1.5 M). The enzyme activity was determined as explained in the L-asparaginase assay.

Statistical Analysis

The Design Expert Software (version 11) was utilised to conduct statistical analysis. Excel and GraphPad Prism 10 were used for graphical presentation.

RESULTS AND DISCUSSION

Results

The L-asparaginase-producing bacterium used in this work was molecularly identified as *Priestia megaterium* GAFA. Accession number PP390497 was assigned to the organism when its sequence was submitted to the National Centre for Biotechnology Information. The phylogenetic analysis of *Priestia megaterium* GAFA is shown in Figure 1. From the analysis, all Bacillus species were of the same origin. Priestia megaterium GAFA, *Priestia megaterium* Tc-3 and Bacillus megaterium MBTD_CMFRI_Ba5 are closely related. The Escherichia coli strains analysed are more distantly related to *Priestia megaterium* GAFA compared with the analysed Staphylococcus aureus strains.

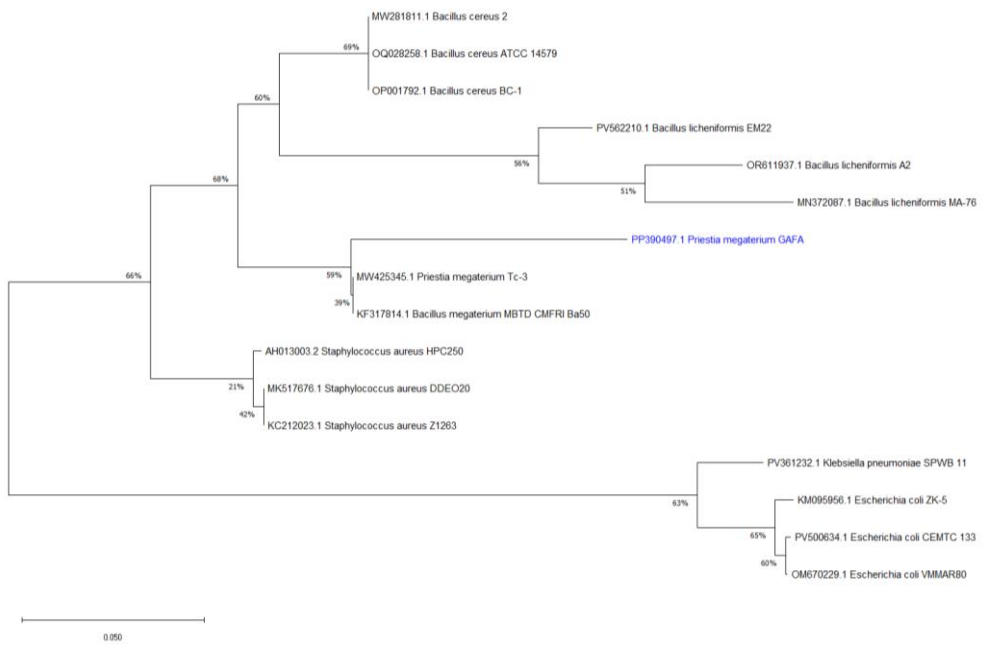

Figure 1: Phylogenetic analysis of *Priestia megaterium* GAFA

The yield of L-asparaginase from the 65 experimental runs ranged from 181.483 U/mL to 10594.100 U/mL, as shown in Figures 2, 3 and 4. The highest yield was recorded in run number 54, where fructose served as the carbon source, peptone as an extra nitrogen source, inoculated with 10% of a 0.5 McFarland standard and fermented for 72 hours at pH 6 (Table 2). The lowest yield occurred in run number 37, when galactose was used as the carbon source, yeast extract as the additional nitrogen source, inoculated with 10% of a 0.5 McFarland standard, and fermented for 24 hours at pH 4. The model generated in equation 1 was confirmed using glucose as a carbon source, L-asparagine as the only source of nitrogen, inoculated with 7.7% of a 0.5 McFarland standard of the organism and fermented for 73 hours at pH 7.8.

