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Optimization of Growth and Urease Production of *Bacillus Tequilensis* Through pH and Temperature Control

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Abstract

This study investigates the influence of pH and temperature on the growth density and urease enzyme production of *Bacillus tequilensis*. Bacterial counts and enzyme activity were measured across a range of pH levels (5.75 to 14.24) and temperatures (24.3°C to 42.7°C). The results showed significant differences in bacterial growth and urease production based on these conditions. The highest bacterial growth (6.34 $\log_{10}$ CFU/mL) occurred at pH 10.00 and 42.7°C, while optimal urease activity was also observed under these conditions, indicating that slightly alkaline pH and high temperature are favorable for both bacterial proliferation and enzyme activity. Conversely, high alkalinity at moderate temperatures significantly inhibited bacterial growth, and acidic conditions combined with high temperatures resulted in minimal bacterial presence. The Response Surface Methodology (RSM) analysis highlighted temperature as the most significant factor affecting urease production, followed by pH. ANOVA confirmed the substantial impact of these variables, with the model explaining 81.83% of the variance. This study highlights the crucial role of pH and temperature in optimizing bacterial growth and enzymatic activity, offering valuable insights for industrial and environmental applications.

1. Introduction

The construction industry, particularly through the production of conventional concrete, is a major contributor to global CO_2 emissions, necessitating urgent and sustainable solutions to mitigate its environmental impact. Cement production alone accounts for approximately 8% of global carbon emissions, with concrete production contributing significantly to the overall carbon footprint of the construction sector [1]. To address this, various innovative approaches and materials are being explored. One promising solution is the development and use of self-healing concrete, which can repair its own micro-cracks, thereby enhancing durability and reducing the need for frequent repairs and replacements, ultimately lowering lifecycle emissions [2]. Additionally, the integration of Supplementary Cementitious Materials (SCMs), such as fly ash and ground granulated blast-furnace slag (GGBS),

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has been shown to significantly reduce CO_2 emissions. Studies have demonstrated that GGBS-based concretes emit fewer pollutants compared to fly ash-based concretes, making them a more sustainable option [3]. However, the availability of fly ash is becoming limited due to the shutdown of coal power plants, prompting the need for alternative SCMs like Volcanic Pozzolan from Iceland (VPI), which has been shown to reduce carbon emissions effectively while maintaining comparable properties to traditional concrete [1]. Another innovative approach involves the use of ground calcium carbonate (GCC) to substitute clinker in concrete, which can reduce carbon emissions by up to 50% while maintaining similar mechanical properties [4].

However, the sustainable construction materials industry has shown significant interest in utilizing *Bacillus tequilensis* bacterial cells to produce bio-foamed concrete bricks (B-FCB). The use of *B. tequilensis* bacterial cells in creating B-FCB has shown promising results in enhancing the mechanical and durability properties of the material [5]. This innovative approach leverages the natural sequestration of CO_2 and the bioreaction of *B. tequilensis* enzymes to optimize the compressive strength and healing properties of B-FCB. Researchers have utilized optimization techniques such as the 2K factorial and RSM analysis to determine the optimal conditions for bio-foamed concrete bricks in terms of carbonation depth, compressive strength, and urease enzyme production [6]. Research has demonstrated that the optimal conditions for achieving a compressive strength of 8.22 MPa include a CO_2 concentration of 10%, a bacterial concentration of 3×10^7 cells/mL, a temperature of 27°C, and a concrete density of 1800 kg/m^3 after 28 days, resulting in a 35.5% improvement compared to traditional foamed concrete bricks (FCB) [7]. Additionally, the self-healing capabilities of B-FCB were significantly enhanced, with a decrease in initial water absorption (IWA) and water absorption (WA) of 52.8% and 29.1%, respectively, compared to FCB. This healing process is primarily attributed to the precipitation of calcium carbonate ($CaCO_3$) within the pores, as confirmed by scanning electron microscopy (SEM), energy dispersive X-ray (EDX), and X-ray diffraction (XRD) analyses [8]. Furthermore, the sequestration process of CO_2 in B-FCB was optimized using a combination of factors, including concrete density, bacterial concentration, temperature, and CO_2 percentage, achieving an optimal carbonation depth of 12.2 mm under specific conditions, which further increased the formation of $CaCO_3$ and improved the material's durability [9]. On the other hand, the incorporation of *B. tequilensis* not only enhances the compressive strength but also contributes to the eco-friendliness of the construction material by reducing CO_2 emissions. This aligns with the broader trend of utilizing microbial activities to induce mineral precipitation and improve concrete properties, as seen in other studies involving different bacterial strains like *Bacillus megaterium* and *Bacillus subtilis*, which have also shown significant improvements in compressive, flexural, and tensile strengths due to microbial-induced calcite precipitation [10], [11]. The use of microbial foaming agents, which are non-toxic and environmentally friendly, further supports the sustainability of this approach [12]. Overall, the integration of *B. tequilensis* in B-FCB represents a significant advancement in sustainable construction materials, offering enhanced mechanical properties, improved durability, and a reduction in environmental impact through effective CO_2 sequestration and self-healing capabilities.

