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Optimization of phenylalanine production by *Bacillus subtilis* isolated from dark soil using response surface methodology

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Abstract

One of the most appealing technologies for the industrial manufacture of amino acids like phenylalanine is microbial fermentation. Phenylalanine has a wide range of industrial uses and is a crucial raw material in the production of aspartame, a low-calorie sweetener that is thought to be at least 150 times sweeter than sucrose and has a sizable global market. Thus, the goal of this study was to identify microorganisms that can produce phenylalanine and to set up the conditions for maximal output. The bacteria were isolated from the soil, identified biochemically and molecularly, and subsequently tested for the ability to produce phenylalanine. Phenylalanine production was initially optimized using a combination of the One-Factor-at-a-Time (OFAT) approach later Response Surface Methodology (RSM) with a Central Composite Design (CCD)." a higher output of 2.89±0.01 mg/mL of phenylalanine was observed by OFAT. At pH 6.5, temperature 40 ^{o}C, agitation rate 125 rpm, and incubation duration 58 hours, RSM produced the highest yield of phenylalanine (3.63 mg/mL), with a significant quadratic model at a p-value less than 0.05 and a several significant effects were identified, including linear and quadratic effects of pH, temperature, and incubation time, along with pH-temperature and temperature-agitation rate interactions. The functional groups (amino group, carboxylic group, and aromatic group) in the manufactured phenylalanine were identical to those in the phenylalanine that was purchased from

a store, according to Fourier Transform Infrared Spectroscopy study. Thin layer chromatography (TLC) analysis of the generated phenylalanine revealed a Retardation factor value (0.6) identical to that of phenylalanine obtained from commercial sources. Using high performance liquid chromatography (HPLC), it was possible to detect bacterially synthesized phenylalanine and measure its concentration (2.8 ± 0.05 mg/mL). According to the study, the isolated bacteria may be able to produce phenylalanine in large quantities.

Introduction

Phenylalanine L. is an essential aromatic amino acid of considerable industrial importance due to its extensive application in the food, pharmaceutical, and nutraceutical industries, particularly as a precursor for the high-intensity sweetener aspartame and for several bioactive compounds (Wendisch et al., 2016). Industrial fermentation process have been developed for large scale production of amino acids. On a commercial scale, fermentation is generally conducted using aerated agitated tank fermenters or airlift tank fermenters in the 50 to 500kl size range. With increases in the demand for amino acids and the needs for cost reduction to remain competitive, there have been a gradual increase in the size of fermenters and this trend will continue. The parameters affecting production yields in the fermentor cultivation include pressure, agitation rate, temperature, pH, feeding rate of sugar and aeration (Khadiga, 2016). Among these, the first four parameters must be given special attention because their optimal conditions could vary considerably depending on the type of the fermentor and the scale of the operation.

Fermentation processes have become a common practice for overproduction of amino acids nowadays as it is cheaper and easier than other processes for commercial production of essential amino acids (Khadiga, 2016). Primary metabolites include amino acids, nucleotides and fermentation end products such as ethanol and organic acids, are considered essential for proper growth of microorganisms (Rajendra et al., 2017). Microbial synthesis is becoming the dominant and optimal process for amino acid production because of its ease to produce enantiomerically pure amino acids at low cost and ecological acceptability (Rajendra et al., 2017).

The production methods of amino acids include extraction from protein hydrolysates, chemical synthesis and microbial fermentations (D'Este et al., 2018). Due to the mild conditions, high yield, as well as economic and environmental advantages, microbial fermentation has become one of the most effective process for the commercial production of phenylalanine recently (D'Este et al., 2018). The fed-batch culture mode is usually adopted in the production of phenylalanine by microbial fermentation.

Although the fermentation process is advanced and widely used, it still has some drawbacks such as requiring highly trained experienced operators due to the uncertain timing of feed addition during the fermentation (Simorgh et al., 2019). Currently, the control points for the production are only empirically determined or based on static parameters of several tests. It is difficult to simultaneously meet the needs of microbial growth and product synthesis, resulting in shortening of the high productivity period of amino acid synthesis and reducing the total output (Yufu et al., 2021). Fungi produce diversified extents of metabolites ranging from antibiotics to mycotoxins. The biosynthetic pathways involve in the synthesis of these molecules are also diverse (Shaily et al., 2016). However it is surprising that these complex structures are synthesized by relatively few building blocks (Shaily et al., 2016).

Bacterial systems are increasingly preferred over fungal platforms for amino acid production due to their faster growth rates, shorter fermentation

cycles, simpler process control, and reduced downstream processing challenges. Unlike filamentous fungi, bacterial cultures do not form complex mycelial structures, thereby minimizing broth viscosity issues and improving oxygen and nutrient transfer during submerged fermentation (Becker, & Wittmann, 2018).. Among bacterial candidates, *Bacillus subtilis* has attracted considerable attention owing to its non-pathogenic nature, metabolic stability, and extensive use in industrial biotechnology (Chen et al., 2015). The organism is classified as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration, making it suitable for applications related to food, pharmaceutical, and nutraceutical production. In addition, *Bacillus subtilis* possesses a well-characterized shikimate pathway that supports efficient biosynthesis of aromatic amino acids, including phenylalanine, and demonstrates high tolerance to variations in environmental and nutritional conditions (Schallmey et al., 2004). These attributes make *Bacillus subtilis* a reliable microbial host for phenylalanine production and an appropriate system for systematic process optimization using Response Surface Methodology.

Previous studies have demonstrated the benefit of natural substances derived from microbes in food, agriculture, and healthcare. Amino acids, enzymes, vitamins, organic acids, and alcohol are examples of primary metabolites that are employed both as nutritional supplements and in the creation of industrial goods through biotransformation (Rajendra et al., 2017; Gopinath et al., 2014), For instance, amino acids come from *Corynebacterium*, *Brevibacterium*, and *Escherichia coli*; vitamins come from *Propionibacterium* and *Pseudomonas*; organic acids come from *Aspergillus*, *Lactobacillus Rhizopus*; and enzymes come from *Aspergillus*, *Bacillus* (Rajendra et al., 2017; Yufu et al., 2021; Hirasawa & Shimizu 2016).

Conventionally, phenylalanine has been produced through chemical synthesis, enzymatic conversion, and microbial fermentation. Chemical synthesis methods are often associated with high energy requirements, racemic product formation, and environmental concerns, which limit their sustainability and economic feasibility (Becker, & Wittmann, 2018). Enzymatic production routes, although offering high specificity, suffer from high enzyme costs, limited operational stability, and challenges related to enzyme recovery and reuse. Microbial fermentation using fungi and bacteria has therefore emerged as a more sustainable alternative; however, fungal systems are frequently constrained by slow growth rates, complex morphology, high broth viscosity, and difficulties in large-scale process control. These limitations have stimulated increasing interest in bacterial platforms, particularly Bacillus subtilis, which combines rapid growth, metabolic robustness, ease of cultivation, and Generally Recognized as Safe (GRAS) status. Consequently, *Bacillus subtilis* represents a promising and industrially relevant microbial host for phenylalanine production and systematic process optimization (Ikeda, 2003). Therefore, the current study objectives were to isolate and improve phenylalanine-producing bacteria as well as to characterize the phenylalanine that was produced using thin-layer chromatography (TLC), Fourier transform infrared spectroscopy (FTIR), and high performance liquid chromatography (HPLC).

Materials and methods

Isolation and preliminary identification were carried out using standard microbiological procedures as described by Cappuccino and Sherman (2014), with confirmatory identification based on Bergey's Manual of Systematic Bacteriology.

Materials

Chemicals and reagents used in the study were of analytical grade and were purchase from Sigma-Aldrich/Merck.

