Research Article

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Optimization of Culture Conditions for L-Glutamic Acid production by Bacillus flexus Using Response Surface Methodology

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Abstract

The body can use L-glutamic acid to make proteins (L-GA). It was formerly derived from plants, however bacteria have subsequently been studied as a potential source of synthesis. Bacillus flexus is a glutamic acid-harvesting bacteria that is well-known. Submerged fermentation was used in the production of L-GA. Response Surface Methodology (RSM) was utilized in this study to statistically optimize fermentation parameters that influence Bacillus flexus yield of L-glutamic acid. The effects of culture conditions, including several medium composition components, were evaluated using Central composite design (CCD) response surface optimization. Inoculum size, pH, substrate concentration, temperature, and time were all found to have substantial effects on glutamic acid production. Production increased considerably ($p<0.05$). Bacillus flexus produced the highest L-glutamic acid production of 4.9 g/L when the substrate concentration was 6%, the inoculum size was 4%, the temperature was 35°C, the pH was 7, and the time was 3 days, as predicted by RSM. This value is very close to the predicted value of 5.02 g/L, proving its validity. RSM proved to be a powerful strategy for improving this organism's L-glutamic acid synthesis.

Key words: *Bacillus flexus*, L-glutamic acid, Response Surface Methodology, Fermentation, Statistical optimization

Introduction

Neurotransmitter transmission, stomach function, taste perception, and intermediatory metabolism are all functions of glutamic acid, a non-essential amino acid. L-GA was also the first amino acid that was commercially available. The vast majority of L-GA is now produced through microbial fermentation. Chemical production of L-GA is discouraged, however, because it can result in the formation of a racemic glutamate combination (i.e., both D and L-GA), which is harmful to humans. A German chemist used acid hydrolysis to extract L-GA from gliadin for the first time [1]. Later, a Japanese scientist discovered that GA was being used to improve "konbu," also known as Laminaria Japonica, which has been used in Japan for millennia to make soup stocks [2]. MSG (monosodium glutamate) is a flavor enhancer that is used to improve the flavor and taste of processed foods, vegetables, meat, and other foods [3]. GA was first commercially available in a salted form in 1909, and the brand name "Ajinomoto" was born [4]. Food processing, biochemical processing, cosmetics, and pharmaceuticals are just a few of the industries that use L-GA [5]. L-GA is the most often imported amino acid in India.The use of fermentation to produce L-GA directly from microorganisms has gained favor in the last decade. Using microorganisms, total global L-GA output is increased to fifteen lakh tons per year [6]. Several Corynebacterium and Brevibacterium strains have been widely used in the conversion process to create various amino acids utilizing GA in the bioprocessing industry [7].

Microbial excretion of L-GA increased when fermentation media using an ammonia-rich substrate became the standard method for producing commercial MSG. The entire cost of amino acid manufacturing, on the other hand, as well as manual process optimization, which takes time and necessitates more trial runs, is a significant barrier for the industry. Over the course of several years, L-GA-producing bacteria were found, and further research led to the invention of a fermentative L-GA production process [8-10]. In submerged fermentation circumstances, agricultural waste materials such as cassava starch and sugar-cane bagasse have been employed to create L-GA using Corynebacterium glutamicum [11-13].

Economic production strategies must be implemented as a realistic means of increasing glutamic acid output. In the majority of previous investigations on the formation of GA, the "one com-

ponent at a time" strategy was used to create the multifactorial experiment [11]. A single-dimensional analysis is often difficult, time-consuming, and useless in achieving an exact ideal because the components are not connected. In order to address this problem, RSM was used to explore the impact of various components and their relationships on glutamic acid production [4, 13-14]. The purpose of this research was to see how independent variables and their interactions affected Bacillus flexus L-glutamic acid production in a submerged fermentation method.

RESULTS

Table 1: Mean and normal results of Shapiro-Wilk test Resilience and anger scores of coronary heart patients.

