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Original Research Article

Optimization of Valine Production Using *Bacillus Cereus* Isolated from Soil

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Abstract

The Production of amino acids such as valine by fermentation has become an essential technology of Industrial microbiology. Valine has extensive industrial applications, which is used as intermediate for the synthesis of agricultural pesticides and semi-synthetic veterinary antibiotics. This research work was aimed to isolate *Bacillus cereus* from the soil capable of valine production and optimize the condition for maximum yield. The valine production was optimized initially by one factor at a time (OFAT) and response surface methodology (RSM). Optimum valine yield (3.53mg/ml) was obtained at pH 7.5, temperature of 40°C incubation time of 58hrs and 125rpm agitation rate, the response surface plots (3D and contour) revealed a significant interactions between pH, temperature and incubation time to valine yield. The results of the characterized valine produced using *Bacillus cereus* shows similar properties with the commercially produced valine by using Fourier transform infrared spectroscopy (FTIR) and boiling point. Based on the study, the isolated *Bacillus cereus* could have a potential for industrial production of valine under optimized conditions.

Key words: Fermentation, valine, optimization, *Bacillus cereus*.

INTRODUCTION

Since 1950s, the production of amino acids by fermentation has become an essential technology of Industrial microbiology. This has led to numerous studies to understand and improve the metabolic conditions driving to amino acid overproduction (D'Este *et al.*, 2018). Industrial fermentation processes have been developed for large scale production of amino acids. On a commercial scale, fermentation is generally conducted using aerated agitated tank fermentors or airlift tank fermentors 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 fermentors and this trend will continue. 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 valine and methionine and other essential amino acids (Khadiga, 2016).

The production methods of valine include extraction from protein hydrolyzates, chemical synthesis, and microbial fermentation (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 attractive processes for the commercial production of valine nowadays; the fed batch culture mode is usually adopted in the production of valine 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. 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). Valine is one of the nine essential amino acids that must be supplied in the diet. It is largely produced through microbial fermentation and the production capacity reached 2 thousand tons per year, with a market value of 45 million dollars. Microbial production of valine has been established using various industrial microorganisms, including *Brevibacterium flavum*, *Escherichia coli*, *Corynebacterium glutamicum*, *Bacillus subtilis* and Fungi. As a well-known workhorse

for the production of amino acids such as glutamate, lysine, isoleucine and threonine, *C. glutamicum* is also viewed as an attractive microorganism for the production of valine (Guoqiang *et al.,* 2020).

The increasing availability of synthetic amino acids continues to make their use industrially and replace a portion of the protein to meet the amino acids needs in industries (Wu, 2009). The chemical compounds used for synthetic production of amino acid are toxic and special equipment and training are needed to handle it (Ramalingam, 2010).

From an industrial application or commercial point of view, amino acid increasing demand for production of wide range of product such as animal feed additives, flavor enhancers in Human nutrition and medical products (D'Este *et al*., 2018).

Fermentative production of amino acid still needs significant improvements, leading to increased productivity and reduction of the production costs (D'Este *et al*., 2018). Although the production process of essential amino acid has been extensively investigated using few microbial isolates in previous studies, extensive researches are needed to identify different microbial isolates to improve the production (Guoqiang *et al.,* 2020). Thus, this research was aimed to optimize the production of valine using *Bacillus cereus* isolated from soil.

MATERIALS AND METHOD

Sample collection

The soil sample was collected at Hotoro Nassarawa LGA, Kano State, Nigeria and collected from a dark loamy soil and transferred safely to the laboratory. 1gm of the soil sample was diluted at 9ml distilled water using test tubes.

Isolation of *Bacillus cereus* from Soil

The soil sample was prepared by serial dilution method. 9ml of distilled water was taken to 4 different test tubes and 2ml of the soil liquid suspension was serially transferred to each 5ml distilled water containing test tubes. The primary medium used for isolation was nutrient agar (2.5g of the agar was dissolved in 100ml of distilled water). The medium was autoclaved at 121℃ and 15lb pressure for 30min. 1ml of the last two test tubes was used for the preparation of culture medium using a pour plate method. Plates were kept in an incubator at 37℃ overnight. The growth of separate colonies was observed. Pure culture was isolated using streak plate technique on the solid prepared agar surface and incubated at 37℃ for 24hours (Oyeleke and Manga, 2008).

Morphological and Biochemical Identification

Bacillus cereus was identified by Gram staining, endospore, motility test, to study morphological characteristics. Different biochemical tests including catalase test, starch hydrolysis test, Voges Proskauer (VP) test, citrate test, urease test, indole test was performed to study the physiological characteristics of *Bacillus cereus* according to Bergy's Manuel of bacteriology (Brenner *et al*., 2015, Amirreza *et al*., 2015, Holt & Krieg, 2000 and Aneja, 2002).

Molecular Identification of *Bacillus cereus*

The genomic DNA was extracted from pure bacterial culture; 24hours grown in nutrient agar medium at 37°C according to the protocol provided by QiaAmp mini Prep DNA extraction Kit (Qiagen, Hilden, Germany). Total genomic DNA was used as a template for the amplification of the 16S ribosomal RNA (16SrRNA) coding region with the universal primers 27F (5´-AGAGTTTGATCCTGGCTCAG-3´) (Abdelhaleem *et al.,* 2019).

Qualitative and Quantitative Analysis of Valine

Valine analysis was done according to Khan *et al.,* (2013). 5ml of fermented broth was centrifuged at 10,000rpm for 15min. Supernatant containing valine was separated and subjected to qualitative and quantitative identification using acid ninhydrin method (Farah *et al.,* 2012). Ninhydrin reagent was prepared by dissolving 0.2g of ninhydrin powder in 20ml Acetone. 2ml of every sample (fermented broth) was taken in test tubes and 0.1ml of acid ninhydrin reagent was added. The tubes were kept in a water bath at 50℃ for 10min and cooled at room temperature, the optical density of each sample was taken at 517nm using a Jenway 6705UV/V Spectrophotometer.