Sample collection and media preparation

Organically rich soil was collected from the top 5 – 10cm layer at multiple sites in Abattoir Kano (Latitude 12.0156N and Longitude 8.5337E). The

soil samples were placed in sterile, labeled polythene bags and transported to the Microbiology Laboratory Bayero University Kano in a cool box to minimize microbial changes. The sub-samples were dried and passed through 1.20 mm diameter sieve. These sub-samples were homogenized and mixed to make a composite sample for each site. The samples were kept at 4°C for subsequent analysis. Test tubes were used to weigh one (1) gram of soil and diluted with 9 milliliters of distilled water. Nutrient Agar was the main medium employed in the isolation of bacteria (2.8g medium was measured and diluted in 100 mL of sterile water). The medium was autoclaved for 15 minutes at 121°C. To prevent contamination, the medium was placed onto the Petri plates in a laminar flow.

Isolation of bacteria from dark soil

The soil suspension was subjected to ten-fold serial dilution by aseptically transferring 1 mL of the homogenized soil suspension in to 9 mL of sterile physiological saline, followed by through mixing. This process was repeated sequentially to obtain dilutions up to 10^{-6}. Aliquots (0. 1 mL) from the soil suspension were spread-plated onto nutrient agar and incubated at 37 °C for 24 hours aerobically to obtain well-isolated bacterial colonies suitable for purification and subsequent characterization (Jahun et al., 2022).

Screening of bacterial isolates for phenylalanine production

The medium was diluted in 100 mL of sterile water and autoclaved for 15 minutes at 121°C with various modifications. A loop of bacterial isolates from 24 hours ago were inoculated into each fermentation medium in 4 distinct conical flasks using the screening medium (50 mL), and each flask was then incubated at 37 °C for 48 hours while being shaken at 125 rpm. Using thin layer chromatography (TLC) and spectroscopy, samples were collected after 48 hours and spun at 10,000 rpm for 15 minutes to further analyze the supernatant for phenylalanine production. Fermentation Media were created with little modification and quantified to test isolates for the synthesis of phenylalanine as stated by (Aliyu et al., 2022).

Morphological and biochemical identification of bacteria

Starch hydrolysis, the catalase test, citrate utilization, Voges-Proskauer, and Gram's staining were used to biochemically and physically identify the bacterial isolate that can produce phenylalanine.

Molecular identification of the bacterial isolate

The Genomic DNA extraction and PCR amplification were carried out at the Instrumental and Molecular Biology Laboratory, Department of Biochemistry, Bayero University Kano, Nigeria. While the 16S rRNA gene sequencing was send to Molecular Genetics Laboratory, Institute of Bioscience, Universiti Putra Malaysia (UPM), Serdang, Malaysia.

DNA Extraction

The pure bacterial isolate was cultured for 24 h at 37 °C on nutrient agar prior to genomic DNA extraction. Genomic DNA was extracted using a commercial bacterial DNA extraction kit following the manufacturer's instructions, with slight modifications as described by Dankaka et al. (2021). Briefly, bacterial cells were harvested and lysed to release genomic DNA, which was subsequently purified to remove proteins and other cellular contaminants. The quality and integrity of the extracted DNA were confirmed by agarose gel electrophoresis, and the DNA was stored at −20 °C until further analysis.

Polymerase chain reaction

The 16S ribosomal RNA (16S rRNA) gene was amplified using the extracted genomic DNA as a template. PCR amplification was carried out in a total reaction volume of 50 µL containing 35.5 µL of nuclease-free water, 5.0 µL of 10× PCR buffer, 1.5 µL of $MgCl_2$, 1.0 µL each of forward and reverse primers (10 mM), 4.0 µL of deoxynucleoside triphosphates (dNTPs), and 1.0 µL of Taq DNA polymerase (5.0 U) using (Reriti™ 96-well Thermal Cycler, Applied Biosystems, Thermo Fisher Scientific USA).

The universal bacterial primers used were:

Forward primer (F): 5′-TGGAGAGTTTGATCCTGGCTCAG-3′
Reverse primer (R): 5′-TACCGCGGCTGCTGGCAC-3′
PCR amplification was performed using a thermal cycler under the following conditions: initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and a final extension step at 72 °C for 10 min (Jahun et al., 2023).

Agarose Gel Electrophoresis and Visualization

The PCR products were analyzed by electrophoresis on a 1.2 % (w/v) agarose gel prepared in Tris–acetate–EDTA (TAE) buffer. Electrophoresis was carried out at constant voltage, after which the gel was stained with ethidium bromide. The amplified DNA fragments were visualized under ultraviolet (UV) illumination and documented using a gel documentation system (Gel Doc™ XR+ System by Bio-Rad Laboratories). The presence of a clear band of the expected size confirmed successful amplification of the 16S rRNA gene (Jahun et al., 2023).

DNA Sequencing and Phylogenetic Analysis

The PCR product of the correct size was purified and sent for DNA sequencing using the forward primer. The obtained nucleotide sequence was compared with existing sequences in the GenBank database using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST). Identification of the bacterial isolate was achieved based on sequence similarity with known 16S rRNA gene sequences

The closest related sequences were used to confirm the taxonomic identity of the isolate (Usman et al., 2023; Abdelhaleem et al., 2019; Horak et al., 2019).

Quantitative and Qualitative Analysis of Phenylalanine

Phenylalanine produced during fermentation was analyzed qualitatively and quantitatively following the ninhydrin-based method described by Khan et al. (2013), with slight modifications.

Sample Preparation

Five milliliters (5 mL) of the fermented broth were centrifuged at 10,000 rpm for 15 min to separate the cell debris. The resulting supernatant, containing soluble amino acids, was carefully collected and used for subsequent analyses.

Preparation of Ninhydrin Reagent

The ninhydrin reagent was prepared by dissolving 0.2 g of ninhydrin powder in 20 mL of acetone, as described by Farah et al. (2012). The reagent was freshly prepared and protected from light prior to use.

Quantitative Determination of Phenylalanine

For quantitative analysis, 2 mL of the clarified supernatant was transferred into a clean test tube, followed by the addition of 0.1 mL of the ninhydrin reagent. The mixture was incubated in a water bath at 50 °C for 10 min to allow color development. After cooling, the optical density of the developed color was measured at 610 nm using a UV–visible spectrophotometer.

A standard calibration curve was constructed using known concentrations of standard phenylalanine (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL) prepared under the same conditions. The concentration of phenylalanine in the samples was determined by extrapolation from the standard curve, as reported by Kawakami et al. (2017).

Determination of the suitable experimental conditions and ranges for phenylalanine production using the one-factor-at-a-time (OFAT) approach

Four factors were taken into consideration for the best growth of the bacterial isolates for the production of phenylalanine. These factors include pH, temperature, agitation speed, and incubation period.

Determination of the pH range

Sodium citrate ($Na_3C_6H_5O_7$), disodium hydrogen phosphate (Na_2HPO_4), and hydrochloric acid (HCl) were used to adjust the pH of the culture medium. Six flasks, each containing 50 mL of Luria–Bertani (LB) broth, were prepared in triplicate to determine the optimum pH. The pH values were adjusted to 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 prior to autoclaving. After sterilization, the flasks were inoculated with the bacterial isolate and incubated at 35 °C for 48 hours with shaking at 130 rpm. Bacterial growth was assessed by measuring absorbance at 610 nm (Jyumpei, 2019).

Determination of the temperature range

Six duplicate flask containing 50ml of luria broth (LB) broth each were added, autoclaved, and inoculated with the bacterial isolate to determine the ideal temperature. Temperatures were altered between 25, 30, 35, 40, 45, and 50°C while the pH was adjusted to 6.5. For 48 hours, the flasks were shaken at 130 rpm on an incubator shaker. The absorbance was measured at 610 nm (Ahmad et al., 2023; Imran and Wei 2019; Jens and Jens 2017).