Statistical Analysis and Model Generation

Equation 1, which is expressed in terms of coded values, is the general model equation produced by the interaction of the dependent and independent variables in this investigation. By comparing the factor coefficients, this mathematical model can be used to determine the relative impact of the factors. Predicting the reaction for specific levels of each factor can also be done with it. The pH, fermentation time, and inoculum load are represented by the coded parameters in terms of a, B, and C, respectively. Conversely, the codes E [1], E [2], E [3], and E [4] stand for nitrogen supplies of asparagine alone, malt extract, peptone, and tryptone, respectively.

Equations 2, 3, 4, 5, and 6 for yeast extract, peptone, tryptone, malt extract, and L-asparagine alone, respectively, provide the model equations in terms of the actual values for various nitrogen sources. As long as the levels are provided in the original units for each factor, these equations can be used to forecast the reaction at certain values of each factor. However, because the intercept is not at the centre of the design space and the coefficients are scaled to the units of each factor, these equations cannot be used to determine the relative impact of each factor.

Modelled Equation in terms of coded values

$$R1 = 6.27 + 6.56a - 0.59B + 3.08C - 1.45E[1] + 1.91E[2] - 0.82E[3] + 0.39E[4] + 0.62aB - 0.30BC + 1.34a^2 - 3.4B^2 - 2.34C^2 + 1.09a^2B - 4.90aB^2 - 2.91B^2C + 0.34BC^2 + 1.62B^2C^2 \quad (1)$$

Modelled Equation in terms of actual values

NB: FP =Fermentation Period, and IL =Inoculum load

i. Nitrogen Source: Yeast Extract

$$Aparaginase\ yield = 16.46 - 1.84pH - 0.91FP - 1.12IL + 0.09[pH * FP] + 0.09[FP * IL] - 0.07pH^2 + 0.008FP^2 + 0.04IL^2 + 0.01[pH^2 * FP] - 0.001[pH * FP^2] - 0.001[FP^2 * IL] - 0.01[FP * IL^2] + 3.54e - 05[FP^2 * IL^2] \quad (2)$$

ii. Nitrogen Source: Peptone

$$Aparaginase\ yield = 19.84 - 1.84pH - 0.91FP - 1.12IL + 0.09[pH * FP] + 0.09[FP * IL] - 0.07pH^2 + 0.01FP^2 + 0.041IL^2 + 0.006pH^2 * FP - 0.001pH * FP^2 - 0.0007[FP^2 * IL] - 0.005[FP * IL^2] + 3.54e - 05[FP^2 * IL^2] \quad (3)$$

iii. Nitrogen Source: Tryptone

$$Aparaginase\ yield = 17.10 - 1.84pH - 0.91FP - 1.12IL + 0.09[pH * FP] + 0.09[FP * IL] - 0.07pH^2 + 0.01FP^2 + 0.04IL^2 + 0.005[pH^2 * FP] - 0.001[pH * FP^2] - 0.001[FP^2 * IL] - 0.01[FP * IL^2] + 3.54e - 05[FP^2 * IL^2] \quad (4)$$

iv. Nitrogen Source: Malt Extract

$$Aparaginase\ yield = 18.30 - 1.84pH - 0.91FP - 1.12IL + 0.09[pH * FP] + 0.09[FP * IL] - 0.07pH^2 + 0.008FP^2 + 0.04IL^2 + 0.006[pH^2 * FP] - 0.001[pH * FP]^2 - 0.001F[P^2 * IL] - 0.01[FP * IL^2] + 3.54e - 05[FP^2 * IL^2] \quad (5)$$

v. Nitrogen Source: *Asparagine only*

$$Aparaginase\ yield = 17.87 - 1.84pH - 0.91\ FP - 1.11IL + 0.09pH * FP + 0.09FP * IL - 0.07pH^2 + 0.008FP^2 + 0.04IL^2 + 0.006pH^2 * FP - 0.001pH * FP^2 - 0.0007FP^2 * IL - 0.005FP * IL^2 + 3.54e - 05FP^2 * IL^2 \quad (6)$$

The plot of predicted against actual values of the experimental data is shown in Figure 3. The fit statistics show that the model, in terms of coded values, has a coefficient estimate (R2) of 0.7892, which implies that the predictor model can account for 78.92% of the variance in the experimental data. Similarly, the adjusted R2 has a value of 0.7002, which implies 70.02% of the variance is explained when adjusting for the number of predictors in the model. These values are sufficiently high and indicate a fairly strong fit for the model. Furthermore, the reduction in the value of R2 to adjusted R2 indicates that when more predictors are added, there might not be a significant improvement in the models. Moreover, the unexplained variance of about 30% suggests that other factors not investigated might be contributing to the value of the response variable.