The TLC solvent system was prepared with n-propanol and distilled water (4:2 v/v). TLC plate was labelled (frontline and baseline). The fermented (screening) broth was spotted on the plate baseline using a capillary tube with standard valine solution simultaneously. The plate was then placed inside the solvent system and allowed to run to the frontline, sprayed with ninhydrin reagent and incubated at 105℃ for 5min for visualization of spots.

Determination of Optimum Growth Conditions for Valine Production

For optimum growth of *Bacillus cereus* for the production of valine, four parameters (Temperature, pH, Incubation period and Agitation rate) were considered.

One Factor at a Time (OFAT) Optimization for Valine Yield

For determination of optimum pH, pH was varied at 5.5, 6.0, 6.5, 7.0, 7.5,8.0 and inoculated with loop full freshly prepared culture of *Bacillus cereus* and incubated at 35ºC for 24h in an incubator shaker at 125rpm, their absorbance was taken at 517nm using a Jenway 6705UV/V Spectrophotometer.

For determination of optimum temperature, 10ml LB broth was prepared and inoculated with loop full of freshly prepared culture of the *Bacillus cereus* for 24hrs, pH of 7.0 at 125rpm in an incubator shaker. The temperature varies at 25, 30, 35, 40 and 45ºC.

For optimum incubation period determination, the prepared LB broth was inoculated and incubated at 24, 48, 72, 96, 120hrs at 35ºC, pH at 7.0 and agitation rate of 125rpm.

For determination of optimum agitation rate, the prepared LB broth was inoculated with *Bacillus cereus*, at constant pH, temperature and incubation period (7.0, 35ºC, 24hrs respectively), the agitation rate was adjusted at 120, 125, 130,135and 140rpm. The absorbance was taken at 517nm using a Jenway 6705UV/V Spectrophotometer.

Response Surface Methodology (RSM) Optimization for Valine Yield

The statistical base optimization was used to study the effect of pH, temperature, and incubation time and agitation rate on valine yield using Central Composite Design (CCD). The optimized conditions were taken as independent variables and the valine yield was chosen as the dependent variables (Table 1), this resulted to thirty experimental runs.

The modeling and statistical analysis were performed using Design expert software, version 6.0.6. All fermentation experiments were carried out and the multiple regression analysis of the observed responses in term of the coded factors resulted in the quadratic model below.

Valine Concentration = +3.30-0.014A+0.11B+0.083C-0.011D-0.76A^2-0.13B^2-0.070C^2-0.037D^2 +0.12 * AB-0.013 * AC+0.034 * BD+0.009841 * CD…… Equation 1.

A, B, C and D represents the independent variables (coded form) of pH, temperature, incubation time and agitation rate respectively.

Table-1: Factors for RSM experimental design

Indicator	Factor	Low level	High level
A	pH	7.0	8
B	Temperature (℃)	35	45
C	Incubation time (hrs)	48	96
D	Agitation rate (rpm)	120	130

Validation of the Second Order Polynomial Model

The second order polynomial model obtained from RSM was validated by adjusting the 3D respond plot to get the highest predicate valine yield and conducting a series of experiments randomly selected from the design in table 2. The experimental output was then compared to the values predicted by the second order model obtained from CCD, to estimate the goodness of fit of the model.

Table-2: Experimental Set Up for Model Validation of Valine Production

Run	pH	Temperature (℃)	Incubation time (hrs)	Agitation rate (rpm)
1	7.5	40	58.5	125
2	7	45	69	120
3	7	35	48	120

STATISTICAL ANALYSIS

The average data and standard deviations were obtained from the triplicate of experiments for each run using Microsoft Excel (Office, 2019). The standard deviation for each value was 5% analysis of variance (ANOVA) was done using Design-Expert software 6.0.6; a confidence level of 95% was used in this study. Any p-vales less than 0.05 were considered significant and vice versa.

Characterization of valine produced

The valine produced using *Bacillus cereus* was further characterized and compared with the physicochemical properties of standard/commercially produced valine using Fourier transform infrared spectroscopy (FTIR) and boiling point.

Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) analysis can be used to elucidate some components of an unknown mixture to identify the types of chemical bonds (functional groups). In the process the identified spot having the same Rf value with the standard on the TLC plate was scratched and then analyze in a FTIR (Perkin–Elmer Spectrum RX1, Shelton, Connecticut), device with the spectrum ranging from 450–4000cm^{-1} at a resolution of 4cm^{-1} (Elazzazy *et al.*, 2015).

RESULTS

Morphological and Biochemical Characterization of *Bacillus cereus*

Table-3: Morphological Characterization of the Bacteria isolates viewed under microscope

Test	Result
Gram Reaction	Positive
Shape	Bacillus
Spore Formation	Positive
Starch Hydrolysis Test	Positive
VP Test	Positive
Cell Diameter (≥1µ Wide)	Positive
Catalase Test	Positive
Motility Test	Positive

The results obtained for morphological and biochemical identification of *Bacillus cereus* were presented in table 3 (Brenner *et al.*, 2005, Amirreza *et al.*, 2015, Holt & Krieg, 2000 and Aneja, 2002). *Bacillus cereus* reacted positively to gram staining, non-capsule forming, rod in shape and spore forming bacteria. *Bacillus cereus* was biochemically positive to starch hydrolysis test, VP test catalase and motility test (table 3).