Variable		Resilience			Anger		
Group	**Test level**	**M**	**SD**	**Sig.**	**M**	**SD**	**Sig.**
Emotion regulation based on Gross model	Pre-test	58.75	2.04	0.438	163.10	3.689	0.446
	Post-test	74.60	1.91	0.126	148.95	3.90	0.553
	Follow up	74.10	1.61	0.599	147.25	3.26	0.757
Benson muscle relaxation exercises	Pre-test	56.90	2.87	0.264	154.35	2.41	0.645
	Post-test	67.40	2.66	0.488	134.25	2.73	0.557
	Follow up	66.55	2.60	0.654	139.10	2.97	0.304
Control	Pre-test	61.65	2.77	0.449	158.60	5.59	0.171
	Post-test	58.75	2.72	0.353	157.70	5.50	0.285
	control	59.75	2.72	0.402	157.50	5.50	0.289

The mean results of Table 1 show that the emotion regulation training methods based on Grass model and Benson muscle relaxation exercises caused by table 1 indicate that the normal Shapiro-Wilk (Sig.) test error distributes resilience and anger scores of patients. Coronary arteries in the emotion regulation groups according to Gross model, Benson muscle relaxation exercises and control in the pre-test, post-test and follow-up stages are higher than the significance level of 0.05, which indicates the normal distribution of data. Therefore, the use of parametric tests to analyze this data is allowed. Table 2 shows the results of Crowt-Machley and Greenhouse Geiser test for intrapersonal validity to increase resilience and reduce anger in patients with coronary heart disease in the post-test and follow-up stages compared to the pre-test stage.

Also table shows the normal Shapiro-Wilk test error (Sig.) of the distribution of resilience and anger scores of coronary heart patients in Gross model-based emotion regulation groups, Benson muscle relaxation exercises, and control in the pre-test stages. Post-test and follow-up is higher than the significance level of 0.05, which indicates that the data distribution is normal. So, the use of parametric tests to analyze this data is allowed. As we can see in Table 2, the results of the Machley-Greenley-Gesser Cervit test for intrapersonal validity to evaluate the sphericity of the covariance matrix error related to the variables between the stages of resilience and anger test in patients with coronary heart disease in Ilam have been presented with the effectiveness of emotion regulation training and muscle relaxation exercises.

Table 2: Results of Machley and Greenhouse Geiser test for intrapersonal credibility with the effectiveness of emotion regulation training and muscle relaxation exercises on resilience and anger in patients with coronary artery disease.

Component		Machley test results					
		Mauchly's W		**Approx. Chi-Square**		**df**	**Sig.**
Resilience		0.852		8.957		2	0.011
Anger		0.872		7.702		2	0.021
component	effect	Greenhouse-Geiser test results					
		Green-house-Geisser	Type III Sum of Squares	df	Mean Square	F	Sig.
Resilience	time	0.0871	2408.078	1.742	1382.017	41.755	<0.01
	time*group		2286.656	3.485	656.166	19.825	<0.01
Anger	time	0.886	5069.033	1.772	2860.181	64.293	<0.01
	time*group		2374.233	3.545	669.826	15.057	<0.01

As we can see the results of Machley test in Table 2, the assumption of sphericity of the covariance matrix error related to the dependent variables converted between the stages of resilience and anger test in patients with coronary artery disease with the effectiveness of emotion regulation training, muscle relaxation exercises and the control group is not approved, because the error value of the chi-square test is less than the significance level of 0.05.

Now, considering the inequality of covariances based on the Machley test, the results of the power view of the variance of the variance-covariance matrix dependent variable in Table 3 indi-

cate that the Greenhouse-Geiser test error of resilience and anger in coronary heart patients with emotion-based training according to Gross model, Benson and control group muscle relaxation exercises are less than 0.05 level. Consequently performing repeated measures analysis of variance is not an obstacle for this data. Table 3 indicates the results of repeated measures analysis of variance to evaluate the effectiveness of emotion regulation training methods and muscle relaxation exercises on resilience and anger of coronary heart patients.