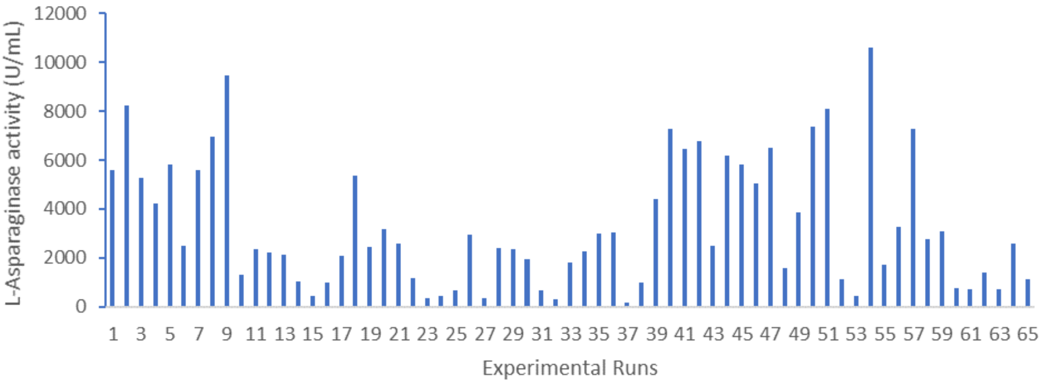

Figure 2: L-asparaginase yield at various experimental runs with different pH and media mixture ratios

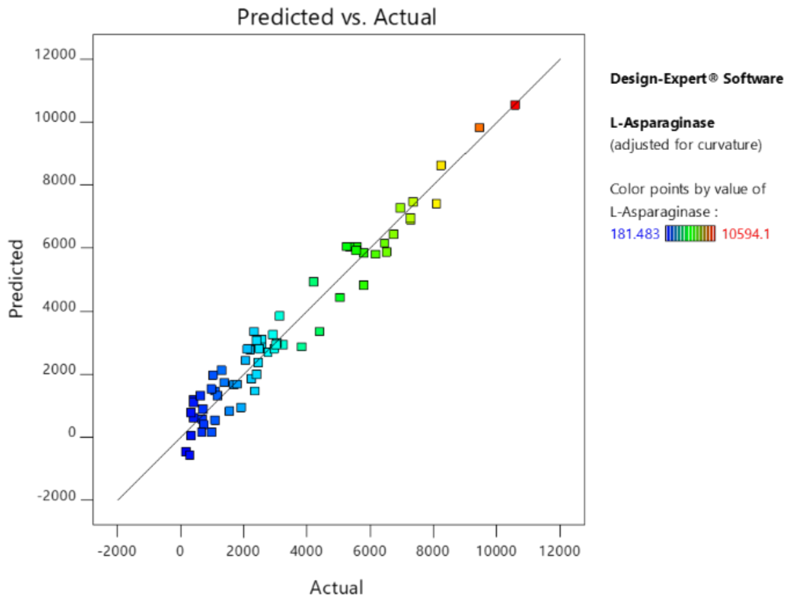

Figure 3: Plot of predicted against actual values of L-asparaginase production

Analysis of Variance (ANOVA)

The analysis of variance based on restricted maximum likelihood (REML) analysis is presented in Table 3. The parameters with p-values less than 0.0500 indicate model terms are significant. In this case, the whole plot is statistically significant, as well as the model terms a, C, E, aB, a^2B, aB^2, and B^2C. On the other hand, the parameters with values greater than 0.1000 indicate the model terms are not statistically significant. The statistically insignificant model terms are potential candidates for model reduction.