Molecular Identification of the Isolated Bacteria

The results for the molecular identification of the *Bacillus cereus* was presented in figure 1 and 2. Figure 1 present the result for the gel electrophoresis of the 16S rRNA gene of *Bacillus cereus* showing 1500 base pairs on the DNA molecular weight ladder, the sequence for the 16S rRNA of the isolate confirmed the identity of the isolate as *Bacillus cereus* with 99% identity with *Bacillus cereus* B4264 with accession number of NC 011725.1 after blasting in NCBI.

Figure 2 present the phylogenetic or the Evolutionary relationships of bacterial isolate using Molecular Evolutionary Genetics Analysis Software version 7.0 (MEGA7) and the result confirm a closely relationship between isolated *Bacillus cereus* with the *Bacillus cereus* B4264 and other neighbouring bacterial cells.

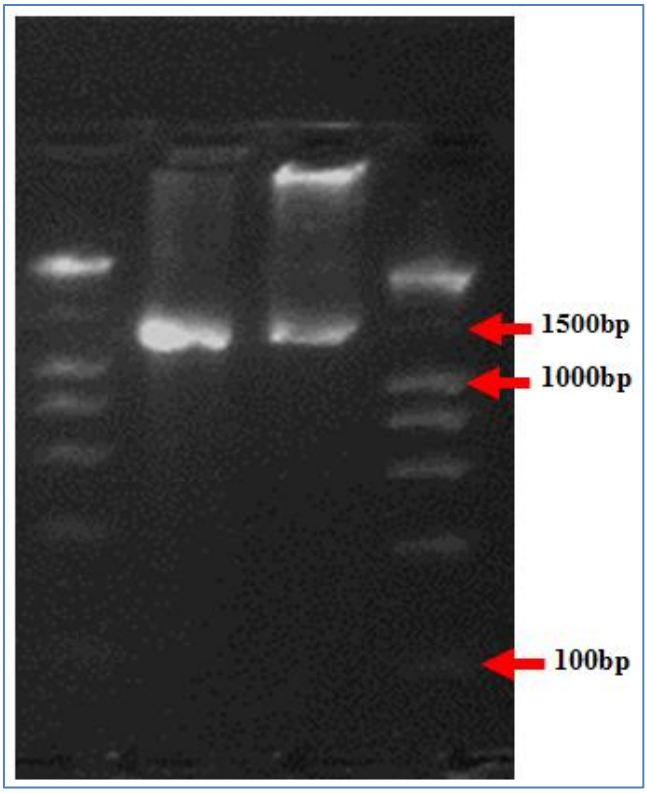

Fig-1: Gel Electrophoresis of the 16SrRNA of the Bacterial Isolate

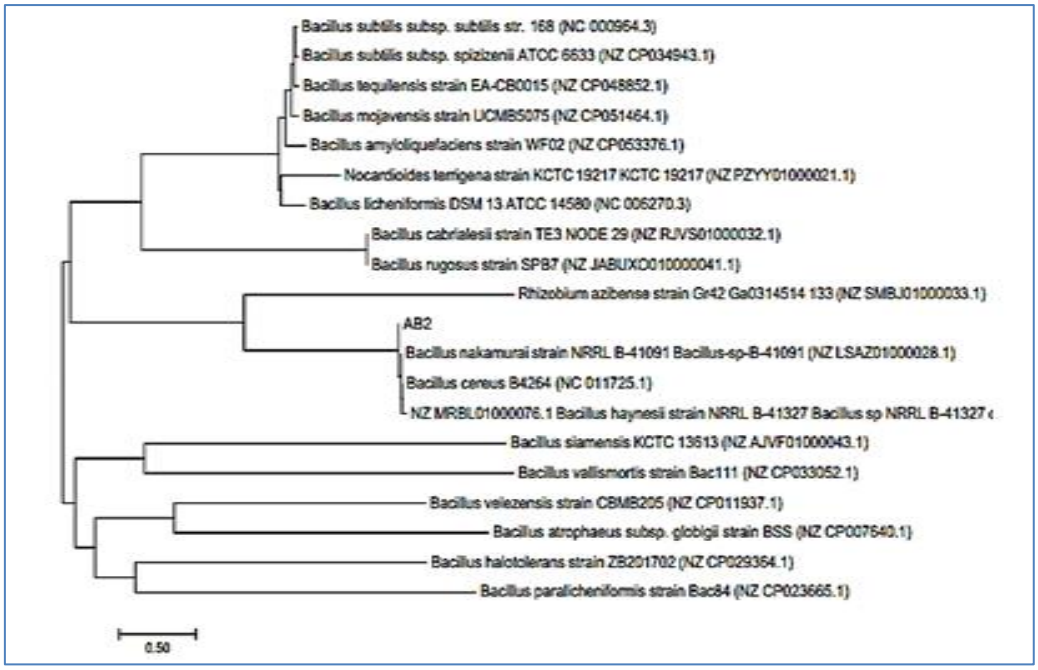

Fig-2: Evolutionary relationships of bacterial isolate using Molecular Evolutionary Genetics Analysis Software version 7.0 (MEGA7)

Qualitative Analysis of Valine

Result obtained for the qualitative analysis was presented in figure 3 below using thin layer chromatography, single band for standard/commercial valine having the same Rf-value with the corresponding band of the valine produced by the *Bacillus cereus* on the TLC plate.

