Research Article

Fermentative Scale-up Production of L-Glutamic Acid Using Response Surface Methodology

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ABSTRACT

Glutamic acid is an industrial important biomolecule because; it is widely used in food, pharmaceutical, medical, agricultural and chemical industries. Glutamic acid was produced with various kinds of raw materials using sub-merged fermentation of palm waste hydrolysate, cassava starch, sugar cane bagasse, date waste etc. The present study is aimed to determine the significant relationship between the dependent variable glutamic acid yield and the independent factors like urea, biotin and salt concentration which strongly influences the glutamic acid production by the microbial culture through Response surface methodology. The bacterium *Corynebacterium glutamicum* DSM 20300^T was used for the glutamic acid fermentation. The fermentation medium contains 1Kg of fruit pulp, urea concentration 2.0g/L, biotin concentration 3µg/L, mineral salt solution concentration 120ml/L (contains K_2HPO_4:0.08 g, KH_2PO_4: 0.08 g, $MgSO_4$: 0.04 g, $MnSO_4$: 0.001 g, $FeSO_4$: 0.001g, Distilled Water: 1L) was carried out at optimum conditions maintained were Time 3d, Temperature 30^{0}C, pH 5.0, Penicillin concentration 1000U/L added after 24h of fermentation. Fermentation was performed in a 5L fermenter. The optimization of glutamic acid production was performed using a statistical design called Response surface methodology. The obtained glutamic acid in the fermentative broth was quantitatively estimated by ninhydrin method and the glutamic acid crystals produced after evaporation were subjected to Fourier transform Infrared spectroscopy (FTIR). The ANOVA of the regression model proves that the model is more significant, as is apparent from the Fischer's F-test (F_{model}=16.51) and a very low probability value (P_{model}>F=0.000). In this report, the determination coefficient value was (R^2=0.884) showed that 94% of the variability in the response could be explained by the model. In addition, the adjusted determination coefficient (Adj R^2=0.884) value was too high which specifies the significance of the model. The final glutamic acid crystals concentration obtained in the fermenter was 30.2g/L. Since the raw materials *Mimusops elengi* Linn fruits are freely available in large quantities, it considerably reduces production cost. These studies highlight the possibility of producing glutamic acid at a commercial level using this inexpensive carbon source.

Keywords: L-Glutamic acid, Fermentation, RSM, Scale-up.

INTRODUCTION

Glutamic acid is a broadly spreaded amino acid and indigenously exists in all living cells. It is one of the richest amino acids present in all known proteins at a larger portion. Both plant and animal proteins contain glutamic acid in a bound form. It has the largest commercial demand among the various amino acids. It is of industrial importance because; it is widely used in food, pharmaceutical, medical, agricultural and chemical industries. For the manufacture of L-Glutamate both chemical and microbial methods were used but the Japanese researchers found that the glutamate produced by chemical methods was a racemic mixture of D and L forms[1]. Later on they developed a direct fermentation process for L-Glutamate using different media and they nearly screened 2000 microorganisms to know the potency of the microorganisms able to produce L-Glutamic acid and found that so many different Bacteria, Streptomycetes, Yeast and Fungi able to secrete L-glutamic acid[2]. The discovery of *C. glutamicum* has also contributed to the development of amino acid fermentation technologies. Consequently, amino acid fermentation has been recognized as one of the big successes in the production of industrially important materials by microbial cells. However, the major glutamate producing strains belonging to the genera *Corynebacterium*, *Brevibacterium*, *Microbacterium* and *Arthrobacter*. These organisms are Gram positive non spore forming and non-motile bacteria and resemble both morphologically and physiologically. L-glutamic acid was mainly produced by microbial fermentations and the chemical mode of synthesis is not widely preferred due to the formation of racemic mixture[3]. In biotechnological processes, *Corynebacterium* species are used for economic production of glutamic acid by submerged fermentation[4]. L-glutamic acid is produced per year using coryneform bacteria. A number of fermentation techniques have been used for the production of glutamic acid[5,6]. Glucose is one of the major carbon sources for production of glutamic acid. Glutamic acid was produced

with various kinds of raw materials using sub-merged fermentation of palm waste hydrolysate, cassava starch, sugar cane bagasse, date waste[7,8].

The other most important intent of the current research was to optimize the basal nutrients and fermentation parameters like urea, biotin, mineral salt solution and penicillin concentrations, pH, temperature, time and agitation for getting high glutamic acid production using empirical optimization strategy or traditional 'one-factor-at-a-time' (OFAT) method. This method is depended on the classical method and involves varying one factor while keeping the other factors unchanged under a specific set of conditions [10]. OFAT method is a closed ended system useful for optimization of both medium ingredients and physical conditions of the fermentation.

