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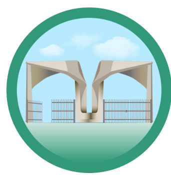

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Original research

Sustainable and Cost-Effective Production of Glutamic Acid by *Corynebacterium glutamicum* PTCC 1532 from Waste Bread using Enzymatic Hydrolysis and Microbial Fermentation

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A B S T R A C T

Food waste generation has increased in recent years due to population growth. The continuous rise in food production for human consumption has resulted in 1.3 billion tons of food waste annually worldwide. Waste bread, an inexpensive substrate with high carbohydrate content, can hydrolyze by proper methods, such as enzymatic hydrolysis, for utilization in fermentation. Glutamic acid, a non-essential amino acid with various applications in pharmaceuticals, food industries, and cosmetics, can be produced by fermentation. In this study, we applied waste bread, as a cost-effective starchy waste, to produce fermentable substances through enzymatic hydrolysis. This process resulted in a significant increase in reducing sugar concentration from 1.285 ± 0.195 g/L to 123.282 ± 0.924 g/L. The obtained hydrolysate was utilized as a carbonic source for the glutamic acid synthesis by *Corynebacterium glutamicum* PTCC 1532. To enhance the glutamic acid yield, response surface methodology was employed to optimize the independent variables. The optimum levels of reducing sugar concentration of hydrolysate, urea concentration, biotin concentration, and inoculum size was 49.889 g/L, 6.812 g/L, 6.57 μg/L, and 5.339% (v/v), respectively. Under these optimized conditions, the experimental glutamic acid production was 21.34 ± 0.204 g/L, which demonstrated a reasonable correlation between the predicted and experimental results. This study illustrated that waste bread can serve as a low-cost carbon source for producing valuable compounds such as glutamic acid.

Keywords: Waste Bread; Glutamic Acid; *Corynebacterium Glutamicum* PTCC 1532; Enzymatic Hydrolysis; Fermentation

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1. Introduction

Food waste generation has increased in recent years due to population growth (Benabda et al., 2019). The continuous rise in food production for human consumption has resulted in 1.3 billion tons of food waste annually worldwide (Ng et al., 2020), which includes cereals, fruits, vegetables, meat, fish, milk, and eggs (Haroon et al., 2016). In Asia, predictions illustrate that food waste production will reach 416 million tons by 2025, up from 278 million tons in 2005 (Uçkun Kiran et al., 2014). Over 2 billion tons of cereals are harvested globally, despite this abundance, many countries still dispose of substantial amounts of food products as waste. This not only represents a missed opportunity to reduce food waste but also emphasizes the necessity for more sustainable practices in food waste management (Sindhu et al., 2019).

Bread, the most consumed bakery product, has a limited shelf life due to physicochemical changes during storage that affect taste and texture. Therefore, bread is often discarded without proper treatment, resulting in economic effects and environmental concerns (V. Kumar et al., 2023). Consequently, suitable waste management is essential for safe waste bread recycling.

Waste bread (WB), an inexpensive substrate with high carbohydrate content, can hydrolyze by proper methods, such as enzymatic hydrolysis, for utilization in fermentation. (Melikoglu & Webb, 2013; Uçkun Kiran et al., 2014). Regarding this matter, WB was employed for fermentative protease and amylase production by

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Rhizopus oryzae using solid-state fermentation (Benabda et al., 2019). Also, Sadaf et al. (2021) indicated that WB hydrolysate can be utilized for lactic acid production by *Lactobacillus paracasei* (Sadaf et al., 2021). In addition, numerous bio-based products including ethanol (Torabi et al., 2021), succinic acid (Gadkari et al., 2021; Zhang et al., 2013), bio-pigments (Haque et al., 2016), glucoamylase and protease (W. Han et al., 2015), and bacteriocin (Haroon et al., 2016) can be produced using WB through fermentation technique.

Fermentation technique is the preferred method for producing amino acids. This method provides mild reaction conditions, economic feasibility, and large-scale production capabilities. Glutamic acid (GLU), a non-essential amino acid, can be produced by this approach, which is suitable for amino acid production instead of other procedures like acidic manner (D'Este et al., 2018). GLU, with various applications in pharmaceuticals, food industries, and cosmetics, has witnessed a significant surge in demand (Bashir et al., 2022). For instance, flavor enhancers that improve food taste classified as food additives in the food industry, and Monosodium Glutamate (MSG), which originated from GLU, is included in this classification. As a result, the annual production of GLU has reached 3 million tons (Wen et al., 2018).

Various bio-resources have been used for glutamic acid production (GAP). Ghazanfari et al., (2023) investigated the potential of dairy sludge and soybean meal as carbon and nitrogen sources for L-glutamate production using the Central Composite Design (CCD) of Response Surface Methodology (RSM) (Ghazanfari et al., 2023). Another study optimized effective parameters such as glucose, biotin, urea concentration, and temperature to enhance glutamate production by *C.glutamicum* PTCC 1532 using RSM (Fahimitabar et al., 2021). Xylose utilization by *C.glutamicum* is challenging in GAP from lignocellulosic biomass due to the coexistence of xylose with inhibitors. Jin et al. (2020) applied pretreated wheat straw solids to glutamate production by simultaneous assimilation of xylose and glucose (Jin et al., 2020). In another study carried out by Reddy et al. (2020), Dioscorea bulbifera L. (Or air potato) was utilized as a cost-effective carbon source for producing GLU through submerged bacterial fermentation with *C.glutamicum* DSM 20300 and *Arthrobacter globiformis* MTCC 4299. Also, In order to enhance GLU productivity, Urea, biotin, and mineral salt solution concentration were optimized using CCD (Reddy et al., 2020). However, previous studies have explored the application of waste materials such as date waste (Abdenacer et al., 2012; Tavakkoli et al., 2012), cassava starch (Jyothi et al., 2005), palm waste (Das et al., 1995), and sugar cane bagasse (Madhavan Nampoothiri & Pandey, 1996) for GAP.