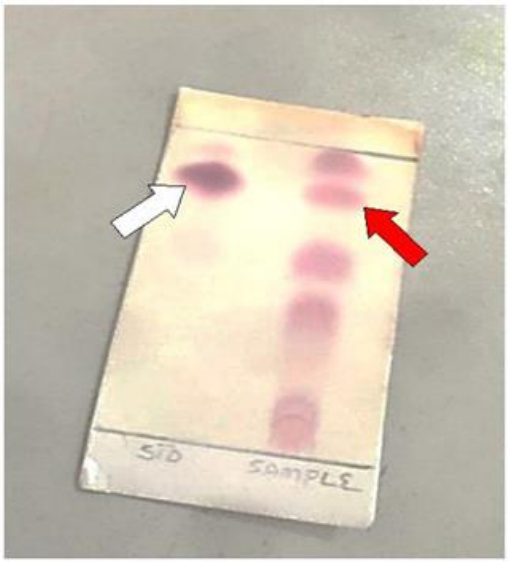

Fig-3: Thin Layer Chromatography (TLC) identifing the bands for Commercially Produced Valine (White) and the Valine produced by *Bacillus cereus* (Red) on the TLC plate

Optimization of valine production

One Factor at a Time Model (OFAT)

Results obtained for the optimum production of valine at different parameters (pH, temperature, incubation period and agitation rate) using one factor at a time (OFAT) technique were presented in Figure 4, 5, 6, and 7. These were achieved by varying one factor and keeping other factors constant.

pH Optimization

Optimum yield of valine was achieved at pH of 7.5 (2.767mg/ml), increase in valine yield was observed with increase in pH from 5.5 to 7.5 and declined at the pH of 8 keeping other factors constant as presented in figure 4.

Temperature Optimization

Figure 5 depicts the effect of temperature on valine yield using *Bacillus cereus*, initial increase in valine yield was observed with increase in temperature from 25℃ to 40^{o}C. However, the yield declined at the temperature of 45^{o}C. Optimum yield of valine (2.78mg/ml) was obtained at 40^{o}C.

Incubation time Optimization

The result for the effect of incubation time was presented in Figure 6. There was gradual increase in valine yield with increasing incubation time from 24hrs to 72hrs and later declined at 96hrs and 120hrs. The optimum valine yield (2.03mg/ml) was obtained at 72hrs.

Agitation rate Optimization

The result for the effect of agitation rate was presented in Figure 7. There was initial increase at 120rpm to 125rpm and gradual decrease at 130rpm to 140rpm. The optimum valine yield (1.13mg/ml) was recorded at 125rpm.

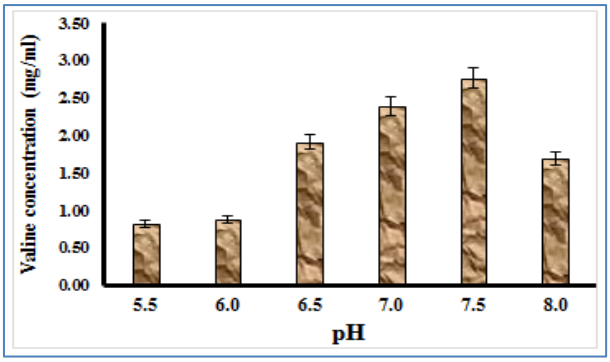

Fig-4: Effect of pH on valine production at constant temperature, incubation period and agitation rate (30℃, 48hrs and 125rpm, respectively)

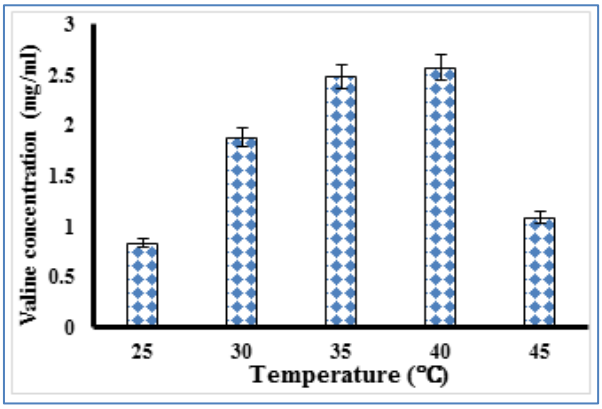

Fig-5: Effect of Temperature on valine production at constant pH, incubation period and agitation rate (7, 48hrs and 125rpm, respectively)

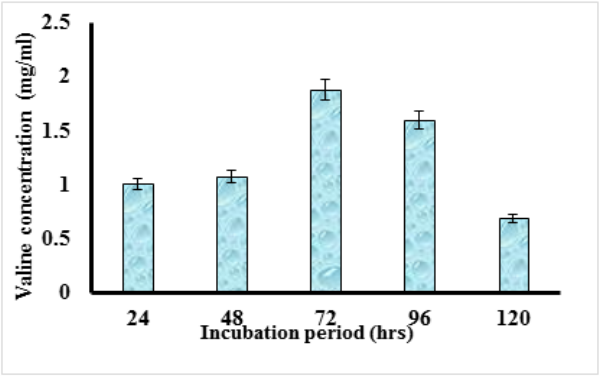

Fig-6: Effect of incubation time on valine production at constant pH, temperature, and agitation rate (7, 30℃ and 125rpm, respectively)

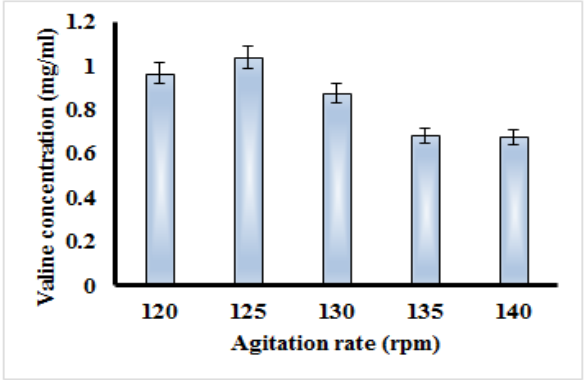

Fig-7: Effect of agitation rate on valine production at constant pH, temperature, and incubation rate (7, 30℃ and 48hrs, respectively)

Response Surface Methodology (RSM)

The results for the optimization study showing experimental and fitted model of valine yield by *Bacillus cereus* using response surface methodology (RSM) were presented in table 4 and 5.