Table 3: Results of repeated measures analysis of variance on resilience and anger in patients with coronary artery disease.

Component	Source of changes	MM	df	MS	F	Sig.	Effect size
Resilience	stable	743565.339	1	743565.339	2398.777	<0.01	0.977
	group	2522.978	2	1261.489	4.070	0.022	0.125
	error	17668.683	57	309.977			
Anger	Stable	4115059.200	1	4115059.200	4349.430	<0.01	0.987
	Group	7408.933	2	3704.467	3.915	0.026	0.121
	Error	53928.533	57	946.115			

The findings of repeated measures analysis of variance in Table 3 indicate that the test error of the effects between the subjects of emotion regulation training based on the Gross model and the control group on the effectiveness of resilience and anger of coronary heart patients in Ilam is less than 05 is / 0. Consequently, there is a significant difference between the effects of subjects on Gross model-based emotion regulation training and control in resilience and anger in patients with coronary heart disease. In other words, Gross model-based emotional regulation training on resilience and anger of coronary heart patients in Ilam city has been effective, so that the effect of Gross model-based emotion regulation training on resilience and anger in patients the order is 0.125 and 0.121, which are significant, significant and desirable values. Table 4 represents the findings of the Benferoni post hoc test to compare emotion regulation training methods based on the Gross model and Benson muscle relaxation exercises on resilience and anger in patients with coronary artery disease.

Table 4: Results of Benferoni post hoc test to compare intervention methods on resilience and anger in patients with coronary heart disease.

Component	Group	Group	Average differences	Sig.
resilience	Emotion regulation based on Gross model	Benson muscle relaxation exercises	3.5667	0.272
		Control	9.1000*	0.019
	Benson muscle relaxation exercises	Control	5.5333*	0.046
anger	Emotion regulation based on Gross model	Benson muscle relaxation exercises	10.5333*	0.197
		Control	-4.8333	1.000
	Benson muscle relaxation exercises	Control	-15.3667*	0.025