Determination of the agitation rate range

Five bottles of 50ml LB broth were made in triplicate at sustain incubation times, pH, and temperature (6.5, 40°C, and 48 hours, appropriately), in order to determine the best agitation rate. 120, 125, 130, 135 and 140 rpm were used to alter the agitation rate. The absorbance measurement was made at 610nm (Bai et al., 2012; Gopinath et al., 2014).

Determination of the incubation time range

Five bottles containing 50mL of LB broth were incubated for 24, 48, 72, 96, and 120 hours at persistent agitation rate, pH, and temperature (6.5, 40°C, and 125rpm, appropriately) to determine the ideal incubation time. At 610 nm, absorbance was measured (Fatima et al., 2023; Zhou et al., 2010).

Phenylalanine yield optimization using response surface method (RSM)

Utilizing Central Composite Design (CCD), the characterization was performed to examine the impact of temperature, pH, agitation rate, and cultivation period on phenylalanine production at both lower and higher ranks depicted in Table 1 In fact, the range of each factor was determined based on the OFAT approach. The dependent variable was determined to be the phenylalanine yield, with the optimal circumstances acting as the independent factors. The program Design-Expert version 6.0.6 produced thirty (30) trial runs as a result depicted in Table 2. The program was used to carry out modeling and data analysis.

Table 1: Experimental Design Using RSM.

Measure	Element	Low rank	High rank
A	pH	6.0	7.0
B	Temperature	35	45
C	Agitation rate	120	130
D	Incubation time	24	72

The second order polynomials' validity

A random selection of trials from the design in Table 2 was used to validate the second order polynomials model that was derived using RSM. The validation of the model is shown in Table 3

Infrared Fourier Transform Spectroscopy (FTIR)

The sorts of chemical bonds (functional groups) in certain components of an unknown combination were clarified using Fourier transform infrared spectroscopy (FTIR) research. FTIR analysis of fermentation broth was conducted using a device having a 450-4000 cm^{-1} spectrum and a 4 cm^{-1} resolution.

High-Performance Liquid Chromatography (HPLC) analysis

HPLC analysis was carried out using an HPLC system equipped with a UV–Visible detector. Separation was achieved on a reverse-phase C18 column under isocratic conditions. The mobile

phase consisted of a suitable solvent system at a constant flow rate of 1.0 mL/min. The injection volume was 20 µL, and detection was performed at an appropriate wavelength. The concentration of the metabolite was determined by comparing the peak area of the sample with that of a corresponding standard.

Table 2: Phenylalanine production experimental design using RSM in standard order

Run	pH	Temp. (ºC)	Agitation rate (RPM)	Incubation time (Hrs)
1	6.0	35	130	24
2	6.5	40	125	48
3	7.0	35	130	72
4	7.0	35	120	24
5	6.0	45	130	24
6	6.5	50	125	48
7	6.0	45	130	72
8	7.0	45	130	24
9	7.0	45	120	72
10	6.5	40	125	24
11	6.5	40	125	48
12	6.5	40	125	48
13	6.5	40	125	48
14	6.5	40	125	48
15	6.5	40	135	48
16	6.5	40	125	96
17	6.0	35	120	72
18	7.0	35	130	24
19	7.0	35	120	72
20	6.5	40	115	48
21	7.5	40	125	48
22	7.0	45	120	24
23	7.0	45	130	72
24	6.5	40	125	48
25	6.0	45	120	24
26	6.5	30	125	48
27	6.0	35	120	24
28	6.0	35	130	72
29	6.0	45	120	72
30	5.5	40	125	48

Data analysis

Using Excel (Office 2019), the mean values and standard deviations for each run were extracted from the triple trials. Using the software Design-Expert version 6.0.6, 5% analysis of variance (ANOVA) was used to calculate the standard deviation for each value. In this investigation, a confidence interval of 95% was employed. Any p-value less than 0.05 were regarded as significant, and the opposite was also true.

Table 3: Validation of the Phenylalanine Second Order Polynomial Model

Run	pH	Temp (ºC)	Agitation rate (RPM)	Incubation time (Hrs)
1	6.5	40	130	58.4
2	6.3	40	125	58.4
3	6.5	40	125	58.4

Results

Morphological and biochemical identification of the bacterial isolate

The morphological and biochemical characteristics of the bacterial isolate coded as E1 are presented in Table 4. Microscopic examination following Gram staining revealed that the isolate was Gram-positive and rod-shaped, occurring singly and in chains. The isolate produced opaque, irregular colonies with rough edges, which are characteristic of *Bacillus* species. The isolate tested positive for catalase activity, starch hydrolysis, citrate utilization, and the Voges–Proskauer test, indicating its ability to produce catalase, hydrolyze starch, utilize citrate as a sole carbon source, and produce acetoin, respectively. These results are consistent with the standard biochemical profile of *Bacillus subtilis*. Based on the combined morphological and biochemical characteristics, the isolate was identified as *Bacillus subtilis*.

Molecular identification of the isolated bacteria

Figures 1 and 2 displays the outcomes of isolated bacteria molecular identification. Figure 1 displays the gel band of the isolate, which indicate 1500 base pairs on gel ladder. The 16S rRNA gene sequence confirmed that the isolate is a *Bacillus subtilis* with 98.73% similarity to *Bacillus subtilis* strain DSM 10. Figure 2 displays the findings for the isolated bacteria evolutionary

connection. Isolate E1 was identified as *Bacillus subtilis* with the highest percentage similarity to strain DSM 10 by sequence alignment and Basic Local Alignment Search Tool (BLAST). The evolutionary relationship were determine using neighborhood joining technique (Jahun et al., 2023, Gupta et al., 2016), and they are expressed in terms of the number of base substitutions per site. 25 closely related species' nucleotide sequences were examined in the study. MEGA 7 was used to conduct evolutionary analysis.

Table 4. Morphological and Biochemical characteristics of the bacterial isolate

Test	Results
Gram staining	Gram-positive rods
Colony morphology	Rough, opaque, irregular shape
Catalase test	+
Starch hydrolysis	+
Citrate utilization	+
Voges-Proskauer test	+

Key: Positive (+)

Optimization of phenylalanine production using the OFAT methodology

Determination of the pH range pH Optimization

At pH 6.5, phenylalanine production (1.810 ± 0.025 mg/mL) was at its highest (Figure 3). When the pH was raised from 5.5 to 6.5, phenylalanine production increased, and when the pH was raised to 7.0 or 8.0, production decreased. All other variables remained unchanged.

Determination of the Temperature Range

Temperatures between 25°C and 40°C were shown to maximize phenylalanine synthesis. The yield, however, decreased when the temperature was increased to 45 °C, as shown in Figure 4. 40°C produced the highest phenylalanine output (2.303 ± 0.03 mg/mL).

Determination of the agitation rate

With increased agitation rate from 120 to 125 rpm, maximum phenylalanine production (2.787 ± 0.002 mg/mL) was achieved (Figure 5). However, when the agitation rate was raised to 140 rpm, productivity fell.

Determination of the incubation time range

Figure 6 displayed the outcome of the optimization of the incubation period. From 24 hours to 48 hours, there was an increase in yield (2.899 ± 0.05 mg/mL), which then decreased from 72 hours to 120 hours.

Methodology for the Response Surface

Tables 5 exhibit the findings of an optimization research that utilized response surface methodology (RSM) and demonstrated experimental and fitted models of phenylalanine production by Bacillus subtilis strain E1. According to various combinations of pH, temperature, agitation rate, and incubation period, the findings for the actual and expected phenylalanine production are shown in Table 3 for your review. Every experiment's results and projected results have a strong correlation. It is evident that increasing the pH, temperature, agitation rate, and incubation period resulted in a drop in phenylalanine production. 48 hours of incubation at 40°C, pH 6.5, and 125 rpm resulted in the highest phenylalanine production (3.47 mg/mL) (Run 12, Table 3) exactly at the center of the tested experimental range.