So, this study aimed to optimize conditions for enhancing urease production from *B. tequilensis*. It involved isolating urease-producing bacterial strains from cement kiln dust and adjusting parameters such as pH (5 to 14), temperature (24°C to 43°C), aeration, agitation, and inoculum size for optimal enzyme production. Statistical methods like RSM and Design of Experiments (DoE) were used for systematic analysis. Additionally, eco-friendly bio-foamed concrete bricks were created using the isolated *B. tequilensis*, and their structural properties, including compressive strength and resistance to penetration, were evaluated.

2. Materials and Methods

2.1 Isolation and Identification of *B. Tequilensis*

B. tequilensis 401 was isolated from cement kiln dust (CKD) collected from the Cement Industries of Malaysia Berhad (CIMA), as previously described [13]. Five CKD samples were obtained from the floor surfaces during and after the burning of cement raw materials at various stages of CIMA. The bacterial isolates were recovered using the direct isolation technique on a selective medium called Kiln Dust Medium (KDM). This medium contained $NaNO_3$ (1 g/L), K_2HPO_4 (0.5 g/L), $KHPO_4$ (0.5 g/L), $MgSO_4$ (1 g/L), yeast extract (1 g/L) as trace elements, and 10 g of CKD as the sole carbon source [9]. Bacterial isolates that produce urease enzymes, survive high CO_2 concentrations, and grow under facultative conditions in a simulated foamed concrete medium were selected. The most potent isolate, coded as 401, was identified as *B. tequilensis* 401 (Accession No. MN865766, https://www.ncbi.nlm.nih.gov/nuccore/MN865766). *B. tequilensis* 401 was then freeze-dried into powder and stored at -20°C for subsequent uses.

2.2 Preparation of *B. Tequilensis* Suspension

The bacterial suspension was prepared by rehydrating freeze-dried bacteria by adding them to a sterile tryptic soy broth (TSB) solution (1 gram of lyophilized bacteria/100 mL of TSB broth). This suspension was then

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incubated at 35°C for 24 hours to allow bacterial growth. After confirming growth through observation of turbidity, a small portion of the culture was streaked onto a TSB agar plate using a sterile loop. Following incubation at 35°C for 24 hours, the morphological appearance of the growing bacterial colonies was studied, and their purity was verified (Fig. 1). Subsequently, a single colony was selected and suspended in a saline solution. The bacterial density was then adjusted using a spectrophotometer to achieve a 0.5 McFarland standard of 0.08–0.1 at 625 nm, corresponding to approximately 1.5 x 10^8 colony-forming units per milliliter (CFU/mL). This bacterial stock solution was stored at 4°C and used within 24 hours for subsequent experiments.

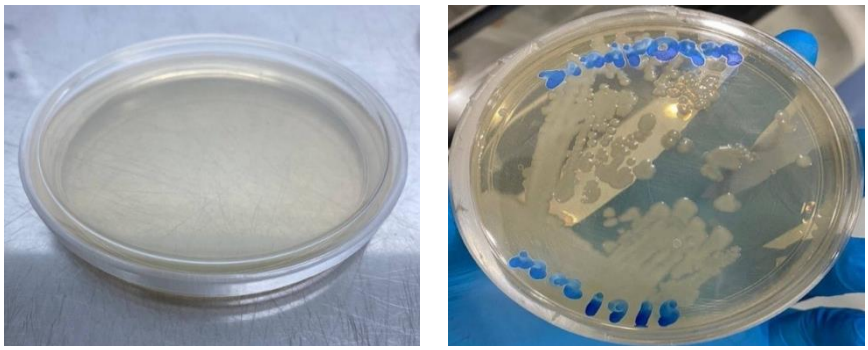


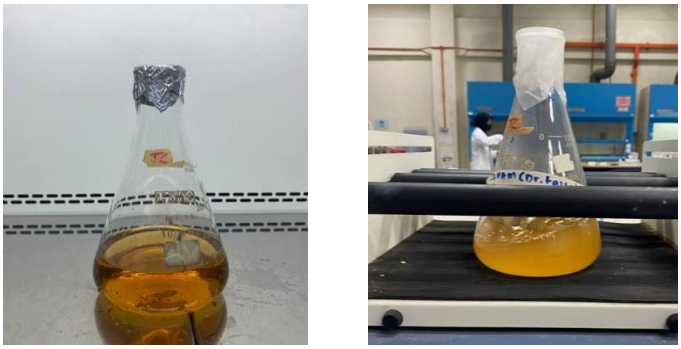
Fig. 1 *Morphological appearance of B. tequilensis colonies on TSB*