The results obtained for the actual and predicted valine yield for different conditions of pH, temperature, incubation time and agitation rate are presented in table 4. The responses obtained for each experimental run and predicted responses were closely related. It can be observed that increase in pH, temperature, incubation time and agitation rate led to decrease in valine yield. Maximum valine yield (3.67mg/ml) was obtained at 58.5hrs of incubation time at 40℃, pH of 7.5 and agitation rate of 125rpm (Run 21, table 4) while the least valine yield (0.23mg/ml) was recorded at 79hrs of incubation time at 50℃, pH of 8.5 and agitation rate of 125rpm (Run 30, table 4).

From the result obtained, table 5 depicts the quadratic model analysis of the valine yield, the Model was significant and highly reliable (P= 0.0001). There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.05 indicate model terms are significant. In this case A2, AB, BC are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

The "Lack of Fitness was not significant at P-value of 0.1676 this indicated that the model was excellent fit without significant noise. R^2 Value and Adj. R^2 Value of 0.9740 and 0.9497 respectively indicated that there was a good correlation between the experimental and the predictate value.

Valine Concentration = -188.77285+43.77338A+0.079233B+0.13857C+0.33908D-3.03618A^2-0.00517841B^2-0.00033428C^2-0.00147766D^2+0.049784A * B+0.00655615A * C-0.00506503A * D -0.00322956B * C+0.00137965B * D+0.000187443C * D ……Equation ii

Where A, B, C and D represents pH, temperature, incubation time and agitation rate respectively. AB, AC, AD, BC, BD, CD are the interactions, and A^2, B^2, C^2, D^2 are the quadratic terms.

Figure 8 (a) depicts a correlation between the observed valine yield and the predicted value in the parity plot. Additionally, figure 8 (b) depicts the parity graph showing the distribution of residual and predicted values of valine yield. The clustered points around the diagonal line indicate goodness of the fit of the model since there is less deviation between the observed and predicted value.

Table-4: Actual and Predicted Valine Yield at Different Condition of pH, Temperatre, Incbation time and Agitation rate

Run	pH	Temperature (℃)	Incubation time (hrs)	Agitation rate (rpm)	Experimental yield (mg/ml)	Predicted yield (mg/ml)
1.	6.50	40.00	48.00	125.00	0.35	2.16
2.	7.50	40.00	58.50	125.00	3.26	1.84
3.	7.50	40.00	58.50	115.00	3.19	2.40
4.	7.00	40.00	48.00	130.00	2.15	2.57
5.	7.00	35.00	69.00	130.00	2.40	2.57
6.	7.00	40.00	48.00	120.00	2.18	2.39
7.	7.00	35.00	69.00	120.00	2.61	2.13
8.	8.00	45.00	48.00	130.00	2.55	2.45
9.	8.00	45.00	48.00	130.00	2.59	2.07
10.	8.00	45.00	69.00	120.00	2.57	2.58
11.	7.50	40.00	37.50	125.00	2.96	2.39
12.	7.50	40.00	58.50	125.00	3.23	2.58
13.	8.00	45.00	69.00	130.00	2.33	2.53
14.	7.00	45.00	69.00	120.00	2.03	2.29
15.	7.50	40.00	58.50	125.00	3.22	2.23
16.	7.00	35.00	48.00	130.00	2.06	2.49
17.	7.50	40.00	58.50	125.00	3.18	0.20
18.	8.00	45.00	48.00	120.00	2.59	0.26
19.	7.50	40.00	58.50	125.00	3.48	3.30
20.	7.50	40.00	58.50	125.00	3.23	3.30
21.	7.50	40.00	58.50	125.00	3.67	2.85
22.	7.00	45.00	69.00	130.00	2.35	3.30
23.	8.00	35.00	48.00	120.00	1.74	3.17
24.	7.50	40.00	58.50	125.00	3.24	3.13
25.	8.00	35.00	69.00	130.00	2.39	3.30
26.	8.00	35.00	69.00	120.00	2.36	3.30
27.	7.50	40.00	58.50	125.00	3.29	3.30
28.	7.50	40.00	58.50	135.00	3.23	3.30
29.	7.00	35.00	48.00	120.00	2.28	3.30
30.	8.50	50.00	79.00	125.00	0.23	3.30

Table-5: Quadratic Model Analysis of Variance of the Valine yield

SOURCE	MEAN SQURE	SUM OF SQUARES	F- VALUE	P>F	
MODEL	17.89	1.28	40.11	< 0.0001	Significant
A	2.300E-003	2.300E-003	0.072	0.7918	
B	0.091	0.091	2.87	0.1111	
C	0.12	0.12	3.78	0.0708	
D	2.649E-003	2.649E-003	0.083	0.7770	
A^2	4.64	4.64	145.71	< 0.0001	
B^2	0.050	0.050	1.57	0.2292	
C^2	0.077	0.077	2.41	0.1413	
D^2	0.036	0.036	1.14	0.3018	
AB	0.16	0.16	4.89	0.0430	
AC	8.946E-003	8.946E-003	0.28	0.6039	
AD	2.078E-003	2.078E-003	0.065	0.8019	
BC	0.22	0.22	6.89	0.0191	
BD	0.013	0.013	0.42	0.5276	
CD	1.413E-003	1.413E-003	0.044	0.8360	
LACK OF FITNESS	0.27	0.046	2.00	0.1676	Not Significant
R-Squared	=0.9740	Adj. R-Squared	=0.9497	Pred. R-Squared	=0.7621