Table 6 Shows the quadratic model analysis of phenylalanine yield based on the results attained ($p = 0.001$). The model was substantial and quite trustworthy. A "model F- value" that is mostly due to noise has a 0.01% probability of happening. With a p-value of 0.2354, it was determined that the model's lack of fit was not statistically significant, indicating that there was no substantial noise. Model terms are considered important when the value of "prob > F" is less than 0.05. A, B, D, A^2, B^2, D^2, AB, and BC are important model terms in this instance. If the value is higher than 0.05, the model terms are not considered relevant. The experimental and projected values showed a strong connection, as demonstrated by R^2 values of 0.9781 and 0.9576 and adjusted R^2 values of 0.9576

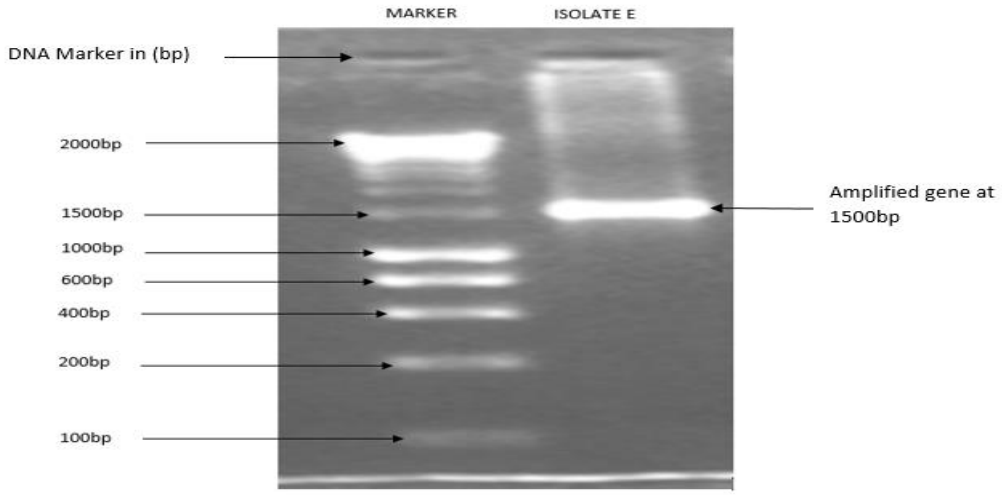

Figure 1: Gel electrophoresis of the 16S rRNA from isolated bacteria E1, displaying the amplified gene at 1500 bp and the marker.

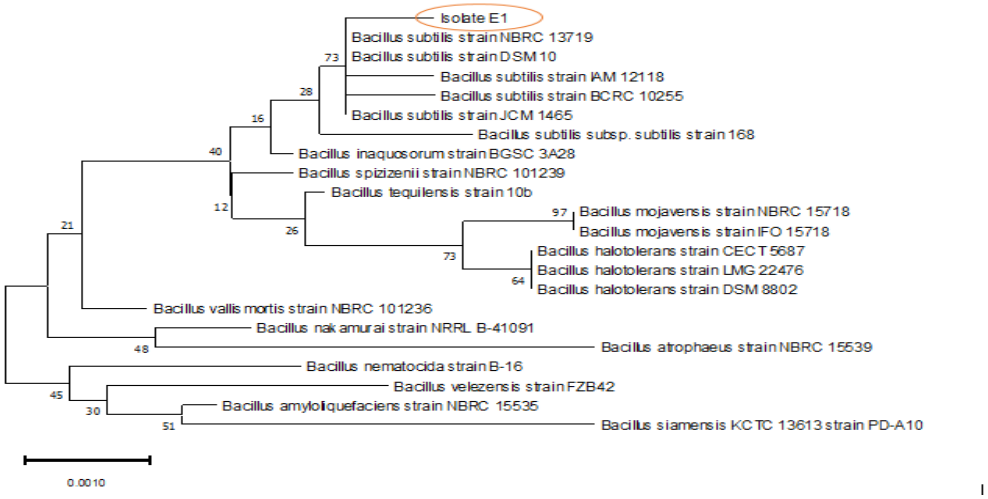

Figure 2: Cladogram indicating the genetic relationship between Bacillus subtilis and referenced related microorganisms based on 16S RNA gene sequence analysis.

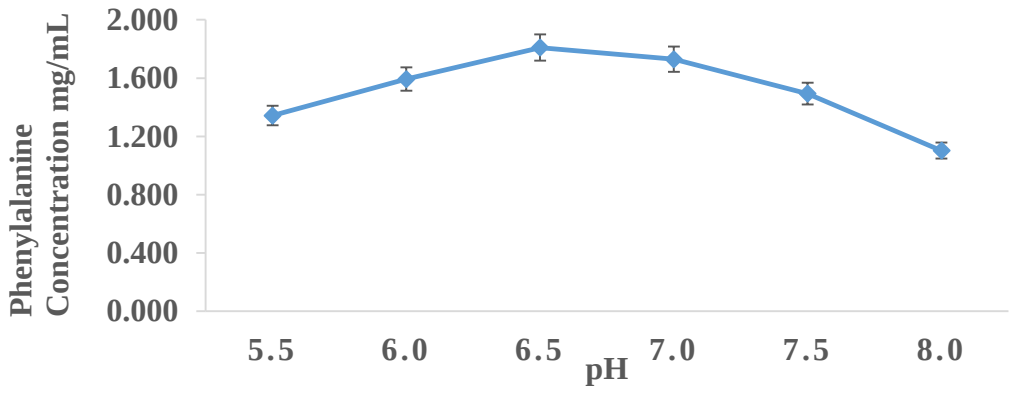

Figure 3: Impact of pH on phenylalanine synthesis at constant inoculum (6%), temperature (30°C), agitation rate (130 rpm), and incubation duration (48 hours) values.

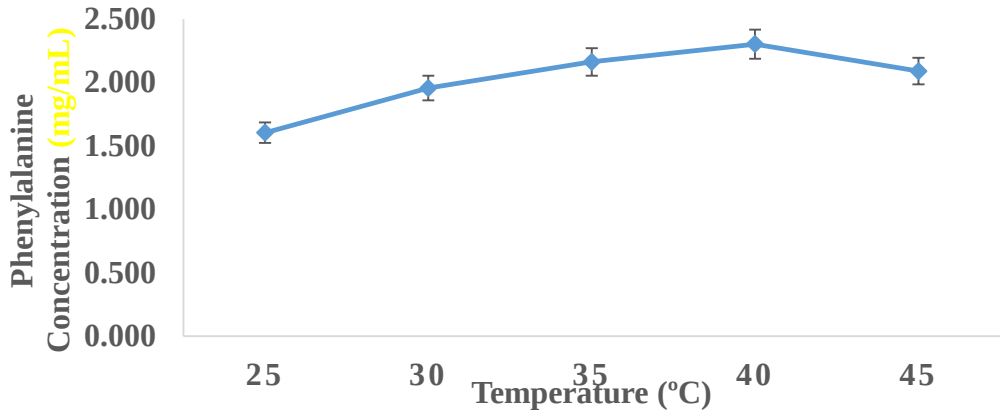

Figure 4: Impact of temperature on phenylalanine synthesis at constant inoculum size (6%), pH (6.5), agitation rate (130 rpm), and incubation duration (48 hours).