2.3 Optimization of Growth Density

The optimization of bacterial growth density was conducted using various pH values and temperatures with RSM. Initially, TSB broth was prepared in 13 flasks, each containing 50 mL of media, with one flask serving as a control at normal pH. To adjust the pH for each flask according to the specified runs shown in Table 1, HCl was used to decrease the pH, and NaOH was used to increase it. A pH meter was used to measure the pH of each flask. The flasks were covered with aluminum foil and sterilized in an autoclave. After sterilization, the flasks were cooled to room temperature before being inoculated with 1 mL of a 0.5 McFarland standard (stock bacterial solution). The flasks were labelled and placed in a shaker incubator set to their respective temperatures (Fig. 2). Bacterial growth density in the flasks was observed after 24 hours.

Table 1 *Experiment runs with a specified pH and temperature*

Run	pH value	Temperature (°C)
1	7.00	27.0
2	13.00	27.0
3	7.00	40.0
4	5.75	40.0
5	5.75	33.5
6	14.24	33.5
7	10.00	24.3
8	10.00	42.7
9	10.00	33.5
10	10.00	33.5
11	10.00	33.5
12	10.00	33.5
13	10.00	33.5



(a) **(b)**

Fig. 2 *Description (a) The soya broth before adding the bacteria; and (b) The turbidity of the broth after 24 hours*

2.4 Bacterial Count

The pour plate technique was used to quantify the bacteria in flasks obtained from the previous experiment. In short, TSB agar was prepared, sterilized by autoclaving, and then kept in a water bath at 48°C. Using test tubes with sterilized TSB broth, serial dilutions were made for each flask containing bacterial turbidity, ranging from 101 to 105. A 0.1 mL sample of each dilution was transferred to empty Petri dishes, and the tryptic-soy agar was poured over them. The dishes were thoroughly mixed and allowed to solidify on a flat surface. They were then incubated at 35°C for 48 hours. After incubation, the colonies in the dishes were counted to determine the bacterial number for each flask. The total bacterial count was expressed in a decimal logarithm of CFU per mL ($\log_{10}$ CFU/mL).

2.5 Urease Enzyme Production

To evaluate the efficiency of the urease enzyme produced by the test bacteria, a urea broth medium was prepared and sterilized using an autoclave. After cooling the media to room temperature, a 40% urea concentration was added. The media was distributed into sterilized test tubes, which were then inoculated with bacteria from the growth density flasks, with each tube representing a separate flask. These tubes were labelled and maintained at the same temperature, as indicated in Table 1. The changes resulting from urease enzyme production in the tubes were observed over a three-day period in the urea broth.

2.6 Statistical Analysis

Duncan's multiple range test was employed to determine statistically significant differences between the samples at a 95% confidence level ($p < 0.05$). The tests were conducted in triplicate, and the mean, standard deviation, and analysis of variance were computed for each test using RSM analysis.

3. Results and Discussion

3.1 Improvement of the Growth Density

Table 2 presents bacterial counts in $\log_{10}$ CFU/mL under various pH levels and temperatures, revealing significant differences in bacterial growth based on acidity and temperature. The highest bacterial growth (6.34 $\log_{10}$ CFU/mL) occurred at pH 10.00 and 42.7°C, demonstrating that slightly alkaline conditions at lower temperatures support significant bacterial proliferation, even more than neutral pH at a slightly higher temperature. The second-highest growth (6.06 $\log_{10}$ CFU/mL) was at pH 7 and 27°C, indicating that neutral pH and moderate temperature are also highly favorable for bacterial proliferation. Conversely, high alkalinity at moderate temperatures (pH 13.00, 27°C) resulted in significantly reduced bacterial growth (2.00 $\log_{10}$ CFU/mL), suggesting that high alkalinity inhibits bacterial proliferation even at moderate temperatures. Similarly, minimal bacterial growth (2.00 $\log_{10}$ CFU/mL) at high alkalinity and moderate temperature (pH 14.24, 33.5°C) indicates that moderate temperatures support some bacterial survival even in acidic conditions, but growth remains significantly inhibited. At pH 5.75 and 40°C, bacterial growth was lower (4.38 $\log_{10}$ CFU/mL) compared to neutral pH and moderate temperature, indicating that higher temperatures negatively affect bacterial proliferation even at neutral pH. Moderate bacterial growth (4.297 $\log_{10}$ CFU/mL) was observed at pH 10 and 42.7°C, indicating that while elevated temperatures reduce bacterial growth, slightly alkaline conditions still permit bacterial presence.