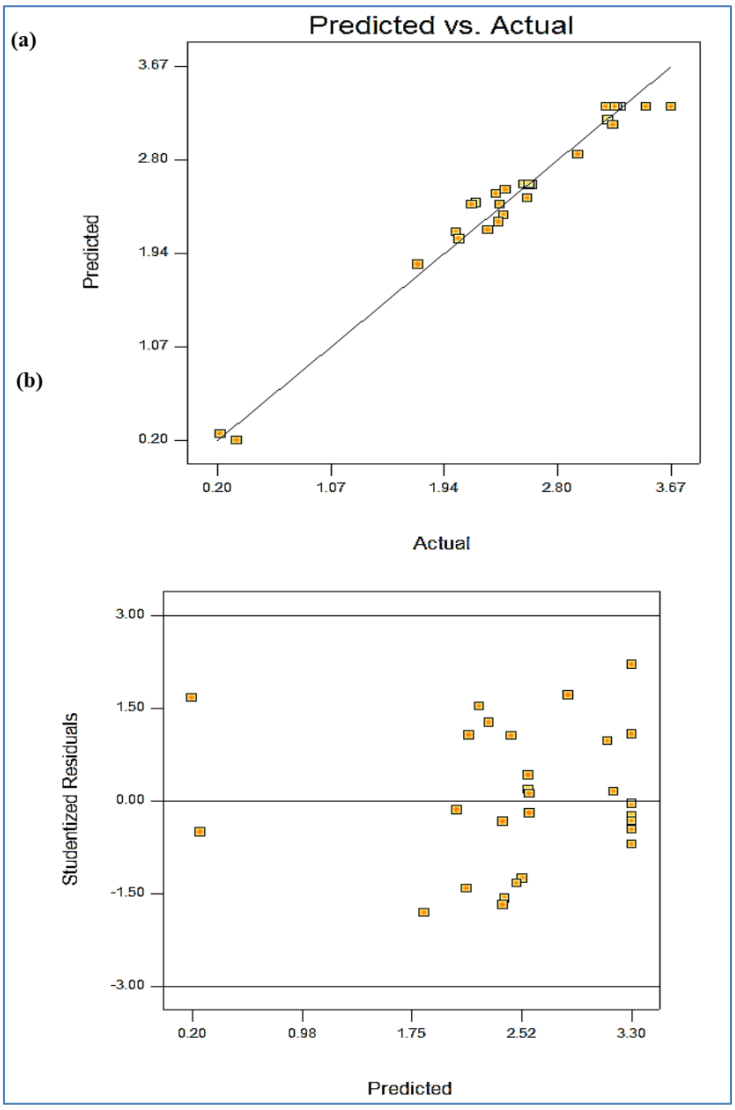

Fig-8: (a) Parity graph showing the distribution of actual against predicted values of valine yield (b) Parity graph showing the distribution of residuals against predicted values of valine yield

3D-Response Surface Plots Representing the Interaction between the Variables

Results obtained for the interaction between temperature and pH response surface (3D and contour) are presented in figure 9. The parabola shape of the 3D plot and the circular shape of contour plots indicated the interaction between temperature and pH was significant, keeping Incubation period and agitation rate constant.

Results obtained for the interaction between incubation time and temperature response surface (3D and contour) are presented in figure 10. The hyperbola shape of the 3D plot and the circular shape of contour plots indicated the interaction between the incubation time and the temperature was significant, keeping agitation rate and the pH constant.

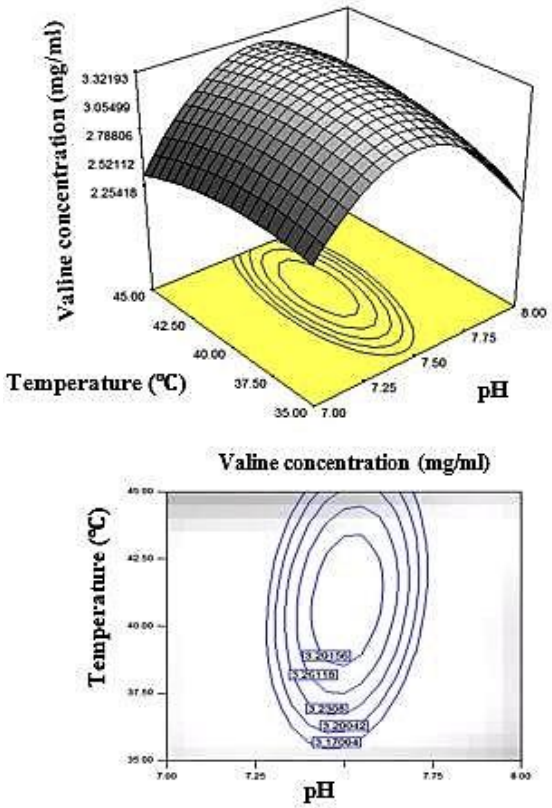

Figure 9: Response Surface Plots (3D and Contour) Showing the Interaction between Temperature and pH affecting the Valine yield.