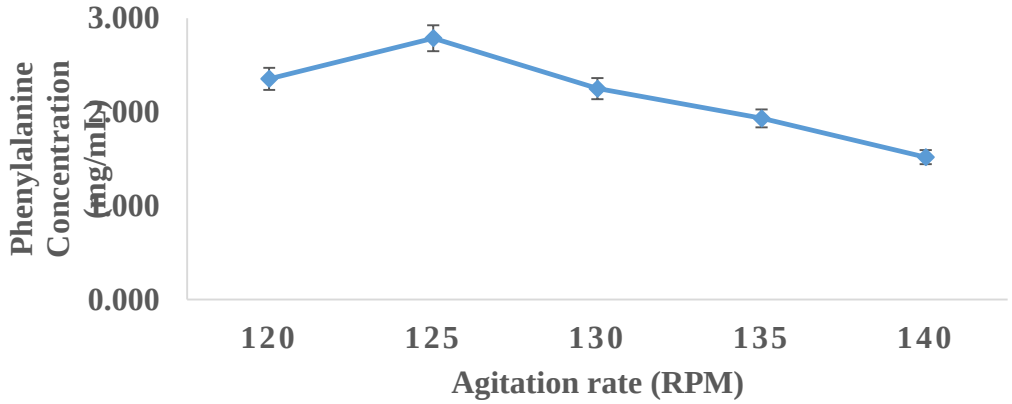

Figure 5: Impact of agitation rate on the generation of phenylalanine under conditions of constant inoculum size (6%), pH (6.5), temperature (40°C), and incubation duration (48 hours).

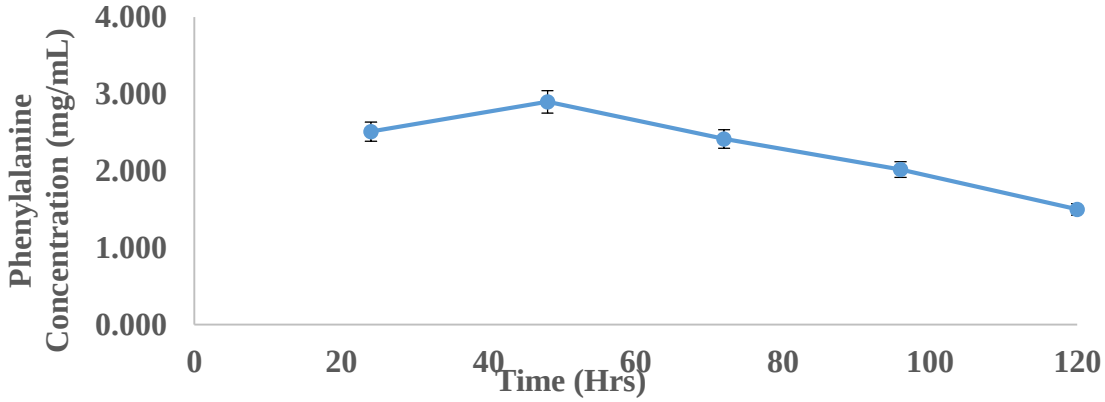

Figure 6. Effect of incubation period on the generation of phenylalanine at constant inoculum size (6%), pH (6.5), temperature (40°C), and agitation rate (125 rpm).

Table 5: Yield of phenylalanine under various pH, temperature, agitation rate, and incubation duration conditions

Run	pH	Temp (ºC)	Agitation rate (RPM)	Incubation time (Hrs)	Experimental value (mg/ml)	Predicted value (mg/ml)
1	6.0	35	130	24	2.16	2.02
2	6.5	40	125	48	3.45	3.39
3	7.0	35	130	72	2.99	2.92
4	7.0	35	120	24	1.99	1.92
5	6.0	45	130	24	2.27	2.11
6	6.5	50	125	48	2.09	2.27
7	6.0	45	130	72	3.09	3.03
8	7.0	45	130	24	1.55	1.44
9	7.0	45	120	72	2.21	2.22
10	6.5	40	125	24	1.63	1.73
11	6.5	40	125	48	3.45	3.39
12	6.5	40	125	48	3.47	3.39
13	6.5	40	125	48	3.39	3.39
14	6.5	40	125	48	3.15	3.39
15	6.5	40	135	48	3.11	3.35
16	6.5	40	125	96	1.85	1.88
17	6.0	35	120	72	2.63	2.62
18	7.0	35	130	24	2.11	2.04
19	7.0	35	120	72	2.63	2.65
20	6.5	40	115	48	3.06	3.09
21	7.5	40	125	48	2.01	2.11
22	7.0	45	120	24	1.79	1.70
23	7.0	45	130	72	2.20	2.11
24	6.5	40	125	48	3.45	3.39
25	6.0	45	120	24	2.19	2.13
26	6.5	30	125	48	2.53	2.61
27	6.0	35	120	24	1.7	1.65
28	6.0	35	130	72	3.19	3.14
29	6.0	45	120	72	2.95	2.88
30	5.5	40	125	48	2.59	2.75

Equation (1) presents the identified model for calculating phenylalanine yield as a function of the four tested factors, represented by the letters A, B, C, and D at their coded levels.

$$\text{Phenylalanine quantity} = +3.39 - 0.16*A - 0.085*B + 0.065*C + 0.41*D - 0.24*A^2 - 0.24B^2 - 0.044*C^2 - 0.58*D^2 - 0.17*A*B - 0.063*A*C - 0.059*A*D - 0.096*B*C - 0.052*B*D + 0.039*C*D \quad \ldots\ldots (1)$$

A-D represent the coded values of A = pH, B = Temperature (ºC), C = Agitation rate (RPM), and D = Inoculation period (h). The quadratic terms are A^2, B^2, C^2, and D^2, and the interaction terms are AB, AC, AD, BC, BD, and CD."

Table 6: Analysis of variance using a quadratic model for the yield of phenylalanine

Source	Sum of Square	Mean Square	F- Value	P > F	
Model	16.470	1.180	47.60	< 0.0001	Significant
A	0.620	0.620	25.36	0.0001	
B	0.170	0.170	6.980	0.0185	
C	0.100	0.100	4.170	0.0590	
D	4.030	4.030	163.65	< 0.0001	
A^2	1.580	1.580	64.38	< 0.0001	
B^2	1.550	1.550	63.05	< 0.0001	
C^2	0.053	0.053	2.160	0.1619	
D^2	9.360	9.360	380.31	< 0.0001	
AB	0.490	0.490	19.77	0.0005	
AC	0.064	0.064	2.590	0.1283	
AD	0.056	0.056	2.290	0.1508	
BC	0.150	0.150	5.950	0.0277	
BD	0.043	0.043	1.750	0.2057	
CD	0.025	0.025	1.010	0.3312	
Lack of Fit	0.29	0.029	1.970	0.2354	not significant
R-Squared	0.9781				
Adjusted R^2	0.9576				
Predicted R^2	0.8929				

A coded level of A = pH, B = Temperature (ºC), C = Agitation rate (RPM), and D = Incubation time (h).