Consistent and substantial bacterial growth, ranging from 4.519 to 5.409 $\log_{10}$ CFU/mL, was noted at pH 10 and 33.5°C across multiple trials, indicating that a slightly alkaline pH at moderate temperatures supports robust

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bacterial proliferation. In contrast, no bacterial growth was observed at pH 5.75 and 33.5°C, or at pH 10 and 24.3°C, demonstrating that the combination of high temperature with acidic pH, as well as extreme alkalinity, regardless of moderate temperature, is lethal to bacteria.

Overall, the results indicated that neutral to slightly alkaline pH (7 to 10) supports bacterial growth more effectively than acidic (5.75) or highly alkaline conditions (13.00 and 14.24), while moderate temperatures (27.0°C and 33.5°C) are generally more favorable for bacterial growth compared to higher temperatures (40°C and 42.7°C). Extreme pH levels, combined with high temperatures, significantly inhibit bacterial growth. A slightly alkaline pH, particularly at lower to moderate temperatures, consistently supports significant bacterial proliferation, with the highest counts observed at a slightly alkaline pH and lower temperatures. However, *B. tequilensis*, a versatile bacterium, has been studied at various pH levels and temperatures, demonstrating its adaptability and potential for diverse applications. For instance, *B. tequilensis* CGMCC 17603, isolated from biological soil crusts, showed optimal growth conditions at pH 8.5 and 31°C, achieving a high-density culture with a biomass increase from 9.62×10^7 to 2.33×10^9 CFU/mL [14]. Similarly, *B. tequilensis* MTCC 9585, known for its protease production, grew well across a pH range of 5 to 12 and temperatures from 4°C to 50°C, with optimal growth at pH 7.0 and 37°C [15]. Moreover, *B. tequilensis* has been shown to enhance the growth and metabolic activity of the microalga *Haematococcus lacustris*, with significant improvements in cell density and chlorophyll content under optimized conditions, suggesting a symbiotic relationship that could be leveraged for biotechnological applications [16]. Collectively, these studies highlight the adaptability of *B. tequilensis* to various pH levels and temperatures, making it a versatile candidate for multiple biotechnological and environmental applications. By optimizing growth conditions and leveraging genetic engineering, the growth density and functional capabilities of *B. tequilensis* can be significantly enhanced, paving the way for its broader utilization in industry and environmental management.

Table 2 *Bacterial counts in log_{10} CFU/mL under various pH levels and temperatures*

No.	log_{10} CFU/mL	pH	Temperature (°C)
1	6.06	7.00	27.0
2	2.00	13.00	27.0
3	4.38	7.00	40.0
4	0	5.75	40.0
5	2.00	5.75	33.5
6	0	14.24	33.5
7	6.34	10.00	24.3
8	4.297	10.00	42.7
9	5.409	10.00	33.5
10	5.393	10.00	33.5
11	5.362	10.00	33.5
12	5.079	10.00	33.5
13	4.519	10.00	33.5

3.2 Urea Enzyme Production by *B. Tequilensis*

Table 3, Fig. 3(a), and Fig. 3(b) outline the production of urease enzymes by bacteria under various pH levels and temperatures, illustrating significant differences in urease activity. The highest urease production was observed under slightly alkaline pH and high-temperature conditions (pH 10, 42.7°C), indicating an extremely positive response (+++++). This suggests that a slightly alkaline pH at high temperatures is optimal for urease activity. Similarly, high urease production (++++) was seen under high alkalinity and moderate temperatures (pH 14.24, 33.5°C), showing that moderate temperatures combined with high alkalinity significantly enhance urease production. Under neutral pH and high temperature (pH 7, 40°C), the bacteria exhibited high urease production (+++), indicating that elevated temperatures also promote urease activity even at neutral pH.