C.glutamicum is a prominent microorganism that widely used to produce GLU. This gram-positive, biotin auxotrophic, rod-shaped, facultative anaerobic bacterium does not produce spores and grows fast in favorable conditions. Furthermore, it recognized as GRASS (D'Este et al., 2018; Ganguly, 2023). The bacterium is distinguished from other gram-positive bacteria by its distinctive outer layer, which comprises mycolic acid with attached alkyl groups that coat its peptidoglycan layer (D'Este et al., 2018; Fahimitabar et al., 2021; R. Kumar et al., 2014). This unique feature can significantly impact cell division and serve as a robust barrier for GLU secretion, which can influence by factors such as biotin deficiency, detergent addition, and beta-lactam antibiotics like penicillin (Ganguly, 2023). According to Hirasawa & Wachi (2017), penicillin addition and biotin deficiency result in a decrease in 2-oxoglutarate dehydrogenase complex (ODHC) activity with no effect on the Glutamate dehydrogenase (GDH) activity (Hirasawa & Wachi, 2017). This issue leads to a restriction in mycolic acid layer synthesis, ultimately causing an increase in glutamate flux, as explained by Schultz et al. 2007. GDH catalyzes the reversible reaction of 2-oxogluratrate to generate glutamate (Schulz et al., 2001).

Applying a cost-effective substrate is essential for industrial biotechnology. A novel aspect of this research was the utilization of WB as a starchy substrate for GAP. Therefore, the current study aimed to employ WB for fermentative GAP using *C.glutamicum* PTCC 1532 and then optimization of effective parameters for enhancing GLU yield. To achieve this, WB hydrolysis was performed using amylolytic enzyme to produce the fermentable compound. Subsequently, the optimum level of the effective variables including reducing sugar concentration of hydrolysate, urea concentration, biotin concentration, and inoculum size was evaluated and statistically authenticated using RSM.

2. Material and Methods

2.1. Preparation of WB

WB was collected from a local bakery shop in Tehran, Iran. After drying at room temperature, the samples were ground in a knife mill grinder. The obtained powder was sieved (1-2 mm mesh size) and stored at -30 °C until the hydrolysis process.

2.2. Enzymatic hydrolysis of WB

Enzymatic hydrolysis (EH) was conducted in a 250-mL Erlenmeyer flask using thermostable α-amylase and glucoamylase from DSM, Netherlands. 15% (w/v) WB solution was gelatinized at 100 °C, and cooled to 50 °C for the liquefaction process. In this step, the pH was adjusted to 5.8 by diluted HCl and NaOH, and then 0.5 mg α-amylase/g-WB was added at 50 °C and 600 rpm for an hour. Due to the inhibitory properties of alpha-amylase on glucoamylase activity, the solution was boiled for 15 minutes to deactivate the enzyme before adjusting the pH to 4.3 for saccharification (Torabi et al., 2021). Glucoamylase was loaded at 0.45 mg/g-WB ratio, and saccharification was carried out at 60 °C and 600 rpm (Torabi et al., 2021). The reducing sugar concentration of hydrolysate was determined by the DNS method.

2.3. Microorganism preparation

C.glutamicum PTCC 1532 was purchased from the Persian Type Culture Collection (PTCC), Tehran, Iran. Freeze-dried bacterium was reconstituted in a nutrient broth and incubated at 30 °C and 120 rpm agitation for 24 h. Activated bacteria were preserved in 2 ml vials containing 400 µL of glycerol at -40 °C.

2.4. Pre-culture preparation

C.glutamicum PTCC 1532 transferred aseptically to a 250-mL Erlenmeyer flask containing 100 mL of sterilized pre-culture with the following composition (g/L): glucose 50, yeast extract 5, $MnSO_4.H_2O$ 0.01, $FeSO_4.7H_2O$ 0.01, $MgSO_4.7H_2O$ 2, KH_2PO_4 1, and K_2HPO_4 1. The optimum pH value for *C.glutamicum* growth is 7-8 (Ganguly, 2023). Therefore, the pH of the pre-culture and main

culture was adjusted to 7 using diluted HCl and NaOH. (Tavakkoli et al., 2012). The inoculated pre-culture was incubated at 35 °C with 200 rpm agitation for 18 hours, and then used for inoculation of the main culture.

2.5. Main culture preparation

The experiments were conducted in a 250-mL Erlenmeyer flask using batch conditions. 100 ml of the main culture was prepared, consisting of $MnSO_4.H_2O$ 0.01, $FeSO_4.7H_2O$ 0.01, $MgSO_4.7H_2O$ 2, KH_2PO_4 1, and K_2HPO_4 1 (g/L) (Tavakkoli et al., 2012). The Reducing Sugar Concentration of Hydrolysate (RSCH), urea concentration, biotin concentration, and inoculum size of the main culture were adjusted as per the experimental design. The fermentation conditions were as follows: temperature 35 °C, agitation 300 rpm, fermentation period 48 h, and pH 7. Penicillin was added to the medium approximately 8 hours after fermentation began to enhance GLU flux (Tavakkoli et al., 2012). Subsequently, the broth was centrifuged (8000 rpm, 15 min) to separate the bacteria cells, and the supernatant was used to determine the GLU content (Fahimitabar et al., 2021). Due to heat sensitivity, urea, and biotin were sterilized using membrane filter.

2.6. Experimental design by RSM

RSM employs statistical techniques to identify the optimal experimental conditions with minimal experiments (Witek-Krowiak et al., 2014). In this research, RSM was utilized to assess the effects of independent variables on GAP, including their interactions. To optimize the GLU productivity by *C.glutamicum* PTCC 1532, four independent variables in 2 levels of CCD with 30 runs were employed (24 runs, and 6 center points). The independent factors selected for optimization were RSCH, urea concentration, biotin concentration, and inoculum size (Table 1). All experiments were conducted in triplicate, and statistical analysis was carried out using Design Expert version 13 trial software (Stat Ease Inc., Minneapolis, MN, USA). The results were used to establish an optimum condition for GAP.