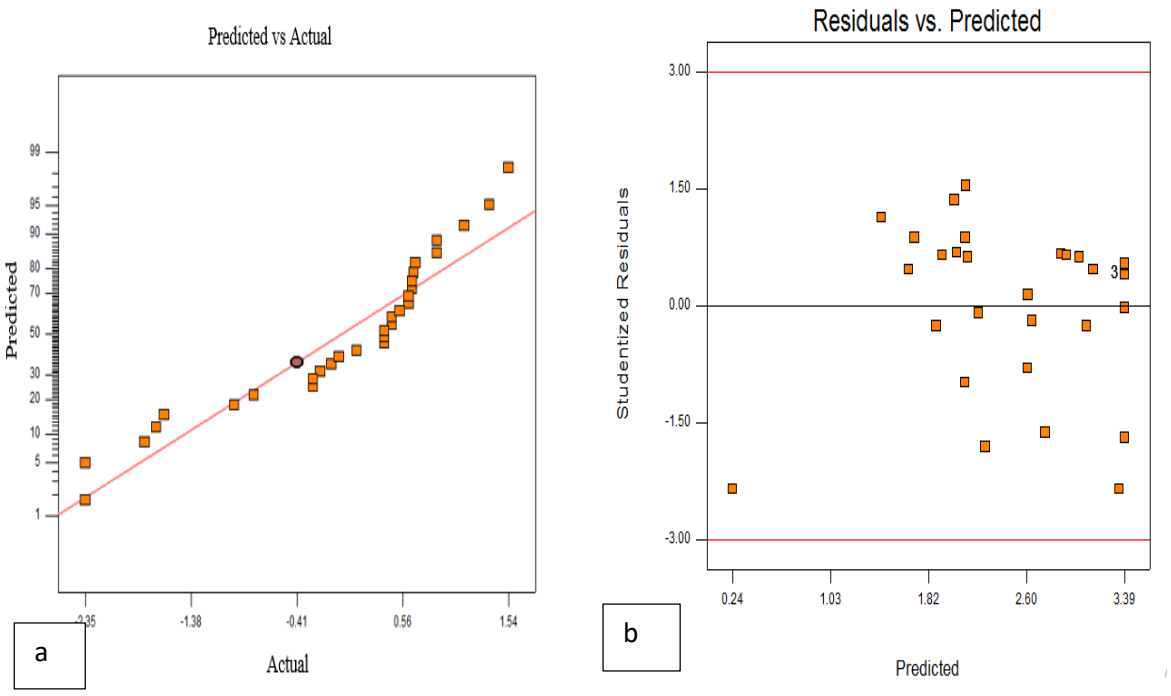

Figure 7: (a) parity graph comparing the distribution of the phenylalanine yield's actual values to predictions (b) Parity graph displaying the distribution of residuals in comparison to projected phenylalanine yield values.

The interaction between variables in 3D response surface plots

Figure 8a displays the results for the 3D and Figure 8b for the contour response surface interaction between pH and temperature. The contour plots' circular form and the 3D plot's panbola shape showed that, while maintaining constant agitation rate and incubation duration, there was a strong interaction between pH and temperature. This was already noted in Table 6, where the pH-temperature interaction showed a highly significant effect, with a p-value < 0.001.

Similarly, Figure 9a displays the outcomes for the 3D and Figure 9b for the contour response surface's interaction with temperature and agitation rate. The 3D plot's hyperbolic form and the contour plots' circular contours showed a substantial interaction between temperature and agitation rate while maintaining a constant pH and incubation duration. This interaction also showed a significant effect, with a p-value < 0.05 (Table 5).

The second order polynomial's validity

Table 7 shows the findings for the validation of the *Bacillus subtilis* strain E1-based second order polynomial of the phenylalanine production. When the observed product of the phenylalanine determined was correlated with the values expected by the second model order, it was discovered there is a strong connection between the experimental and predicted values, which supports the models' validity.

Chromatogram of the phenylalanin production using Fourier Transform Infrared (FTIR)

Results for the Fourier transform infrared (FTIR) of the produced phenylalanine using *Bacillus subtilis* and commercial phenylalanine was presented in Table 7. The spectrum of the produced phenylalanine revealed the presence of different functional groups such as carboxylic group, amino group and aromatic ring (phenyl group).

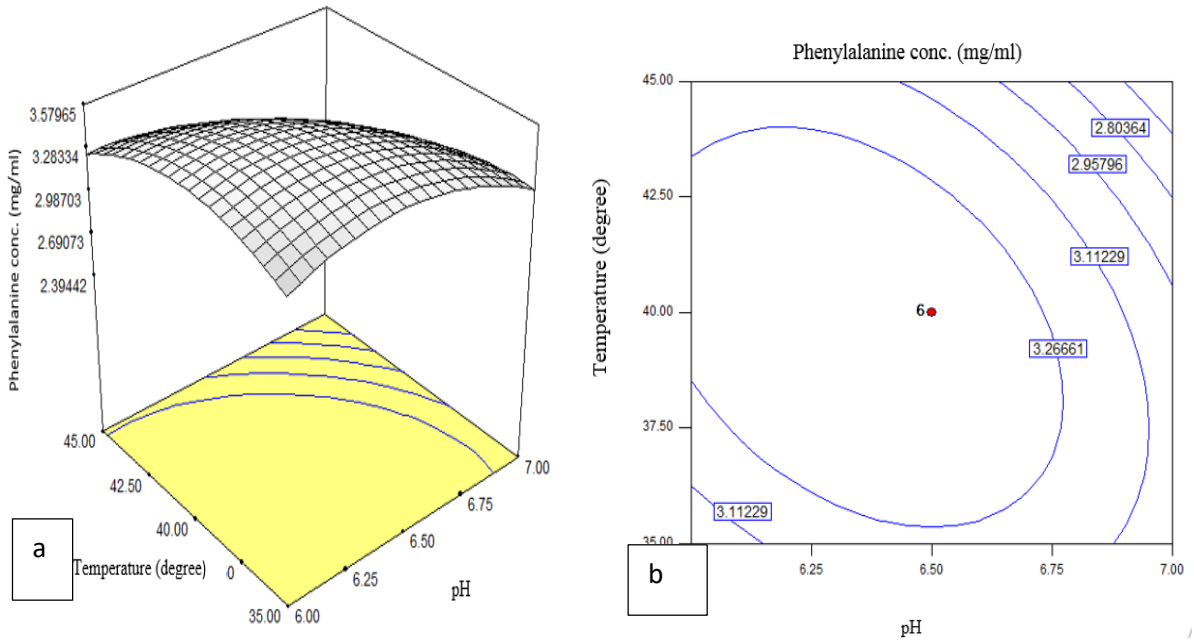

Figure 8: Response surface plots (a) 3D and (b) contour plots illustrating the influence of temperature and pH on the production of phenylalanine.

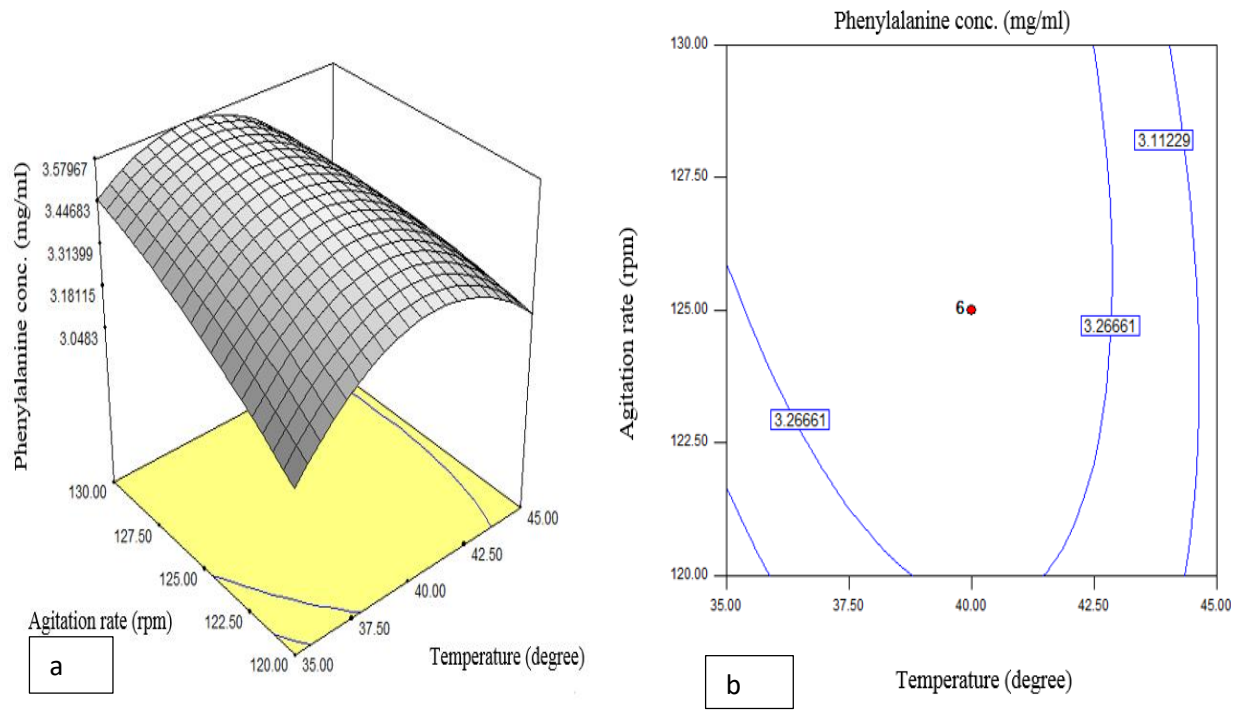

Figure 9: Plots of (a) 3D and (b) contour depicting how the combination of temperature and agitation rate affects the yield of phenylalanine.