Table 3 *The production of urease enzyme by B. tequilensis under various pH levels and temperatures*

No.	Urease Enzymes	pH	Temperature (°C)
1	–	7.00	27.0
2	–	13.00	27.0
3	+ + +	7.00	40.0
4	–	5.75	40.0
5	+ + + +	14.24	33.5
6	–	10.00	33.5
7	–	10.00	24.3
8	+ + + + +	10.00	42.7
9	+ + + +	10.00	33.5
10	+ + +	10.00	33.5
11	+ + + +	10.00	33.5
12	+ + + +	10.00	33.5
13	+ + +	10.00	33.5

*Note: the results descriptions in the table are as follow: (-) negative, (+) slightly positive, (++) moderately positive, (+++) positive, (++++) highly positive, and (+++++) extremely positive.

(a) **(b)**

Fig. 3 *Description (a) The result after 24 hours; and (b) The result after 3 days*

Additionally, multiple trials at a slightly alkaline pH and moderate temperature (pH 10, 33.5°C) showed urease production ranging from positive (+++) to highly positive (++++). These consistent results indicate that a slightly alkaline pH at moderate temperatures is generally favorable for urease enzyme activity. Conversely, conditions such as neutral pH and moderate temperature (pH 7, 27°C), high alkalinity and moderate temperature (pH 13, 27°C), acidic pH and high temperature (pH 5.75, 40°C), and slightly alkaline pH and lower temperature (pH 10, 24.3°C) resulted in negative (-) urease production. This indicates that these conditions did not support urease production, suggesting the presence of inhibitory enzyme activity.

Generally, elevated temperatures enhance urease activity, as shown by the positive results at neutral pH and 40°C, as well as slightly alkaline pH and 42.7°C. On the contrary, acidic pH at high temperatures (pH 5.75, 40°C) and neutral pH at moderate temperatures (pH 7, 27°C) are not conducive to urease production. While moderate temperatures, combined with high alkalinity (pH 14.24, 33.5°C) and a slightly alkaline pH (10.00, 33.5°C), generally support urease production, lower temperatures (24.3°C) do not. A slightly alkaline pH at high temperatures (pH 10, 42.7°C) is optimal for maximizing urease enzyme production. Furthermore, the optimal conditions for urease enzyme activity closely aligned with the typical climate conditions in Malaysia, suggesting that environmental factors play a crucial role in enhancing enzymatic efficiency under local weather conditions.

However, studies have been conducted on *B. tequilensis* to investigate its ability to generate enzymes at different pH levels and temperatures. For instance, *B. tequilensis* ZMS-2, identified from desert soils, exhibited maximum enzyme production at pH 8 and 37°C, with notable proteolytic activity [17]. Another strain, identified as *B. tequilensis*, isolated from mangrove soils, exhibited maximum antimicrobial activity at pH 6 and 30°C after 36 hours of incubation. Additionally, *B. tequilensis* P15, isolated from the Jakhau coast, produced a solvent- and detergent-tolerant protease optimally at pH 8 and 30°C, with a 6.4-fold increase in protease production [18]. Another strain, *B. tequilensis* HFSI-5, isolated from the fermented intestine of H. scabra, produced a fibrinolytic protease with high thrombolytic activity, although specific pH and temperature conditions were not detailed [19].

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B. tequilensis MTCC 9585, isolated from soil, produced protease optimally at pH 10 and 60°C, with 90% of production occurring within 6 hours, and demonstrated stability over a wide pH (5–12) and temperature range (4–50°C) [15]. Another strain, *B. tequilensis* RG-01, isolated from soil samples, showed maximum amylase production at pH 7 and 55°C, with significant stability in various organic solvents and surfactants [20]. Additionally, *B. tequilensis* SALBT, isolated for pectinase production, achieved maximum enzyme activity at pH 7.5 and 40°C, indicating its adaptability to slightly alkaline conditions [21]. Also, the strain of *B. tequilensis* ON1, isolated from shrimp pond sludge, exhibited optimal protease activity at pH 8 and 50°C, further demonstrating the strain's versatility in alkaline and moderately high-temperature environments [22]. Overall, these studies highlight the ability of *B. tequilensis* to produce urease at a range of pH levels and temperatures, making it a promising candidate for various industrial, agricultural, and environmental applications.

3.3 Analysis of Response Surface Methodology (RSM) Results

The Pareto chart of standardized effects, as shown in Fig. 4, provides a clear visualization of the impact of different factors on the response variable, which is the production of the urease enzyme. Factors A and B in this chart represent pH and temperature, respectively. The chart also includes interaction and quadratic terms: AA for the quadratic effect of pH, BB for the quadratic effect of temperature, and AB for the interaction effect between pH and temperature. The red dashed line at 2.365 marks the significance level ($\alpha = 0.05$), indicating that any effects surpassing this line are statistically significant.