Optimisation of Asparaginase Yield

The optimal production of asparaginase yield is shown in the response surface plot displayed in Figure 4. The optimum process conditions are a fermentation medium containing maltose as the carbon source, peptone as an added nitrogen source with L-asparagine, at pH 7.9, inoculated with 9.7% of a 0.5 McFarland standard of *Priestia megaterium* GAFA, and fermented for 54 hours. This prediction was confirmed in the laboratory by validating, using glucose as a carbon source, L-asparagine as the only source of nitrogen, inoculated with 7.7% of 0.5 McFarland standard of the organism and fermented for 73 hours at pH 7.8 as suggested by Design Expert.

Table 3: Analysis of Variance (Restricted Maximum Likelihood) analysis Kenward-Roger p-values

Source	Term df	Error df	F-value	p-value	
Whole-plot	2	3.98	13.38	0.0171	significant
a-pH	1	46.92	24.05	< 0.0001	
a^2	1	1.84	7.54	0.1215	
Subplot	15	44.67	7.06	< 0.0001	significant
B-Fermentation Period	1	45.20	0.5594	0.4584	
C-Inoculum Load	1	45.05	9.02	0.0043	
E-Nitrogen Source	4	46.24	9.00	< 0.0001	
aB	1	46.07	8.82	0.0047	
BC	1	45.57	2.42	0.1269	
B^2	1	45.34	2.72	0.1063	
C^2	1	45.09	1.38	0.2466	
a^2B	1	46.02	4.82	0.0331	
aB^2	1	46.63	13.02	0.0007	
B^2C	1	45.09	7.68	0.0081	
BC^2	1	45.74	0.2410	0.6259	
B^2C^2	1	45.37	0.5770	0.4514	

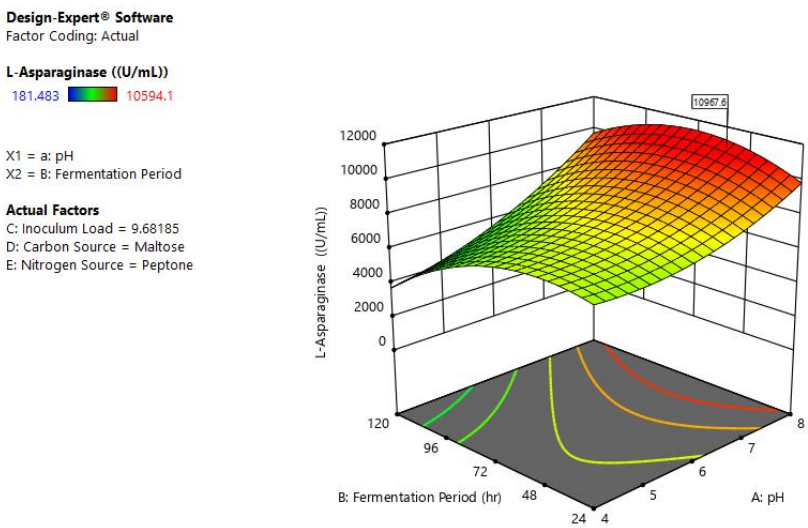

Figure 4: 3D Response surface plot for the optimisation of Asparaginase

Characterisation of L-asparaginase

The calcium cation was the best among the cations used that supported the activity of L-asparaginase (13021.40 U/mL), as shown in Figure 5. It was closely followed by Mn^{2+} (8467.32 U/mL) and Mg^{2+} (8342.55 U/mL). The activity of L-asparaginase without metal ions (control), 7406.78 U/mL, was higher than the activities obtained with K^{+} (7242.31 U/mL) and Fe^{2+} (6652.49 U/mL).