The method is simple, easy and can also be useful to study the effects of process parameters in the form of graphs. It is the most popular method of optimization. Most of the previous works related to glutamic acid production were conducted using OFAT method but recently an experimental design called Response surface methodology (RSM) was used by Mahmoud Tavakkoli *et al*. (2012) to study the main and interaction effects of various factors and the production of glutamic acid[9]. This was the first report of using RSM for the glutamic acid fermentation. It is a statistical method used to find out the relationships among at least one dependent response and a number of input factors. RSM comprises full factorial CCD and regression analysis.

Furthermore, the method also determines the effective factors and building models to study the interaction and select optimum conditions of variables for a desirable response. Hence RSM is a sequential procedure of leading the experimenter rapidly and efficiently to the general vicinity of the optimum. In recent times, the experimental factorial design have been efficiently applied to optimize the medium and cultural conditions for the production of a variety of enzymes, primary and secondary metabolites[10]. The downstream process often involves the determination of glutamic acid by ninhydrin method or chromatographic methods and for the glutamic acid confirmation different analytical methods like Infrared spectroscopy, Nuclear magnetic resonance spectroscopy, Mass-spectroscopy, and High-pressure liquid chromatography are generally employed. *Mimusops elengi* L. (sapotaceae) is native to Western peninsula and popularly known as bullet wood or Spanish cherry or Medlar. It is found in South India in dry ever green forests and also extends to Andaman, Martaban, Tennasserim, Burma and Western Ghats. In the Eastern Ghats it is found in dry areas, frequently on laterite and in comparatively small in size. It is mostly found in North Western Himalayas, Western Ghats, Eastern Ghats, Central Deccan plateau, East coast, West coast, and Indo-gangetic plain and outlying Islands. Nazarudeen (2010) had reported that bakul fruit contain Moisture 79.27%, Protein 1.29%, Fat 2.76%, Total sugar 15.2%, Fiber 1.13%, Vitamin-C 3.27mg, Mineral content 0.32%, Fe 0.59mg, Na 5.16 mg, and K 98.54 mg[11].

The present study is aimed to determine the significant relationship between the dependent variable glutamic acid yield and the independent factors like urea, biotin and salt concentration which strongly influences the glutamic acid production by the microbial culture. The current study was designed specifically to enhance the glutamic acid yield rates by scaling the production in a 5L Sartorius B production bioreactor or fermentor with the higher yielding strain of microorganism and the study also includes the statistical analysis through one-way ANOVA was performed for all the experiments and Response surface methodology (RSM) was done for *Mimusops elengi* fruits using STATISTICA 24.0 version.

MATERIALS AND METHODS

Microorganisms

The bacterium *Corynebacterium glutamicum* DSM 20300^T was obtained from the National chemical laboratory, Pune, India. The bacterial cultures was maintained on a basal nutrient agar slopes and stored in a refrigerator at 4^{0}C or also maintained in a glycerol stocks at -70^{0}C.

Fermentation medium and Fermentation conditions

Fresh ripened fruits of *M.elengi* were collected and cleaned with tap water. The seeds were removed and the pulp was sterilized in an autoclave. The fermentation medium contains 1Kg of fruit pulp, urea concentration 2.0g/L, biotin concentration 3µg/L, mineral salt solution concentration 120ml/L (contains K_2HPO_4:0.08 g, KH_2PO_4: 0.08 g, $MgSO_4$: 0.04 g, $MnSO_4$: 0.001 g, $FeSO_4$: 0.001g, Distilled Water: 1L),was carried out at optimum conditions maintained were Time 3d, Temperature 30^{0}C, pH 5.0, Penicillin concentration 1000U/L added after 24h of fermentation. Fermentation was performed in a 5L fermenter. In the present research, a Sartorius B 5L fermenter was used to transferred the research process developed in shake flasks from an independent development laboratory and rapidly scaled up to a fermenter.

The reactor culture system includes total vessel volume-5L, agitation-2 impellers, Impeller-3 large blades 45^{0}pitch down, pH control-Active: CO_2/HCO_3^- ,DO control-Active:O_2 enrichment and the Gas flow control-solenoid valve and Rota meter, Sparge gassing-DO and pH control, sparger design-1x6mm id, O_2 enrichment-pulsed and CO_2 enrichment-pulsed. At the end of the fermentation, the fermented broth samples were subjected to precipitation at isoelectric pH (3.2) of glutamic acid and the resultant glutamic acid cake was severally washed with water to remove impurities and quantitative analysis was performed with ninhydrin method. Upon drying in a vacuum desiccator, glutamic acid crystallizes out and the obtained crystals were subjected to Fourier transform Infrared spectroscopy (FTIR).