The chart directly relates to the optimization procedure described in section 3.4, where pH and temperature conditions were manipulated in 13 different flasks to optimize urease enzyme production. According to the Pareto chart, the most significant factor is temperature (B). The chart shows that the bar representing temperature extends well beyond the significance threshold of 2.365, indicating that temperature variations have a substantial impact on urease enzyme production. This highlights the critical importance of temperature optimization in the production process, as it significantly influences the biochemical reactions involved in enzyme activity.

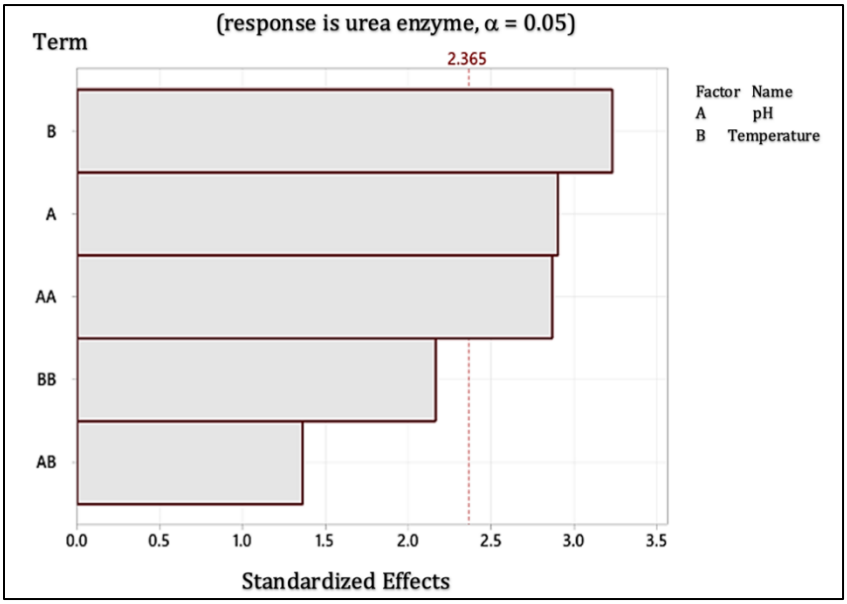

Fig. 4 *The Pareto chart of standardised effects*

Following temperature, pH (A) is the second most significant factor. The bar for pH also exceeds the significance threshold, underscoring its crucial role in urease enzyme production. The pH of the medium affects both enzyme activity and stability, making its careful adjustment essential for maximizing production efficiency. The quadratic effect of pH (AA) is another significant factor, with its bar surpassing the significance threshold. This suggests a nonlinear relationship between pH levels and enzyme production, indicating an optimal pH range where urease production is maximized. Deviations from this optimal range, either higher or lower, decrease enzyme production, indicating the importance of precise pH control.

Next in significance is the quadratic effect of temperature (BB). Although its bar is close to but not extending beyond the significance threshold, it indicates a nonlinear effect of temperature on enzyme production. This suggests that, similar to pH, an optimal temperature range maximizes enzyme production, and deviations from this range can reduce production efficiency. The practical implications of these findings are significant. The validation of the optimization procedure confirms that focusing on pH and temperature is crucial for optimizing

urease enzyme production. The significant quadratic and interaction effects suggest that further refinement of the experimental conditions might be necessary. Future experiments could benefit from using a response surface methodology (RSM) approach to identify the optimal levels of pH and temperature, considering both linear and quadratic effects as well as their interactions.

In conclusion, the Pareto chart of standardized effects provides critical insights into the factors affecting urease enzyme production. It underscores the importance of pH and temperature, as well as their quadratic and interaction effects, in optimizing enzyme production. This analysis suggests that a more refined approach could further enhance the optimization process, leading to more efficient production of urease enzymes.

3.4 Residual Plot or Urease Enzyme Production

The residual plots for urease enzyme production are used to evaluate the statistical reliability prior to the optimization process. The Normal Probability Plot shows that the data points align closely along the straight line (see Fig. 5), indicating the normality of the residuals and confirming the reliability of the urease enzyme model. Only a few points deviate slightly from the line, demonstrating a high level of accuracy in the model. In the Residuals versus Fits plot, the data points are scattered randomly around the zero line, with no visible patterns. This randomness indicates that the residuals are independent and have no systematic bias. Most points fall within the limits of -1 and 1, suggesting that the prediction errors are minor.