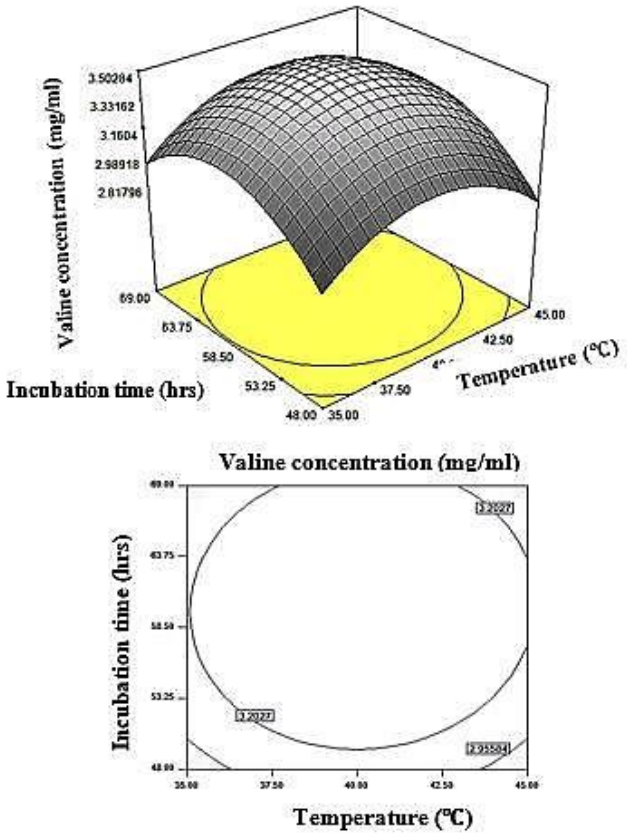

Figure 10: Response Surface Plots (3D and Contour) showing the Interaction between Incubation time and Temperature affecting Valine yield.

Table-6: Validation of Second Order Polynomial Model Between Experimental and Predicted Value of the Valine yield

Run	pH	Temperature (°C)	Incubation time (hrs)	Agitation rate (rpm)	Experimental yield (mg/ml)	Predicted Value (mg/ml)
1	7.5	40	58.5	125	3.53	3.32
2	7	45	69	120	2.82	2.29
3	7	35	48	120	3.03	3.30

Validation of the Second Order Polynomial

Results for the validation of the second order polynomial of the valine yield using *Bacillus cereus* are presented in table 6. The experimental yield of valine produced are compared with the predicted values by the second order model indicated that there was very good correlation between experimental and predicted values and in turn prove the validity of the models.

Characterization of valine produced using *Bacillus cereus*

Results for the characterization of valine produced using *Bacillus cereus* were presented in table 7 and figure 5. Table 7 present the Fourier transform infrared (FTIR) of the produced valine using *Bacillus cereus* and the commercial valine. The spectrum of the produced valine revealed the presence of four different functional groups such as hydroxyl group (OH), amino

group (NH_2), carbonyl group (C=O) and methyl group (CH_3). The result revealed that the spectrums of the produced valine were similar to that of the commercial produced valine as presented in table 3 below. Result for the characterization of valine using boiling point was presented in figure 11, showing that there was a good correlation between the boiling point of the valine produced by *Bacillus cereus* and the commercially produced valine with no significant difference at P>0.05 (0.4296).

Table-7: Characteristics of Valine Produced by *Bacillus cereus* using Fourier Transform Infrared Spectroscopy (FTIR)

Functional Group	Valine Produced Valine Using *Bacillus cereus* (CM^{-1})	Commercially Produced Valine (CM^{-1})
OH	3257	3260
NH_2	1640	1640
C=O	2113	2100
CH_3	2903	2989

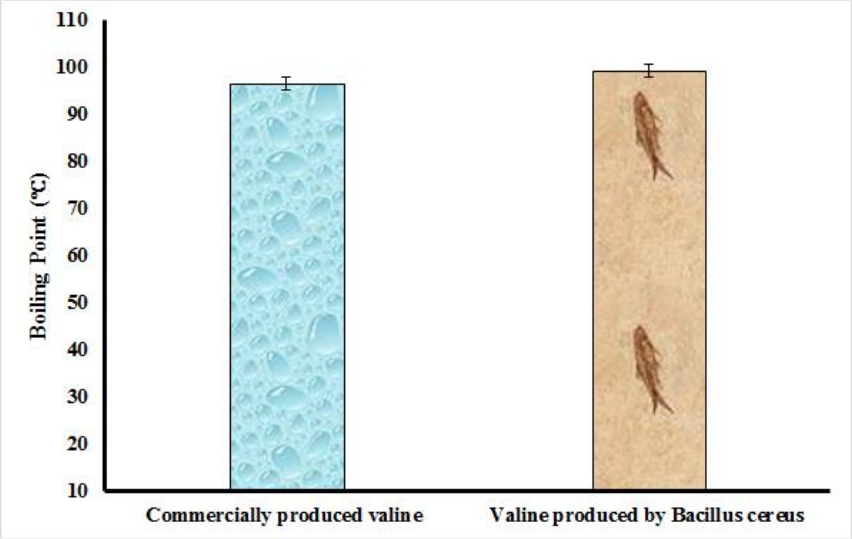

Fig-11: Comparism between the Boiling Point of Valine Produced Using *Bacillus cereus* and Commercially Producing Valine

DISCUSSION

In the present study research, *Bacillus cereus* was isolated and identified according to Brenner *et al.*, (2005), Amirreza *et al.*, (2015), Holt & Krieg, (2000) and Aneja, (2002). Genomic DNA was extracted according to the protocol provided by QiaAmp mini Prep DNA extraction Kit (Qiagen, Hilden, Germany) and the 16srRNA gene was amplified using PCR with 1500 base-pairs on the DNA molecular weight ladder. The nucleotide sequence confirm the identity of the isolate as *Bacillus cereus* with 99% identity with *Bacillus cereus* B4264 with accession number of NC-011725.1. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987).The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 563 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2016).

Optimum valine yield was obtained by *Bacillus cereus*, this is in line with the finding of Imran and Wei (2019), reported that *Bacillus cereus* produce valine and other essential amino acids, the ability of these isolates to produce valine in submerged fermentation is in agreement with the work done by Ezemba *et al.*, (2016), which reported maximum production amino acid including valine using *Bacillus cereus* in a submerged medium.