Table 1. Independent variable levels utilized in the CCD.

Factor	Name	Units	Min	Center	Max	Coded Low	Coded High
A	RSCH	g/L	20	40	60	-1 ↔ 20.00	+1 ↔ 60.00
B	Urea Concentration	g/L	4	7	10	-1 ↔ 4.00	+1 ↔ 10.00
C	Biotin Concentration	µg/L	0	5	10	-1 ↔ 0.00	+1 ↔ 10.00
D	Inoculum Size	% (v/v)	2	6	10	-1 ↔ 2.00	+1 ↔ 10.00

The experimental variables were coded using Eq. 1, where X_i represents the actual value of each independent variable, X_0 represents the actual value at the center point, ΔX_i represents the step change value, and x_i represents the coded value of each independent variable.

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

The following quadratic equation was used to describe the model behavior. Where y is the predicted value, b_0 is a constant coefficient, b_i, b_{ii}, and b_{ij} are first-order, second-order, and interaction coefficients, x_i is the independent variable of i, $x_i x_j$, and x_i^2 are the interaction between independent variables and the second-order coefficient respectively.

$$y = b_0 + \sum_{i=1}^{n=3} b_i x_i + \sum_{i=1}^{n=3} b_{ij}\, x_i x_j + \sum_{i=1}^{n=3} b_{ii} x_i^2 \quad (2)$$

2.7. Analytical methods

2.7.1. Chemical composition of WB

The moisture content by oven drying (105 °C, 3h), ash by incinerating a certain amount of the sample, and total protein (N × 6.25) by the Kjeldahl method, were determined according to the AOAC methods (AOAC, 2019).

2.7.2. Measurement of reducing sugar content

The reducing sugar content was determined by 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). 1 mL sample was mixed with 3 mL of DNS reagent in a 25 mL test tube, then heated in boiling water for 5 minutes. The absorbance of the sample was recorded at 540 nm using UV–vis spectrophotometer (Cecil CE2041 UV/Vis Spectrophotometer Single beam, China). The reducing sugar content was calculated from glucose standard curve. All experiments were performed in triplicate.

2.7.3. Analysis of GAP

GLU was measured using the ninhydrin method (Spies, 1957). Ninhydrin reagent reacted with free alpha-amino acid, and Ruhemann's purple appeared. To do this, 1 ml of culture media supernatant was mixed with 1 ml of ninhydrin reagent in the test tube, and placed in a boiling water bath for an hour. After that, 1 ml of acetic acid was added and the absorbance was measured at 570 nm using UV-Vis spectrophotometer (Cecil CE2041 UV/Vis Spectrophotometer Single beam, China). The standard curve was drawn by measuring the absorbance of different concentrations of GLU.

2.7.4. Optical density measurement

The optical density (OD_{600}) of the culture broth at 600 nm was measured to determine the bacterial growth using UV-visible spectrophotometer (Cecil CE2041 UV/Vis Spectrophotometer Single beam, China) (Yang et al., 2021).

3. Results and Discussion

3.1. Chemical composition of WB

Table 2 depicts the chemical composition of WB. The chemical composition of WB depends on the extraction degree of flour. Similar to our results, Torabi et al. (2021) reported that WB contains 9.8% moisture and 13% protein (Torabi et al., 2021).

3.2. EH of waste bread

Hydrolytic enzymes have been shown to effectively break down starch into fermentable sugars, as demonstrated in previous studies (AČANSKI et al., 2014; Benabda et al., 2018; Demirci et al., 2019; Ebrahimi et al., 2008; Hudečková et al., 2017; Pietrzak & Kawa-Rygielska, 2014; Sükrü Demirci et al., 2017; Torabi et al., 2021). As described in section 2.2, the initial step involved the utilization of heat-resistant α-amylase to liquefy the starch of the WB. The α-1,4-glycosidic bonds in the amylose and amylopectin chains were broken down to generate dextrin during liquefaction and decrease the viscosity of the gelatinized WB slurry. In the second step, glucoamylase was applied for saccharification, to obtain fermentable sugar such as glucose (Pietrzak & Kawa-Rygielska, 2014).

Table 2. Chemical composition of WB.

Parameters	Values
Moisture Content	10.663 ± 0.334 *
Total Protein (dry base)	14.598 ± 0.375
Ash (dry base)	1.1004 ± 0.116

*Mean of three replicates ± Standard deviation

The maximum reducing sugar concentration achieved was 123.282 ± 0.924 g/L, while the initial quantity was 1.285 ± 0.195 g/L. Therefore, EH demonstrated a high potential to produce fermentable sugar from WB for utilization in fermentation as an economical carbon source. The reducing sugar production obtained in this research was higher than previous reports. Hudečková et al. (2017) investigated the efficiency of enzymatic hydrolysis using a 15% (w/v) of WB and varying factors such as temperature, pH, and time. They reported that the final amount of produced glucose under optimal conditions was 70.28 g/L (Hudečková et al., 2017). Similarly, W. Han et al. (2017) applied *Aspergillus* hydrolytic enzymes to generate utilizable sugar for ethanol production and achieved a maximum glucose production of 40.58 g/L after 24 hours at 55 °C (W. Han et al., 2017). Furthermore, WB was reused as a substrate for cultivating bakery yeast. 25% (w/v) of the WB was transformed into a gel-like substance by heating it up to 60 °C for 15 minutes. Then, the resulting solution was subjected to a hydrolysis process using amylase and glucoamylase enzymes, with the inclusion of protease in the second step. Ultimately, total amount of produced reducing sugar was 57.6 g/L (without protease) and 60.6 g/L (with protease) (Benabda et al., 2018).