The Histogram of residuals further supports the normality assumption by displaying a roughly symmetric distribution centered around zero. This symmetry signifies that the residuals are evenly distributed, reinforcing the accuracy and reliability of the model. Lastly, the Residuals versus Order plot demonstrates that the residuals are randomly distributed over time, showing no discernible pattern. This randomness indicates that the residuals are independent and that there is no autocorrelation.

Overall, the residual plots collectively confirm that the urease enzyme production model is statistically reliable, with normally distributed errors, no significant bias, and independence of residuals. This high accuracy level ensures the validity of the optimization process for urease enzyme production.

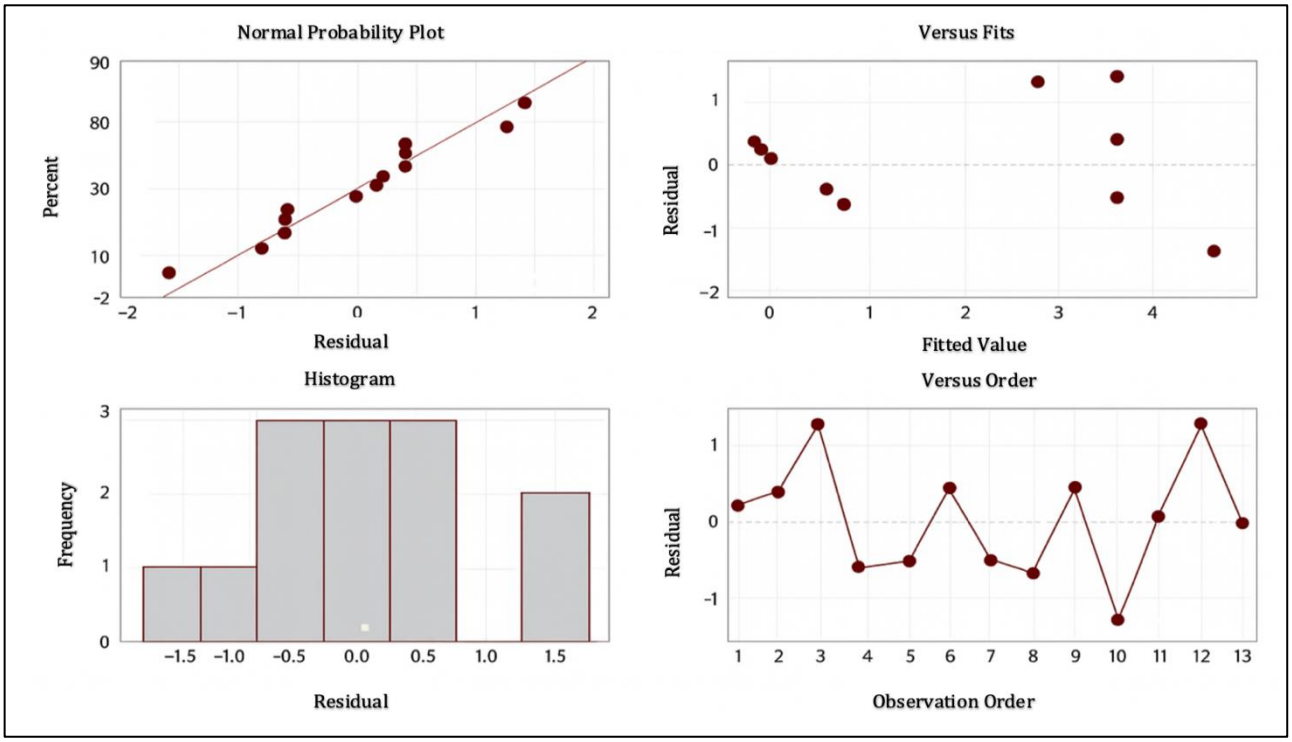

Fig. 5 *The residual plot for urease enzyme production*

3.5 Analysis of Variance (ANOVA) for Urease Enzyme Production

The analysis of variance (ANOVA) reveals significant effects of pH and temperature on the urease enzyme production (Table 4), as indicated by P-values < 0.05 for the linear terms of pH and temperature. Specifically, the P-values for pH and temperature are 0.023 and 0.014, respectively, demonstrating their significant impact. The quadratic terms pH*pH and Temperature*Temperature also exhibit significant effects with P-values of 0.024 and 0.067, respectively. Although the interaction terms pH*Temperature and Temperature*Temperature have P-

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values greater than 0.05, they still show notable effects with P-values of 0.216 each. This suggests a less pronounced, yet potentially important, interaction effect. The model explains 81.83% of the variance in the training data (R^2 = 0.8183), which indicates a good fit since it exceeds 80%.