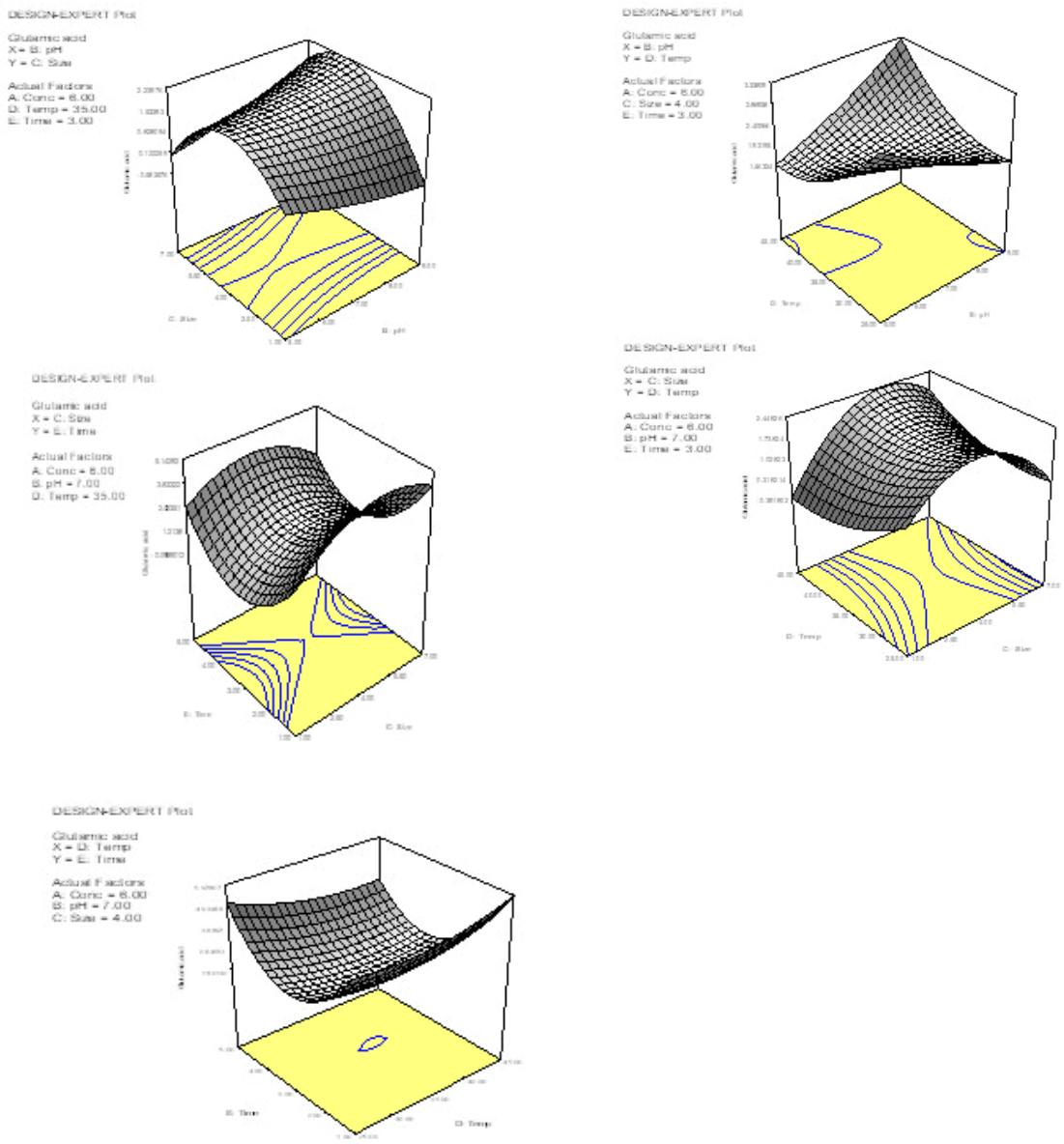

Figure 1: Response surface curve for glutamic acid production representing the interaction between pH, temperature, inoculum size and substrate concentration of whole cell of Bacillus flexus using acid treated cowpea waste as substrate

Discussion

RSM for optimizing L-glutamic acid production by CCD

The strain chosen, as well as the growth conditions and downstream purification technologies employed, have a big influence on the amount of beneficial metabolites produced during submerged fermentation [15].

Inoculum size, temperature, substrate concentration, pH, and time are all essential parameters in Bacillus flexus L-glutamic acid synthesis, according to early research. The effects of these five elements were then optimized using RSM in line with CCD. Table 1 shows the actual and expected responses for 32 runs with different combinations of the five variables (in three equi-distance levels). The quadratic regression models were shown to be significant after an ANOVA, with a computed F-value of 3.87 for L-glutamic acid production and a P-value (Prob > F) of less than 0.01. (See Table 2) If the coefficient of variance is lower, the experiment is more dependable (P 0.01). The coefficient of determination was used to measure the model's fitness (R2).

Statistical analysis of the CCD model

Response models can explain 87.56 % of total L-glutamic acid production fluctuations, with the rest being attributable to chance, according to this study's R2 values of 0.8756. All variables strongly contributed to these answers, according to the quadratic impact analysis (P 0.05). For L-glutamic acid, the models exhibit a statistically insignificant lack-of-fit of 3.87. By all statistical indices, the models were shown to be acceptable for describing the genuine link between the selected components for Bacillus flexus L-glutamic acid production. It was also discovered that the interaction of any two factors on L-glutamic acid was promising. The most significant (P 0.05) mutual interactions for L-glutamic acid production, according to Anova data (Table 2) and response surface-contour plots, were between

inoculum size and pH, temperature and pH, inoculum size and temperature, inoculum size and temperature, inoculum size and temperature, inoculum size and time, temperature and time (Fig. 1). To arrive at an ideal fermentation environment, the Design expert 12 numerical optimization subroutine was used to explore the design space with the fitted quadratic model. The ideal variables were determined using a desirability objective function, which assigns relative value to the responses.