3.3. Analysis of GAP by RSM

Table 3 represents a summary of the experimental and predicted GAP, with the highest and lowest yields obtained in runs 7 (22.656 ± 0.063 g/L) and 9 (10.204 ± 0.083 g/L), respectively. Table 4 reports the degree of freedom, sum of squares, mean squares, significant level (*P*-value), and Fisher test (*F*-value) for each term to assess their statistical significance. GLU responses were modeled using linear, two-factor interaction (2FI), quadratic, and cubic models. After statistical analysis, the quadratic model was identified as the most fitting model (p-value < 0.05 and Lack of Fit p-value > 0.05) (Table 5).

3.4. Checking of model adequacy

In general, once an acceptable optimization model is obtained, it is necessary to verify its predictive ability to confirm that the model is adequate for approximating the system. Various statistical expressions can be used to check the adequacy of the model (Breig & Luti, 2021). Therefore, the coefficient of determination (R^2), adjusted determination coefficient ($R^2_{adjusted}$), predicted determination coefficient ($R^2_{predicted}$), *F*-value, *p*-value, and lack of fit were considered. Table 6 represents the statistical parameters for the quadratic model.

The GAP model was found to be significant, as approved by the *F*-value of 44.74 and p-value < 0.05. Additionally, linear coefficients (A, B, C, D), quadratic term coefficients (A^2, B^2), and interaction term coefficients (AD, BD) were all found to have *p*-value less than 0.05, indicating their significant effects on GAP. The impact of each variable on GAP can be determined by its corresponding *F*-value (Witek-Krowiak et al., 2014). The RSCH with the highest *F*-value has the greatest influence on the GAP.

A model with an R^2 value close to 1.0 indicates excellent prediction efficiency (Breig & Luti, 2021). This model showed a high coefficient of determination (R^2) of 0.9766, which means that most of the variations in the data could be explained, and a close correlation between effective variables and response and their key role in the prediction of the GAP model. Moreover, $R^2_{adjusted}$ was found to be nearly equal to R^2, indicating that the model is suitable for predicting experimental data associated with GAP. $R^2_{predicted}$ determined by PRESS describes the model ability to predict new responses. PRESS value is a measure utilized to assess model ability to predict new responses (Breig & Luti, 2021). The preferable result is a lower value of PRESS, which in current study, the PRESS was obtained as 32.18. A signal-to-noise ratio greater than 4 is considered ideal by Adeq Precision. The calculated Adeq Precision of 20.0449 indicates a satisfactory signal (Table 6).

The model accuracy can be assessed through a lack of fit value. This index determines the model inadequacy by comparing pure error to residual error from the experimental design points of replication and center points in the design (Breig & Luti, 2021). The lack of a fit should not be significant (p-value > 0.05). According to the ANOVA findings, the *p*-value of lack of fit was less than 0.05 (Table 4). In statistical studies, residuals are the differences between the observed and predicted values of the model. A normal plot of residuals is a useful graph for evaluating whether a dataset conforms to a normal distribution (Breig & Luti, 2021). The normal plot of residuals, shown in Fig. 1-A, suggests that the regression model has a normal distribution. Also, plotting the predicted versus experimental responses constructs a helpful plot for model evaluation. In ideal mode, the data establishes a 45° line and any aggregation of points above or below this line indicates areas of over or underprediction. Fig. 1-B, illustrates the predicted values compared to the experimental values, indicating an acceptable agreement between the data. Residual vs. predicted and residual vs. runs plots are displayed in Figures 1-C and 1-D, respectively. These plots demonstrated well-distributed data obtained from the GAP model.

3.5. GAP model

The relationship between variables and responses related to GAP was expressed through the development of second-order equations using multiple regression analysis on the experimental data (Date not shown). The equations, which include linear, quadratic, and interactive terms, are presented in terms of coded factors in Eq 3.

$$GAP = 21.19 + 2.3\,A - 0.4378\,B + 0.5259\,C - 0.4391\,D - 0.2808\,AB - 0.2455\,AC + 0.5402\,AD + 0.5745\,BC + 0.1306\,BD - 0.3878\,CD - 2.08\,A^2 - 3.38\,B^2 - 0.6693\,C^2 - 0.9388\,D^2 \quad (3)$$

Table 3. CCD with experimental and predicted values of GAP.