Table 7: Validation of second order polynomial model between experimental and predicted value of phenylalanine yield

Test	pH	Temperature (ºC)	Agitation rate (RPM)	Inoculation time (Hrs)	Experimental value (mg/mL)	Predicted value (mg/mL)
1	6.5	40	130	58.4	3.35 ± 0.017	3.57 ± 0.01
2	6.3	40	125	58.4	3.48 ± 0.01	3.57 ± 0.01
3	6.5	40	125	58.4	3.63 ± 0.01	3.69 ± 0.01

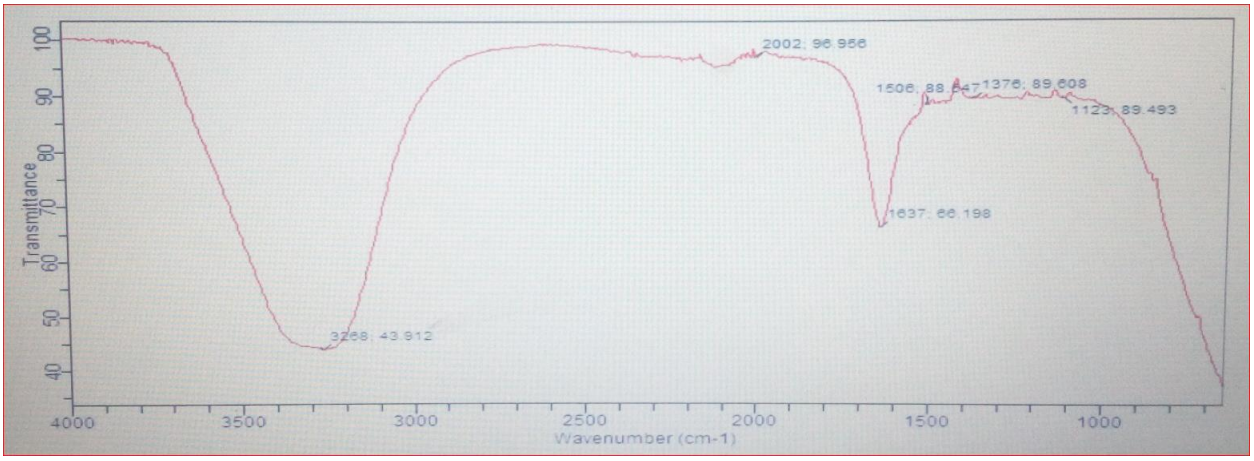

Figure 10: The Fourier transform infrared (FTIR) with different functional group for the produced phenylalanine using bacterial isolate *Bacillus subtilis*

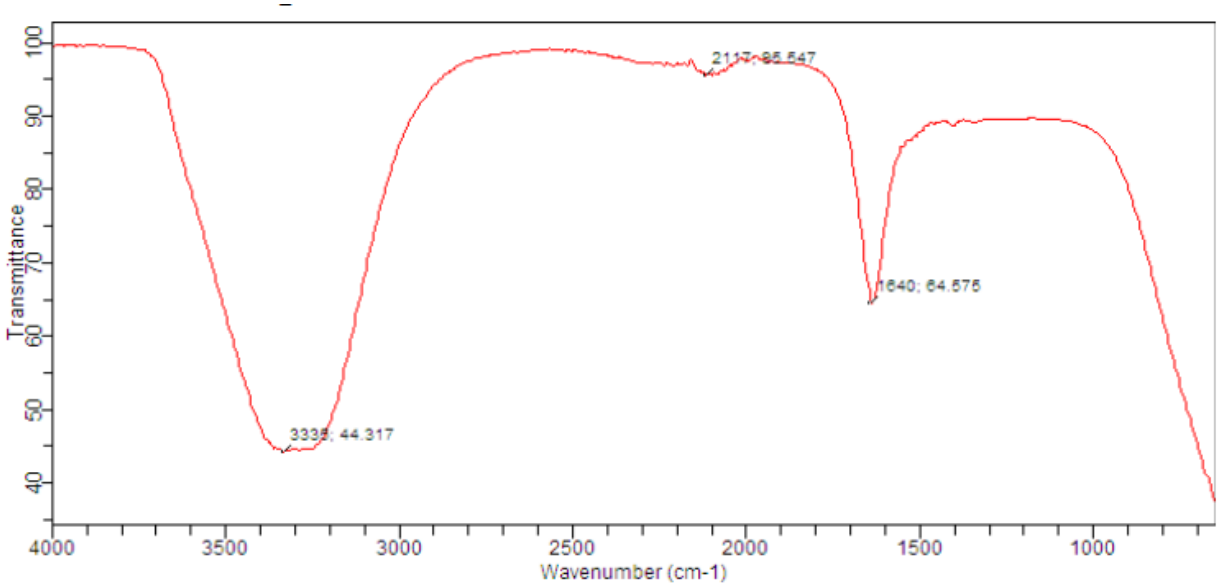

Figure 11: The Fourier transform infrared (FTIR) with different functional group for the commercial (Standard) phenylalanine

Characterization of phenylalanine using Fourier Transform Infrared (FTIR)

As a result of utilizing *Bacillus subtilis* and commercial phenylalanine, Table 8 shows the results of the Fourier transform infrared (FTIR) analysis of the generated phenylalanine. Different functional groups, including carboxylic groups, amino groups, and aromatic rings (phenyl groups), were detected in the spectrum of the synthesized phenylalanine.

Table 8: Fourier Transform infrared spectroscopy (FTIR) was used to characterize the phenylalanine generated by *Bacillus subtilis*.

Major group	Phenylalanine synthesized by *Bacillus subtilis*	Commercial phenylalanine
Amino	3268	3335
Aromatic ring	1637	1640
Carboxylic	2002	2117

Characterization of phenylalanine using Thin Layer Chromatography (TLC)

In Figure 12, the results of the thin layer chromatography used to characterize phenylalanine are shown. Using a TLC plate, a single band of normal phenylalanine had the similar Retardation factor value (0.6) as the comparable band of phenylalanine generated by *Bacillus subtilis* strain E1.