Suggestions for solutions with a higher attractiveness level When the substrate concentration was 6%, the inoculum size was 4%, the temperature was 35°C, the pH was 7, and the time was 3 days, Bacillus flexus produced the most L-glutamic acid, 4.9 g/L. Under these settings, three duplicate confirmation experiments were conducted, and the findings were in good agreement with the predicted model, showing the model's suitability [16-17]. The model's F-value of 3.87 shows that it is significant. Due to noise, an F-value of this magnitude has a 0.01 percent probability of occurring. P-values of less than 0.05 indicate that model terms are significant. In this circumstance, significant model terms include E, A2, C2, E2, BC, BD, CD, CE, and DE. If the value is more than 0.1000, the model terms are irrelevant.

If your model has a lot of insignificant model terms, model reduction may help you improve it (not including those required to support hierarchy). The Lack of Fit F-value of 3.87 implies that noise has a 1.25 % chance of causing a large Lack of Fit F-value. The model should not be overly big or too little. This low probability (less than 10%) is troubling. RSM was applied to optimize fermentation conditions using Streptomyces albus y07, a high yielding strain, according to Yang et al. (2014). [18] compared medium optimization for L-lysine-methionine production by a newly found strain Pendiococcus pentosaceus RF1 using one component at a time, Response Surface Methodology, and artificial neural networks [18]. In our investigation, RSM proved to be a really effective technique. [16] employed RSM to optimize the medium for Streptomyces diastatochromogenes to synthesize -poly-L-lysine. [15] recently cited RSM as a good statistical strategy for enhancing culture conditions for Corynebacterium glutamicum nCIM2168 L-glutamic acid synthesis, despite the yield being only 16.499 g/L. In batch fermentation, [17] employed RSM to maximize L-glutamic acid production by Corynebacterium glutamicum. In this study, we determined that RSM appears to be a very useful strategy for optimizing the mutant's L-glutamic acid synthesis while also taking into account multifactorial interactions [17].

Materials and methods

Collection of samples

Soil samples from Camp, Abeokuta were considered for sample collection to isolate Bacillus flexus.

Substrates

Cowpea waste was bought from a market at Osiele in Abeokuta, Ogun State. The cowpea waste sample was packaged into polythene bag, labeled appropriately and then transported to the Department of Microbiology, Federal University of Agriculture, Abeokuta for analysis.

Proximate Analyses of the Sample

Approximately 8 g of each acid-treated and alkali-treated dried unfermented cowpea waste sample was used to conduct proximate analyses at the Department of Microbiology, Federal University Of Agriculture, Abeokuta, in order to determine the total carbohydrate content, crude protein content, crude fat, crude fiber, ash content, and moisture content as percentage compositions of the substrate using the methods proposed by [19].

Isolation and characterization of bacteria

Isolation of glutamic acid producing bacteria

Isolation of *Bacillus flexus*

After the samples collection, it was brought to the laboratory, one gram of each sample will be ground in a sterile motar and re-suspended in 5 ml of sterile distilled water. Bacillus sp will be isolated on nutrient agar medium by inoculating 0.1 ml of the soil suspension onto nutrient agar and incubating for 24 h at 37oC. Resulting colonies will be characterized in terms of Gram stain, spore production, catalase reaction, haemolysis, gelatin liquefaction, and starch hydrolysis and plasmid profile. Presumed Bacillus sp colonies were stored in nutrient agar slants at 4oC for further use.