Table 5 illustrates the impact of coded factors on urease enzyme production, with a focus on pH and temperature. The findings offer valuable insights into how these variables influence urease enzyme production. The coefficient for pH (-1.149) indicates a negative relationship with urease enzyme production, suggesting that as pH decreases (becomes more acidic), the production of urease enzyme decreases. This negative effect is statistically significant (T-Value = -2.90, P-Value = 0.023), highlighting the enzyme's sensitivity to acidic environments. Moreover, the quadratic effect of pH (pH*pH) is also significant (Coefficient = -1.249, P-Value = 0.024), indicating a nonlinear relationship. This suggests that urease enzyme production initially increases with pH up to an optimal point, after which it declines.

Similarly, the coefficient for temperature (1.259) suggests a positive effect on urease enzyme production, indicating that as temperature increases, the production of urease enzyme tends to increase as well. This effect is statistically significant (T-Value = 3.23, P-Value = 0.014). However, the quadratic effect of temperature (Temperature*Temperature) is not statistically significant (Coefficient = -0.902, P-Value = 0.067), indicating no significant nonlinear relationship within the studied temperature range. The interaction effect between pH and temperature (pH*Temperature) shows a non-significant coefficient (-0.750) and P-value (0.216), suggesting that the combined influence of pH and temperature does not significantly impact urease enzyme production in this study.

Understanding these coded factors is critical for optimizing conditions to enhance urease enzyme production. Controlling pH levels and temperature can potentially improve enzyme production efficiency in industrial and research settings. This analysis provides a comprehensive overview of how pH and temperature affect urease enzyme production, highlighting significant findings and areas for further exploration in enzyme production processes.

Table 4 *ANOVA for urease enzyme production*

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	5	38.271	7.6543	6.31	0.016
Linear	2	22.914	11.4570	9.44	0.010
pH	1	10.236	10.2358	8.43	0.023
Temperature	1	12.678	12.6783	10.44	0.014
Square	2	14.118	7.0590	5.81	0.033
pH*pH	1	9.986	9.9863	8.23	0.024
Temperature*Temperature	1	5.686	5.6864	4.68	0.067
2-Way Interaction	1	2.250	2.2500	1.85	0.216
pH*Temperature	1	2.250	2.2500	1.85	0.216
Error	7	8.498	1.2140		
Lack-of-Fit	3	7.298	2.4327	8.11	0.036
Pure Error	4	1.200	0.3000		
Total	12	46.769			
Standard deviation	=	1.10181			
R^2	=	81.83%			
R^2 adjusted	=	68.85%			
Predicted R^2	=	0.00%			

Table 5 *Ranking of factors according to ANOVA*

Term	Coef	SE Coef	T-Value	P-Value	VIF	Ranking
Constant	3.603	0.492	7.32	0.000		
pH	-1.149	0.396	-2.90	0.023	1.00	3
Temperature	1.259	0.390	3.23	0.014	1.00	1
pH*pH	-1.249	0.436	-2.87	0.024	1.02	2
Temperature*Temperature	-0.902	0.417	-2.16	0.067	1.01	4
pH*Temperature	-0.750	0.551	-1.36	0.216	1.00	5

4. Conclusion

This study elucidates the significant impact of pH and temperature on the growth density and urease enzyme production of *B. tequilensis*. Optimal bacterial growth and urease activity were achieved under slightly alkaline conditions (pH 10) and high temperature (42.7°C). These findings suggest that a slightly alkaline pH, combined with high temperatures, significantly enhances both bacterial proliferation and enzyme activity. High alkalinity at moderate temperatures and acidic conditions, combined with elevated temperatures, were found to inhibit bacterial growth and urease production, highlighting the sensitivity of these processes to environmental conditions. The RSM analysis confirmed the critical importance of temperature and pH, with ANOVA results supporting their significant effects. These insights are valuable for optimizing microbial processes in industrial and environmental applications, emphasizing the need for precise control of pH and temperature to maximize efficiency.

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Conflicts of Interest

The authors declare that they have no conflict of interest regarding the publication of this paper.

Author Contribution

The authors confirm their contributions to the paper as follows: ***Writing-original draft, formal analysis, validation, and investigation:*** *Abdullah Faisal Alshalif;* ***Investigation, writing-review & editing, conceptualization, validation, visualization, and supervision:*** *Qais Ali Al-Maqtari;* ***conceptualization, data curation, and review editing:*** *Norzila Othman;* ***Writing-review and editing:*** *Mohammed A. Mansour.*

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