Run	Space Type	Uncoded and coded values of variables				GAP	
		A	B	C	D	Experimental	Predicted
		g/L	g/L	µg/L	% (v/v)		
1	Axial	40 (0)	7 (0)	5 (0)	10 (+1)	19.103 ± 0.578*	19.81
2	Center	40 (0)	7 (0)	5 (0)	6 (0)	20.204 ± 0.104	21.19
3	Factorial	20 (-1)	10 (+1)	0 (-1)	10 (+1)	10.398 ± 0.133	9.86
4	Axial	40 (0)	4 (-1)	5 (0)	6 (0)	18.244 ± 0.209	18.25
5	Factorial	60 (+1)	10 (+1)	0 (-1)	10 (+1)	15.808 ± 0.140	15.46
6	Factorial	20 (-1)	4 (-1)	0 (-1)	10 (+1)	11.122 ± 0.511	11.07
7	Center	40 (0)	7 (0)	5 (0)	6 (0)	22.656 ± 0.063	21.19
8	Axial	20 (-1)	7 (0)	5 (0)	6 (0)	16.460 ± 0.197	16.82
9	Factorial	20 (-1)	10 (+1)	0 (-1)	2 (-1)	10.204 ± 0.083	10.79
10	Factorial	60 (+1)	10 (+1)	10 (+1)	10 (+1)	16.293 ± 0.226	16.4
11	Center	40 (0)	7 (0)	5 (0)	6 (0)	19.638 ± 0.132	21.19
12	Axial	40 (0)	7 (0)	10 (+1)	6 (0)	21.297 ± 0.194	21.05
13	Axial	40 (0)	7 (0)	5 (0)	2 (-1)	21.221 ± 0.097	20.69
14	Center	40 (0)	7 (0)	5 (0)	6 (0)	21.844 ± 0.653	21.19
15	Axial	40 (0)	7 (0)	0 (-1)	6 (0)	19.566 ± 0.756	20
16	Factorial	60 (+1)	10 (+1)	0 (-1)	2 (-1)	14.188 ± 0.120	14.23
17	Factorial	60 (+1)	4 (-1)	0 (-1)	2 (-1)	17.257 ± 0.157	17.07
18	Factorial	60 (+1)	10 (+1)	10 (+1)	2 (-1)	16.466 ± 0.089	16.71
19	Factorial	60 (+1)	4 (-1)	0 (-1)	10 (+1)	17.379 ± 0.153	17.79
20	Axial	40 (0)	10 (+1)	5 (0)	6 (0)	17.189 ± 0.118	17.37
21	Axial	60 (+1)	7 (0)	5 (0)	6 (0)	21.587 ± 0.160	21.41
22	Factorial	20 (-1)	10 (+1)	10 (+1)	10 (+1)	11.407 ± 0.112	11.78
23	Factorial	20 (-1)	10 (+1)	10 (+1)	2 (-1)	14.897 ± 0.094	14.25
24	Center	40 (0)	7 (0)	5 (0)	6 (0)	21.652 ± 0.068	21.19
25	Factorial	60 (+1)	4 (-1)	10 (+1)	2 (-1)	16.959 ± 0.115	17.26
26	Factorial	60 (+1)	4 (-1)	10 (+1)	10 (+1)	16.819 ± 0.114	16.43
27	Factorial	20 (-1)	4 (-1)	10 (+1)	2 (-1)	13.147 ± 0.184	13.68
28	Factorial	20 (-1)	4 (-1)	10 (+1)	10 (+1)	10.954 ± 0.741	10.68
29	Factorial	20 (-1)	4 (-1)	0 (-1)	2 (-1)	12.849 ± 0.072	12.51
30	Center	40 (0)	7 (0)	5 (0)	6 (0)	21.710 ± 0.140	21.19

*Mean of three replicates ± Standard deviation.

Table 4. Analysis of Variance for GAP.

Source	Sum of Squares	df	Mean Square	*F*-value	*p*-value	
Model	415.69	14	29.69	44.74	< 0.0001	*
A-RSCH	94.84	1	94.84	142.9	< 0.0001	*
B-Urea Concentration	3.45	1	3.45	5.2	0.0377	*
C-Biotin Concentration	4.98	1	4.98	7.5	0.0152	*
D-Inoculum size	3.47	1	3.47	5.23	0.0371	*
AB	1.26	1	1.26	1.9	0.1881	
AC	0.9643	1	0.9643	1.45	0.2467	
AD	4.67	1	4.67	7.04	0.0181	*
BC	5.28	1	5.28	7.96	0.0129	*
BD	0.2728	1	0.2728	0.4111	0.5311	
CD	2.41	1	2.41	3.63	0.0762	
A^2	11.18	1	11.18	16.85	0.0009	*
B^2	29.67	1	29.67	44.71	< 0.0001	*
C^2	1.16	1	1.16	1.75	0.2059	
D^2	2.28	1	2.28	3.44	0.0834	
Residual	9.96	15	0.6637	-	-	
Lack of Fit	3.57	10	0.3568	0.2793	0.9592	
Pure Error	6.39	5	1.28	-	-	
Cor Total	425.64	29	-	-	--	

* Significant at 5% level.

Where *GAP* is response and *A*, *B*, *C*, and *D* are RSCH, urea concentration, Biotin concentration, and inoculum size respectively.

3.6. Effects of independent variables and Their Interactions on GAP

An effective method for analyzing the relationship between independent variables and GAP is utilizing 3D surface and 2D contour plots. These plots provide a visual representation of the regression equation and illustrate how two variables impact the response, while the remaining variables are maintained the center point (Breig & Luti, 2021). Fig. 2-4 depicted the interaction of RSCH and Urea concentration, RSCH and Biotin concentration, RSCH and Inoculum size, Urea concentration and Biotin concentration, Urea concentration and Inoculum size, and Biotin concentration and Inoculum size on GAP respectively.

Table 5. The suggested model for GAP.

Source	Sequential *p*-value	Lack of Fit *p*-value	Adj-R^2	Pre-R^2	
Linear	0.112	0.0057	0.1309	-0.0679	
2FI	0.9857	0.0029	-0.0903	-1.6812	
Quadratic	< 0.0001	0.9592	0.9548	0.9244	Suggested
Cubic	0.8713	0.8922	0.9349	0.8445	

Table 6. Statistical parameters for the quadratic model of GAP.

R^2	0.9766	**Std. Dev.**	0.8147
Adjusted R^2	0.9548	**Mean**	16.95
Predicted R^2	0.9244	**C.V. %**	4.81
Adeq Precision	20.0449	**PRESS**	32.18

3.6.1. Effect of RSCH on GAP

Different carbohydrates like glucose, fructose, and sucrose can be consumed by *C.glutamicum* (Uhde et al., 2013), but glucose has been recognized as the most suitable carbon source according to the previous researches (Alharbi et al., 2019; Das et al., 1995). Based on ANOVA results, it can be concluded that RSCH (as glucose-rich slurry) has the most heightened effect on GLU synthesis, with an *F*-value of 142.9 and *p*-value < 0.05 (Table 4). These findings were in line with those found in the literature (Alharbi et al., 2019; Bashir et al., 2022; R. S. Kumar et al., 2013; Sunitha et al., 1998).