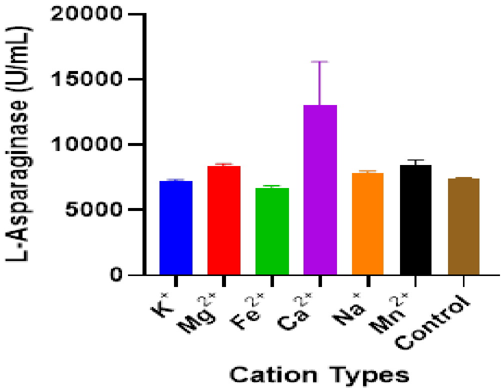

Figure 5: Effect of cations on activity of L-asparaginase of *Priestia megaterium* GAFA

Figure 5 shows the effect of various pH levels on the activity of L-asparaginase produced by *Priestia megaterium* GAFA. Among the pH ranges used, the highest activity of L-asparaginase (7619.45 U/mL) was observed at pH 6, which was closely followed by the activity (7406.78 U/mL) recorded at pH 8. The lowest (5552.25 U/mL) activity was recorded at pH 7.

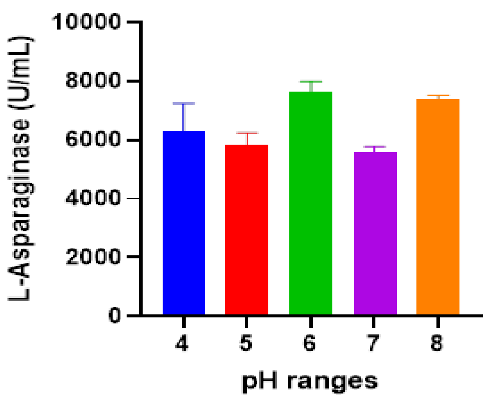

Figure 6: Effect of pH on the activity of L-asparaginase of *Priestia megaterium* GAFA

The effect of temperature and time on L-asparaginase activity of *Priestia megaterium* GAFA is shown in Table 4. When L-asparaginase was incubated for 15 minutes, the lowest (4871.69 U/mL) and highest (8441.80 U/mL) activities of L-asparaginase were recorded at 50 °C and 60 °C, respectively. When incubated for 30 minutes, an increase in the activities of L-asparaginase occurred with rising temperature from 25 °C (5038.99 U/mL) to 50 °C (7823.62 U/mL), before a decline in activity was observed at 60 °C (6142.21 U/mL). After 45 minutes of incubation, L-asparaginase activity also increased with temperature, moving from 3331.92 U/mL (25 °C) to 7480.51 U/mL (50 °C) before decreasing with a further rise in temperature to 6068.34 U/mL (60 °C). When incubated for 60 minutes, the lowest activity (6337.73 U/mL) occurred at 37 °C, while the highest activity (9400.26 U/mL) was recorded at 60 °C.

Table 4: Effect of Temperature and Time on the L-asparaginase (U/mL) of *Priestia megaterium* GAFA

Temperature (°C)	Time (minutes)			
	15	30	45	60
25	6510.71	5038.99	3331.92	7143.06
37	6780.09	6391.61	7194.10	6337.73
50	4871.69	7823.62	7480.51	6751.74
60	8441.80	6142.21	6068.34	9400.26

Discussion

Priestia megaterium was formerly known as Bacillus megaterium (Phuong et al., 2024), a name also confirmed by phylogenetic analysis, which revealed that Priestia megaterium and Bacillus megaterium are the same species. The production of L-asparaginase by *Priestia megaterium* has been reported by some researchers (Lu et al., 2019; Phuong et al., 2024). *Priestia megaterium* is one of the bacteria that are generally recognised as safe (Park et al., 2025). The use of an organism that is generally recognised as safe to produce L-asparaginase is advantageous because such an organism can be used for commercial production, as its safety is guaranteed. Some authors have reported using response surface methods to optimise the synthesis of L-asparaginase (Abhini et al., 2022; Moguel et al., 2022; Lefin et al., 2025). A highly dependable prediction was indicated by the R^2 produced in this investigation, which was close to 1 (Abhini et al., 2022). Maltose was identified in this study as the carbon source that would maximise the synthesis of L-asparaginase. Given that several researchers have found that glucose is the preferred carbon source for L-asparaginase synthesis (El-Aziz et al., 2021; Baraka et al., 2023), the preference for maltose in this study may be species- or substrate-specific. Nevertheless, the Design Expert software recommended glucose as the carbon source to validate the model created for this investigation.