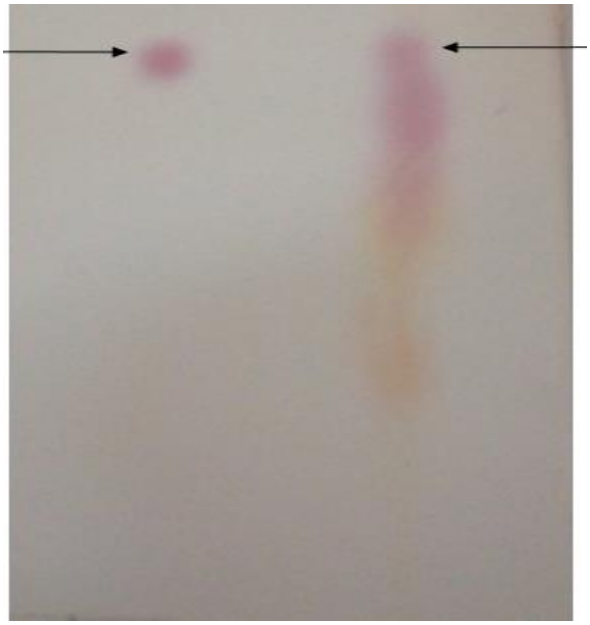

Figure 12: Bands on the TLC plate for commercially manufactured phenylalanine (A) and phenylalanine produced by isolate E1 (B) showing similar retardation factor using thin layer chromatography (TLC).

High Pressure Liquid Chromatography (HPLC)

Figure 13 shows the outcomes of phenylalanine production by *Bacillus subtilis* using high performance liquid chromatography (HPLC) it was possible to detect the isolated bacteria's production of phenylalanine and measure its concentration (2.8 ± 0.05 mg/mL).

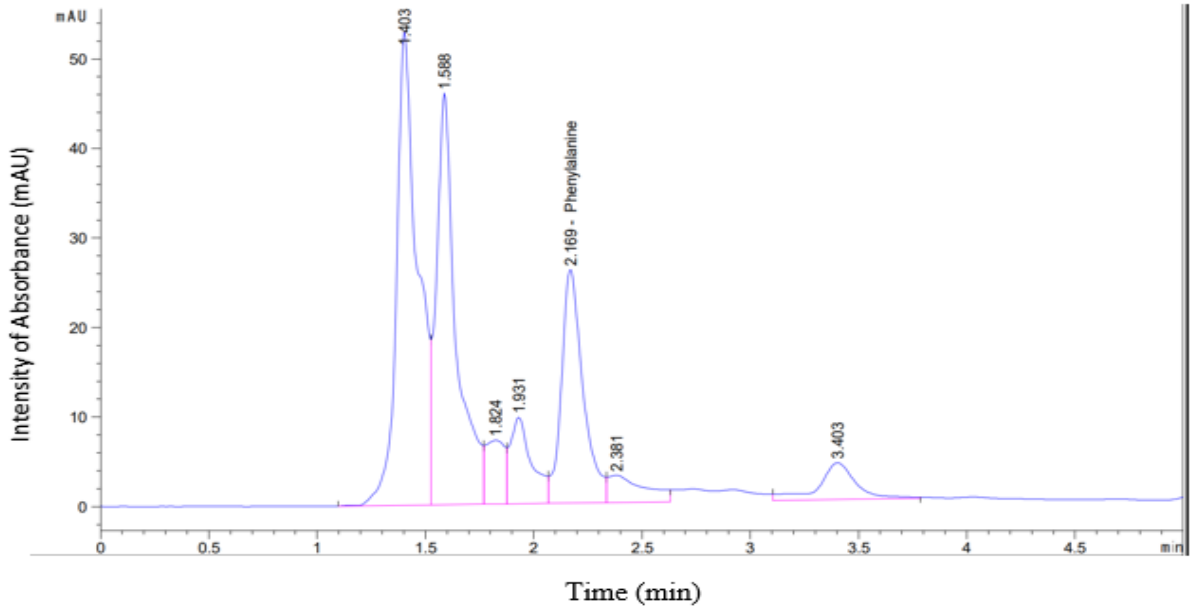

Figure 13: Analysis of phenylalanine generated by *Bacillus subtilis* with the peak value observed at the retention time of approximately 2.169 Minutes which correspond to the retention time of phenylalanine standard using high performance liquid chromatography (HPLC).

Discussion

The present study demonstrates that *Bacillus subtilis* isolated from dark soil can effectively produce phenylalanine, an essential aromatic amino acid with significant industrial applications. Morphological, biochemical, and molecular identification confirmed the isolate as *Bacillus subtilis*, consistent with prior reports highlighting its GRAS status and suitability for amino acid biosynthesis (Schallmey et al., 2004; Becker & Wittmann, 2018). The isolate's Gram-positive, rod-shaped morphology and biochemical profile (catalase positive, starch hydrolysis, citrate utilization, Voges-Proskauer positive) aligned well with standard descriptions of *B. subtilis*, confirming the reliability of both classical and molecular identification methods.

Optimization using the OFAT approach revealed that phenylalanine production was highest at pH 6.5, 40°C, an agitation rate of 125 rpm, and 48 hours of incubation. These results are consistent with findings by Ikeda (2003) and Wendisch (2016), who emphasized that moderate pH and temperature favor both microbial growth and metabolic activity in bacterial fermentation for amino acids. Excessively high or low pH, temperature, or prolonged incubation led to reduced yields, likely due to enzyme denaturation or metabolic stress. Application of Response Surface Methodology (RSM) further improved phenylalanine yield to 3.63 mg/mL under optimized conditions. The quadratic model demonstrated high significance ($p < 0.0001$) and excellent fit ($R^2 = 0.9781$), indicating the robustness of the model in predicting phenylalanine production. The significant interactions between pH and temperature, as well as temperature and agitation rate, suggest that these variables synergistically influence

metabolic pathways in *B. subtilis*, which is in agreement with recent studies on microbial amino acid production (Ahmad et al., 2023; Yufu et al., 2021). The decline in production at extreme conditions emphasizes the importance of precise control of fermentation parameters to maximize yields.

Qualitative analysis using FTIR and TLC confirmed that the chemical structure of the produced phenylalanine matched commercial standards, showing the presence of amino, carboxyl, and aromatic functional groups, with an Rf value of 0.6. Quantitative HPLC analysis validated the production level, corroborating the accuracy of the ninhydrin-based spectrophotometric method. These results collectively indicate that *B. subtilis* can be exploited for the cost-effective and eco-friendly microbial production of phenylalanine, which could serve as a sustainable alternative to chemical synthesis.

Overall, the study highlights the potential of *Bacillus subtilis* as a microbial cell factory for phenylalanine. The findings underscore the relevance of combining classical optimization approaches (OFAT) with statistical modeling (RSM) to achieve higher yields, minimize experimental runs, and identify significant interactions between process variables. This approach aligns with current trends in bioprocess engineering where precision and scalability are critical for industrial applications (D'Este et al., 2018; Becker & Wittmann, 2018).

Conclusion

This study successfully isolated and identified *Bacillus subtilis* from dark soil as an efficient phenylalanine-producing bacterium. Optimization of fermentation conditions using OFAT and RSM led to a maximum phenylalanine yield of 3.63 mg/mL at pH 6.5, 40°C, 125 rpm, and 58 hours of incubation. FTIR, TLC, and HPLC analyses confirmed that the produced phenylalanine was structurally and quantitatively comparable to commercial standards. The significant interactions among pH, temperature, and agitation rate indicate the importance of fine-tuning environmental conditions to enhance amino acid production. These findings demonstrate that *Bacillus subtilis* can serve as a reliable, eco-friendly, and cost-effective platform for industrial phenylalanine production, potentially reducing dependence on chemical synthesis. Future work should focus on scaling the process to pilot and industrial levels and exploring genetic or metabolic engineering strategies to further improve yields and process efficiency.

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Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contribution

Jahun Bashir Muhammad: Writing – review & editing, Writing Methodology, Investigation, Formal analysis. Amina Ado Gwaram: Writing – review & editing, Original draft, Visualization, Resources, Funding acquisition, Conceptualization. Fatima Yusuf: Formal analysis, Data curation, Conceptualization. Sadiq Bashir Babuga: Methodology, Data curation. Muntari Bala: Resources, Funding acquisition, Data curation, Project administration, Supervision, Writing – review & editing.

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