Physical factors such as pH, temperature and incubation time and agitation rate were considered among the most important fermentation parameters due to their effect on growth of microorganisms, fermentation efficiency and by-product formation (Yufu *et al.*, 2021). Therefore, maintenance of these parameters is therefore of great significance in fermentation for better yield.

The production of valine using *Bacillus cereus* was initially optimized using one factor at a time (OFAT) as conventional technique and further by response surface methodology (RSM) as a statistical technique.

Effect of initial medium pH experiment was carried out ranging from pH 5.5 to 8. Optimum valine yield was obtained by pH of 7.5 as presented in figure

4, which was in agreement with the work of Farah *et al.,* (2012) that reported maximum yield of valine of 2.13 g/L at pH 7.5. Most microbes grow best around neutral pH values (6.5 -7.5), some microorganisms produce acid as they grow. This acid is excreted and brings down the pH of the surrounding environment.

The effect of temperature on valine yield was studied by varying temperatures ranging from 25^{o}C to 45^{o}C and keeping all other variables constant. Optimum valine yield was observed at the temperature of 40°C (figure 5), increase in temperature lead to decrease in valine yield which could be due to the fact that high temperatures are lethal to microorganisms thereby decreases fermentation processes and denaturation of enzymes. This was related to the finding of Mohanta *et al.,* (2017), for maximum valine yield. That reported 2.16 g/L at 30^{o}C.

The effect of incubation time on valine yield was observed by varying time ranging from 24hrs to 120hrs and keeping all other variables constant. Optimum valine yield was determined at 72hrs of incubation and declined with increase incubation period as presented in figure 6, which is in agreement with the result obtained by Guoqiang, *et al.,* (2020) which reported high yield after 72hrs of fermentation. The decrease in valine yield was due to the relationship between the *Bacillus cereus* and sugar contents in the medium and the bacterial growth curve this was in agreement with results reported by Javed *et al.,* (2011), decline in valine production after 3 days could be attributed to the age of the bacteria, depletion of sugar content and decreased available nitrogen in the fermentation medium. Microbial production of metabolites usually starts after a lag phase of one day and reaches maximum at the onset of stationary phase or late.

The effect of agitation on valine production was observed by varying agitation rate ranging from 120rpm to 140rpm and keeping all other variables constant as presented in figure 7. As agitation speed increased from 120 to 125 rpm, valine yield increases rapidly and declined at 130rpm, maximum yield of valine was obtained at 125 rpm which was in agreement with the work of Farah *et al.,* (2012) that reported maximum yield of 8.0g/l at 125rpm (Lee *et al.,* 2012), suggested that agitation rates above 200rpm will prompt denaturation of enzymes with low production of metabolites. The influence of differing agitation rate on growth and valine production was evaluated. Agitation is very important in fermentation flask since oxygen is low solubility nutrient. Oxygen transfer capabilities in the flask controls the growth and fermentation.

Findings from the preliminary optimization using one factor at a time (OFAT) experiment were then applied to RSM modeling. Four factors to be optimized (pH, temperature, incubation time and agitation rate) were optimized, in which they were assigned to a number of runs as determined through central composite design (CCD). The statistical analysis for significances of all factors was described by analysis of variance (ANOVA) in Table 5. Based on the results obtained, the model of the analysis was confirmed significant and highly reliable at P-value less than 0.05, and the non-significant of lack of fitness indicated that the model was excellent fitted with no significant noise. The experimental and the predicated value were relatively closed (figure 8) due to the closeness of the R^2 and adj. R^2 value were closed to 1 (9740 and 0.9497 respectively) as indicated in table 5. Additionally, this shows that the extraneous factor terms in a derived model equation will affect in some reduction in the calculation of the error sum of squares (Mohamed *et al.,* 2013).

Optimum valine yield (3.53mg/ml) was obtained at pH 7.5, temperature of 40°C incubation time of 58hrs and 125rpm agitation rate, the response surface plots (3D and contour) revealed that the interactions between the factors (pH, temperature and incubation time) as depicted on figure 9 and 10 were all significant to valine yield. Additionally, the significant of the interaction between pH and temperature and that of temperature and incubation time were presented in the ANOVA result (table 5) with P-value of 0.043 and 0.019 respectively. This indicated that the valine yield was dependent on three factors (pH, temperature and incubation time). This was related to the work done by Guoqiang, *et al.,* (2020) and Farah, *et al.,* (2012), pH 7.5, incubation period of 72hours and 40°C for valine production.

However, the valine produced by *Bacillus cereus* was characterized using Fourier transform infrared (FTIR). Four different functional groups (hydroxyl group, amino group, carbonyl group and methyl group) were identified. The spectrum of the functional groups identified in the commercial valine were relatively closed to the spectrum in the valine produced by *Bacillus cereus* (table 7). This revealed that what was produced was valine.

Qualitatively, the produced valine shows the same Rf value with the commercially produced valine on the TLC plate (figure 3). Boiling point (figure 11) of the valine produced by *Bacillus cereus* was determined to show the similarities between the produced valine and the commercial valine and were found to be comparable without a significant difference at P>0.05 (0.4296). Thus confirming what was produced was valine.

CONCLUSION

Base on the results obtained from this study, *Bacillus cereus* was isolated from the soil, identified and possessed potential for industrial production of

valine. pH, temperature, incubation time and agitation rate were found to exert effect on valine yield.

By optimizing these conditions, pH, Incubation time and temperature were found to exert more positive effect on valine yield compared to agitation rate. The response surface plots (3D and contour) revealed that the interactions between pH with temperature and incubation time with temperature were significant to valine yield.

Various methods of characterization (Boiling point, Fourier transform infrared spectroscopy FTIR,) were determined and confirmed the present of valine produced by *Bacillus cereus* isolated from the soil.

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