Microbial growth and metabolite production are significantly impacted by the availability of a nitrogen source (Yap et al., 2021; Fasiku et al., 2023; Odjoji et al., 2026). The ability of L-asparaginase to function as an inducer while peptone acts as a nitrogen source in the synthesis of L-asparaginase may be the basis for the optimal production of L-asparaginase with peptone in addition to L-asparaginase as a nitrogen source. Additionally, it has been noted that organisms that produce L-asparaginase are substrate-specific (Yap et al., 2021). This study's optimal yield of L-asparaginase utilising L-asparagine as an inducer is comparable to that reported by El-Aziz et al. (2021) and Yap et al. (2021), where L-asparagine triggered the production of L-asparaginase. The use of peptone as a nitrogen source for the optimum yield of L-asparaginase was reported by Baraka et al. (2023).

Microbial production is impacted by inoculum load (Fasiku and Wakil, 2022). L-asparaginase synthesis was significantly impacted by inoculum load. *Priestia megaterium* GAFA produces different amounts of L-asparaginase depending on the number of cells. The synthesis of L-asparaginase depends on the concentration of hydrogen ions (pH). *Priestia megaterium* was predicted to produce the most L-asparaginase at pH 7.9. Bacteria typically develop best at neutral pH levels, while acidic pH inhibits bacterial growth (Al-kaabi and Chelab, 2024). Aspergillus tubingensis produced the most L-asparaginase at pH 6.0 (Chinnadurai and Govindasamy, 2024), but Fusarium proliferatum, a fungus, produced the most L-asparaginase at pH 5.0 (Yap et al., 2021). The optimum fermentation period was 54 hours. Most bacteria have their optimum production of primary metabolites between 48 and 96 hours (Fasiku and Wakil, 2021). Although *Priestia megaterium* GAFA's L-asparaginase was active across a broad pH range, pH 6 showed the highest activity. In this experiment, the maximum activity of L-asparaginase was found at pH 6, which is near the pH of food. Though the enzyme was produced at pH 7.8, the highest L-asparaginase activity observed at pH 6 was not significantly different from the activity at pH 8. While Cunha et al. (2021) found the optimal activity of asparaginase from Aspergillus oryzae at pH 5, El-Aziz et al. (2021) and Al-Harbi et al. (2025) showed the maximum activity of L-asparaginase from Pseudomonas aeruginosa and Bacillus xiamenensis ASPJ1-4, respectively, at pH 9.

L-asparaginase activity was enhanced by certain metal ions. Certain metal ions may have stimulatory activity because they attach to the active site of L-asparaginase as cofactors, which makes substrate binding easier. On the other hand, chelation with the sulfhydryl groups of protein structures may be the cause of suppression by other metal ions (Al-Harbi et al., 2025). In this work, Ca2+, Mg2+, Mn2+, and Na+ activated enzyme activity. The Ca2+ increased the activity of L-asparaginase of *Priestia megaterium* GAFA by 76%, while Na+ enhanced enzyme activity by just 6% when compared with the control (without metal ion). Luhana and Bariya (2025) reported that metal ions such as iron, copper and zinc led to reduced L-asparaginase activity, while manganese and magnesium had no impact on L-asparaginase activity.

Each enzyme has a temperature at which its optimum activity is observed. Any temperature below or above the optimum temperature will lead to a decrease in the enzyme activity (Fasiku et al., 2023). In this work, the L-asparaginase of *Priestia megaterium* GAFA was active at all the temperatures used, which meant that it can be utilised over a wide range of temperatures. The highest activity was recorded at 60 °C. El-Aziz (2021) reported the highest activity of L-asparaginase at 35 °C, while Al-Harbi et al (2025) had the highest activity of asparaginase produced by Bacillus xiamenensis ASPJ1-4 at 40 °C.

CONCLUSION

Production of L-asparaginase by *Priestia megaterium* GAFA was optimised using response surface methodology, which represents the first response surface methodology optimisation of this strain. Factors such as metal ions, pH and temperature affected the activity of L-asparaginase of *Priestia megaterium*. Further studies should evaluate the therapeutic scale-up and industrial potential of this optimised process.

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