High substrate concentration has an adverse effect on bacterial growth owing to the establishment of hypertonic environment (Das et al., 1995; R. S. Kumar et al., 2013). Khan et al. (2005) found that *C.glutamicum* growth was enhanced at elevated substrate concentrations (glucose) up to 50 g/L without any inhibitory effect. However, when the glucose concentration exceeded 50 g/L, *C.glutamicum* growth and GAP were decreased (Khan et al., 2005). Also, Alharbi et al. (2019) stated that an obvious correlation exists between GAP and glucose concentrations. Their research demonstrated that an increase in glucose concentrations up to 40-50 g/L results in an increase in GAP (Alharbi et al., 2019). The effect of RSCH on GAP is depicted in Fig. 5-A. According to this plot, by increasing RSCH up to 50 g/L, GAP was enhanced, and after that was decreased.

Based on the RSM results, the optimum RSCH level was 49.889 g/L, which is consistent with the earlier findings (Alharbi et al., 2019; Bashir et al., 2022). Bashir et al. (2022) explored the optimization of fermentation parameters such as temperature, agitation speed, and carbon source concentration to enhance GAP using *C.glutamicum*. They revealed that the optimal glucose level was 50 g/L, leading to a maximum GAP of 14.2 g/L under optimized conditions (Bashir et al., 2022). In a study conducted by Alharbi et al. (2019) the impact of process parameters on the GAP was examined. After analyzing the results, it was found that the optimal glucose concentration was 50 g/L (Alharbi et al., 2019).

However, Jin et al. (2020) reported that *C.glutamicum* GJ04 produced 39.8 g/L of glutamate from 60.3 g/L of glucose and 38.8 g/L of xylose (Jin et al., 2020). Another study was conducted on optimizing fermentation parameters in GAP. They found that the optimal glucose level was 61.5575 kg/m^3 (Sunitha et al., 1998).

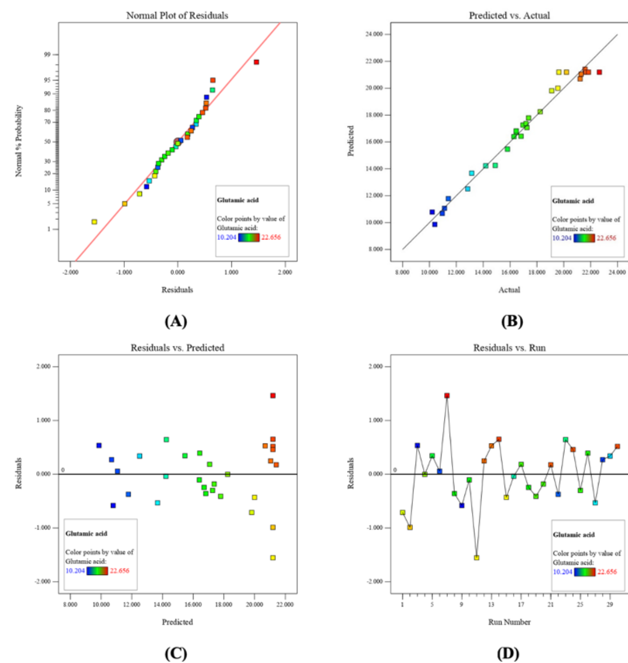

Fig. 1. A) Normal plot of residuals, B) Predicted values vs. actual values plot, C) Residual vs. predicted values plot, and D) Residual vs. run number.

3.6.2. Effect of urea concentration on GAP

The impact of nitrogen sources on amino acid synthesis is significant since there is a direct correlation between nitrogen metabolism and amino acid production (Schulz et al., 2001). Urea was identified as the preferred nitrogen source for *C.glutamicum* (Alharbi et al., 2019), which may be attributed to the robust urease activity of the bacterium (Ganguly, 2023). Besides, Yang et al. (2021) conducted a study that revealed *C.glutamicum* has a higher preference for organic nitrogen sources, such as urea, compared to inorganic nitrogen sources like $(NH_4)_2SO_4$ (Yang et al., 2021). Based on the results of the statistical analysis, it was determined that the urea concentration has a significant impact on the GLU accumulation (*p*-value < 0.05). Nevertheless, a study reported that an adequate concentration of urea leads to an increase in GAP. However, excessive concentration of urea can impede cell growth (Alharbi et al., 2019; Jyothi et al., 2005). As depicted in Figure 5-B, GLU accumulation increased, with the increase in urea concentration within the range of 5-7.5 g/L. It declined at higher concentrations owing to release of surplus ammonium ions during urea hydrolysis (Jyothi et al., 2005).

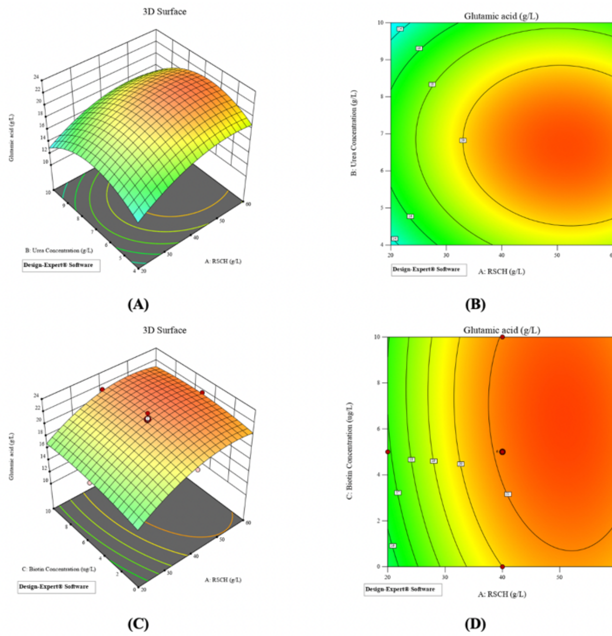

Fig. 2. 3D and, 2D plots of interaction of independent variables on GAP. (A) and, (B): RSCH and Urea concentration, (C) and, (D): RSCH and Biotin concentration (Other variables held in center point).