Fermentation medium

MgSO4.7H2O 0.2, FeSO4.7H2O 0.001, MnSO4.H2O 0.001, KH2PO4 0.1, K2HPO4 0.1 are the components of the glutamate synthesis medium (all of them g.dL-1). Sodium hydroxide or hydrochloric acid were used to alter the pH of the medium to 7.0. The fermentation took place in a 100 mL Erlenmeyer flask with 50 mL of medium. 10 mL of the overnight culture was added to the fermentation media. For 48 hours, the manufacturing medium was shaken at 180 rpm at controlled temperatures in an orbital shaker. 4 U/mL penicillin G was added after 20 hours of incubation, releasing cellular glutamate into the solution [21].

Shake Flask Fermentation

About 50ml of the basal medium was mixed separately with 13g each of the substrate (4:1 v/v) in 250 ml Erlenmeyer flasks and labeled appropriately. Four (4) ml each of the 18hours-old culture was added appropriately and incubated on a rotary flask shaker at (180 rpm) at 37°C for 96 hrs [19]. The qualitative and quantitative analyses of the glutamic acid produced were carried out and results recorded accordingly.

Colorimetric assay

Qualitative Estimation of Glutamate

The paper chromatographic technique was used for qualitative analysis of L-Glutamate, as stated by (Hassan et al., 2003).

Quantitative Estimation of Glutamate

Two milliliters of the supernatant from each fermented screening media was taken individually in test tubes, two milliliters of 5% ninhydrin in acetone was added, and the test tubes were cooked in a boiling water bath for 15 minutes. The tubes were subsequently cooled to room temperature, and glutamate was quantified using a spectrophotometer to take readings at 570 nm, as stated by (Hassan et al., 2003).

HPLC analysis

The glutamate content was evaluated using reversed phase high-performance liquid chromatography (RP-HPLC) according to Yang et al's method. A C18 column (250 x 4.6 mm) and a UV detector are included in this HPLC setup. Solution A (aqueous solution of 10.254 g sodium acetate, 0.5 ml tri-ethylamine, and 0.7 ml acetic acid in 1000 ml, pH 5.8), 12 percent solution B (acetonitrile), and 28 percent solution C (deionized water) made up the mobile phase. The injection flow rate and volume were 0.6 ml/min and 20 l, respectively. Phenyl iso thio cyanate (PITC) was used to derivatize amino acids in the precolumn, and the resulting Phenyl iso thio carbamate-glutamate (PITC-glutamate) had a UV absorbance of 254 nm [20].

Design of experiments

Central Composite Design (CCD)

CCD is a statistical strategy for optimizing variables in a process that is based on the multivariate nonlinear model. The CCD was used in this study to investigate the interactions of numerous parameters affecting amino acid fermentation and to establish the best conditions for glutamate synthesis using Bacillus flexus. Design-Expert software version 12.0.3.0 was used for the experiment design and statistical analysis (dx-12, State-Ease Inc). Five variables (substrate concentration, pH, inoculum size, temperature, and time) were allocated to five levels: plus and minus alpha (axial points), plus and minus 1 (factoral points), and the center-point. Table 2 shows the 32 experimental runs that made up the CCD. Bacillus flexus was found to be capable of producing glutamate under all of the circumstances tested [17].

Conclusion

Findings showed that the CCD could be applied for evaluating of the variables influencing on glutamate fermentation and determining the mentioned process optimization condition. The optimal conditions were evaluated as: temperature 35°C, Substrate concentration 6 mg/L, Inoculum size 4 µg/L, Time 3 days and pH 7. The highest glutamate production was 4.9%

Declarations

Author contribution statement

Adegoke Olaposi was in charge of conducting the experiments, analyzing and interpreting the results, and writing the paper. Kareem Sarafadeen: Conceived and designed the experiments; analyzed and interpreted the data; provided reagents, materials, analytical tools, or data and wrote the paper.Balogun Saka: Analyzed and interpreted data; provided reagents, materials, analysis tools, and data.Adeogun Abideen: Analyzed and interpreted data; provided reagents, materials, analysis tools, or data [22-24].

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Data availability statement

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper

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