The optimal urea concentration was determined to be 6.814 g/L. This finding is in line with those found in the literature (Fahimitabar et al., 2021; Sunitha et al., 1998). Fahimitabar et al. (2021) demonstrated the significant impact of urea on GAP. They reported that 0.3 g/dL was the optimal urea concentration, which resulted in 19.84 mg/mL GAP under optimized conditions (Fahimitabar et al., 2021). Sunitha et al. (1998) utilized RSM to investigate the optimal urea concentration. Their findings showed that the optimum urea concentration was 7.3272 Kg/m^3.

3.6.3. Effect of biotin concentration on GAP

Biotin, as a cofactor for acetyl-CoA carboxylase, plays a crucial role in fatty acid synthesis in *C.glutamicum*. This biotin auxotrophic bacterium is unable to produce GLU under normal growth conditions in the presence of excess biotin (Wen et al., 2018). Biotin is essential for *C.glutamicum* growth in trace quantities (X. Han et al., 2019). However, the absence of biotin suppresses cell growth, while excessive biotin supply hinders GLU secretion by reducing the permeability of the bacterial cell wall (Fahimitabar et al., 2021; Tryfona & Bustard, 2004). To overcome this issue, certain surfactants such as Tween 40, β-lactam antibiotics such as penicillin can be used to disrupt the over-strengthened cell structure and activate GLU secretion (Hirasawa & Wachi, 2017), as well as redirect the carbon flux to GLU synthesis (Wen et al., 2018).

Based on the RSM findings, it was determined that the optimal level of biotin concentration in the GAP was 6.570 µg/L. This finding is consistent with a study conducted by Fahimitabar et al. (2021), which investigated the parameters affecting GAP by *C.glutamicum* PTCC 1532. They reported that under optimized conditions, 9 µg/L was the optimum biotin concentration for achieving 19.84 mg/mL GAP. Moreover, Yang et al. (2021) demonstrated that a concentration of 10 g/L of biotin is sufficient for the growth of *C.glutamicum* ATCC 13032 and for glutamate secretion (Yang et al., 2021).

It was observed that the addition of 5.6 µg/L biotin led to a faster glucose consumption and cell growth in *C.glutamicum*, while simultaneously, GAP was suppressed. Besides, when biotin concentration was reduced to 2.5 µg/L, glucose consumption, and cell growth were restricted, but GLU generation increased. However, when the biotin concentration was further reduced to 0.0625 µg/L, GLU generation declined (X. Han et al., 2019). These findings are consistent with our results, which showed that higher concentrations than optimal biotin level (6.570 µg/L) can decrease GLU secretion (Fig. 5-C).

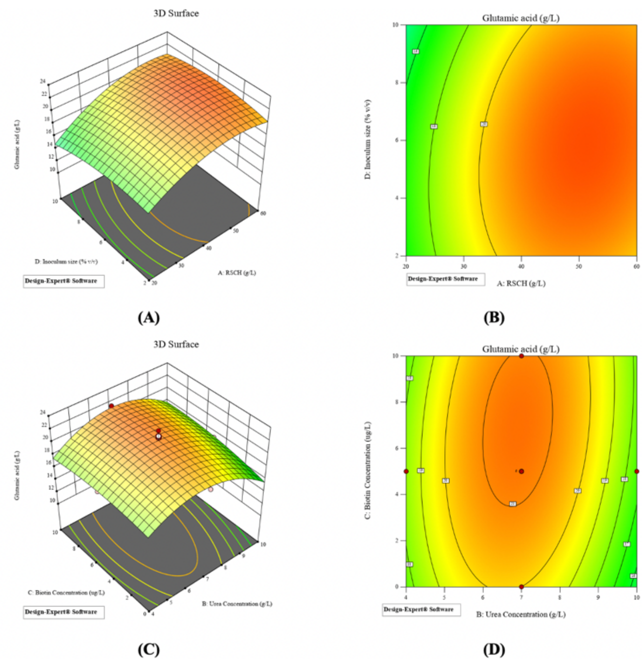

Fig. 3. 3D and, 2D plots of the interaction of independent variables on GAP. (A) and, (B): RSCH and Inoculum size, (C) and, (D): Biotin concentration and Urea concentration (Other variables held in center point).

3.6.4. Effect of inoculum size on GAP

The inoculum size is a critical factor in GAP, which depends on the bacterial strain and the culture condition, as stated by Tavakkoli et al. (2012). In the current study, the optimal inoculum size was determined 5.564% (v/v) which is in agreement with the results reported by (Alharbi et al., 2019; Das et al., 1995; R. S. Kumar et al., 2013).

According to Jyothi et al. (2005), the amount of GLU produced is directly proportional to the inoculum size, ranging from 3-7%, and an inoculum size of more than 10% can inhibit the production of GLU (Jyothi et al., 2005). Similarly, Alharbi et al. (2019) found that increasing the inoculum size resulted in higher GAP efficiency. They reported that the optimal level of inoculation was 5% (v/v) (Alharbi et al., 2019). Das et al. (1995) reported that the highest GAP was obtained under optimum inoculum size of 6% (v/v) (Das et al., 1995). However, the results of Tavakkoli et al. (2012) do not completely align with those of the other studies mentioned, as they

did not observe significant changes in GLU production with increasing inoculum size (Tavakkoli et al., 2012).

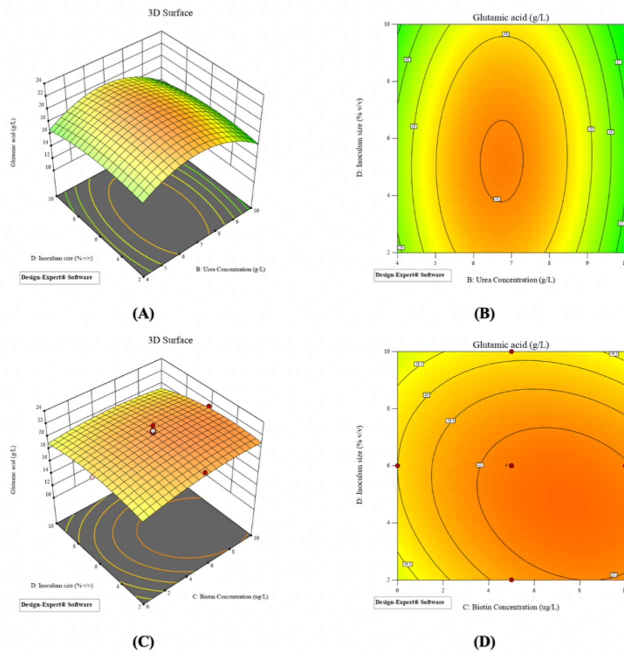

Fig. 4. 3D and, 2D plots of interaction of independent variables on GAP. (A) and, (B): Urea concentration and Inoculum size, (C) and, (D): Biotin concentration and Inoculum size (Other variables held in center point).

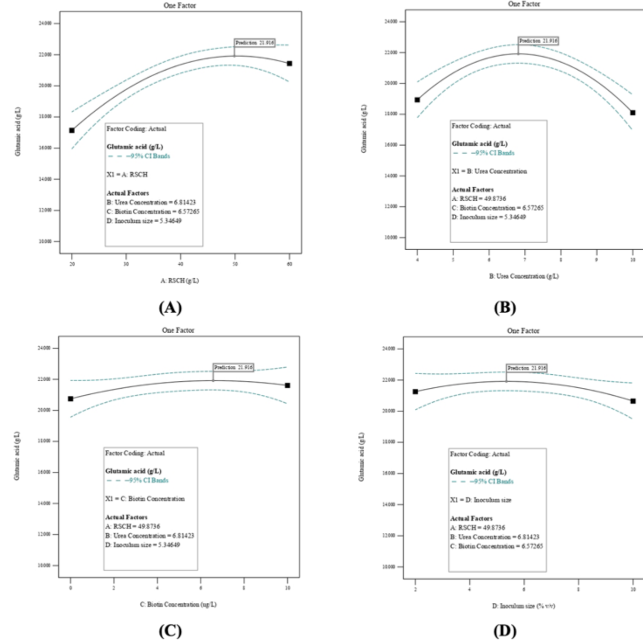

Fig. 5. Optimum points of effective factors on GAP. (A): RSCH, (B): Urea concentration, (C): Biotin concentration, (D): Inoculum size.

3.7. Verification of the Fitted Model and Optimum Point

Table 7 illustrates the optimum level of independent variables including RSCH, urea concentration, biotin concentration, and inoculum size. The verification of the optimization model was conducted through experimentation with the optimized conditions of independent variables. Predicted GAP was 21.916 g/L under optimized conditions, which was validated through experimental values performed in triplicate. The GLU yield was 21.34 ± 0.204 g/L under the optimized conditions. The desirability of the model was determined to be 0.839, which signifies an ideal response value for the obtained model.

Table 7. The optimal levels of effective variables in GAP.

Independent Variables				GAP		Desirability
A	B	C	D	Predicted	Actual	
49.889	6.812	6.57	5.339	21.916	21.34 ±0.204	0.839

A: RSCH (g/L)
B: Urea Concentration (g/L)
C: Biotin Concentration (µg/L)
D: Inoculum size (% v/v)

As shown in Fig. 6, bacterial cell growth, sugar consumption, and GAP were monitored during the fermentation process under optimized conditions. After about 10 hours of fermentation, *C.glutamicum* entered the exponential phase, which was followed by the stationary phase. In the stationary phase, most of the sugar in the environment was consumed, leading to an increase in GAP. However, the plot indicates that not all the sugar in the environment was utilized, and approximately 15 g/L of glucose remained in the medium after 24 hours of fermentation.

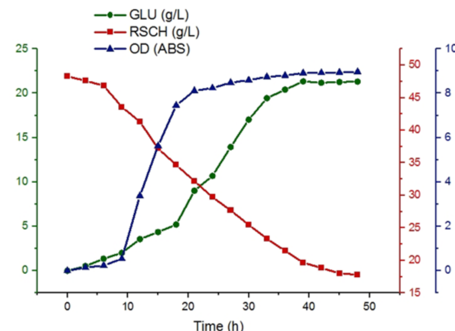

Fig. 6. Profile of GAP, sugar consumption and bacteria cell growth during fermentation process.

4. Conclusion

In this study, we applied WB, as cost-effective starchy waste, to produce fermentable substances through EH. This process resulted in a significant increase in reducing sugar concentration from 1.285 ± 0.195 g/L to 123.282 ± 0.924 g/L. Subsequently, the obtained hydrolysate was utilized as a carbonic source for the GLU synthesis by *C.glutamicum* PTCC 1532. To enhance the GLU yield, RSM was utilized to optimize the independent variables. The optimum levels of reducing sugar concentration of hydrolysate, urea concentration, biotin concentration, and inoculum size was 49.889 g/L, 6.812 g/L, 6.57 µg/L, and 5.339% (v/v), respectively. Under these optimized conditions, the experimental GAP was 21.34 ± 0.204 g/L, which demonstrated a reasonable correlation between the predicted and experimental results. In conclusion, the results illustrated that WB

can serve as a low-cost carbon source for producing valuable compounds such as GLU. This approach not only reduces food waste but also decreases production costs.

Acknowledgments

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Conflict of interest

The authors declare that there is no conflict of interest.

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