Statistical methodology by Central composite design

The optimization of glutamic acid production was performed using a statistical design called Central composite design matrix. Three independent variables namely urea concentration, biotin concentration and mineral salt solution concentration were selected to know their effect on glutamic acid production a dependent variable. Each factor was examined at three levels low, basal and high. A complete or a fraction of a 2^K factorial design in which each factor level can be generally coded to -1, +1 values and is termed as factorial portion of the design. A star design is an additional design where the experimental points are situated at a distance from its centre. No central point. Thus the total number of design points in CCD consisting of K variables is given as $N= 2^K+2k+n_0$. A 2^3 full-factorial experimental design is the principal class of second order design a CCD, was employed for the RSM in the experimental design. The CCD assigns a suitable amount of data for checking lack of fit while not including a huge number of design points and CCD is ideal for sequential experimentation. To make the design rotatable, the variance of the predicted response remains constant at the points which are equidistant from the design center. For a CCD to be rotatable, the star arm α is chosen from the condition such that $\alpha=2^{K/4}$. Centre points are repeated for every block of experiments to have a check on whether the experiments are conducted under the same conditions in every block.

Based upon the equation ($Xi= (X_i- xi) /\Delta xi$) the variables are coded. In order to calculate the predicted response a second order polynomial equation was used which includes all interaction terms. Twenty experiments were done with different combinations of the variables as indicated by the CCD for each set.

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_{11}X_1^2+ b_{22}X_2^2+b_{33}X_3^2+b_{12}X_1X_2+ b_{23}X_2X_3+b_{31}X_1X_3$$

Where Y is the dependent variable (Glutamic acid yield); X_1, X_2 and X_3 are the independent variables as mentioned above; b_0 is the regression coefficient at the centre point; b1, b2 and b3 are linear coefficients; b12, b13 and b23 are second order coefficients; and b11, b22, b23 and b33 are quadratic coefficients. The values of the coefficients and the optimum levels were calculated using SAS 9.0 software version. The quality of the fit of the polynomial model equation was expressed as the coefficient of determination (R^2).

RESULTS

The glutamic acid concentrations with 20 experimental runs and with different combinations of urea, biotin and mineral salt solution concentration were evaluated. 2^3 full factorial design with five coded levels were showed in the Table 1. The CCD matrix was given in Table 2. The calculated predicted values from the above equation for glutamic acid concentration as well as with the experimental values were depicted in the Table 3. The coefficients which were computated by multiple linear regression analysis (equation above) were showed in the Table 4 which specifies that it includes 3 linear, 3 quadratic and 3 interaction terms and one block term. For each coefficient, their significance was estimated by student's t-test and p-test and the values were represented in the Table 4. The corresponding coefficient was found to be more significant if it have a smaller 'p' value and larger magnitude of 't' value. This implies that the first order and second order main effects of urea, biotin and mineral salt concentration were highly significant and is proved from their relevant 'p' values. They were found to be highly significant at the second order. This clearly demonstrates that they can act as limiting nutrients and even a very narrow change in their concentrations will vary the rate of product formation or growth rate or both to a great extent. The interaction effect of biotin x urea, urea x mineral salt concentration and biotin x mineral salt concentration were insignificant ($p\leq0.05$). The second order response surface model results were fitted in the form of ANOVA and showed in the Table 5. It is essential to analyze the significance and adequacy of the model. The Fischer variance ratio, the F-value ($=S^2_r/S^2_C$) is a statistically valid measure of how properly the factors explain the variation in the data about its mean. The larger the F-value is from unity, the more absolute is that the factors describe adequately the variation in the data about its mean, and the determined factor effects are real.

The ANOVA of the regression model proves that the model is more significant, as is apparent from the Fischer's F-test ($F_{model}=16.51$) and a very low probability value ($P_{model}>F=0.000$). The determination coefficient (R^2) was used to check the goodness of the fit of the model. The R^2 value presents a measure of much variability in the observed response values can be elucidated by the experimental variables and their interactions. The R^2 value is always between 0 and 1. If the R^2 value is closer to 1, indicates the model is stronger and the better it predicts the response. In this report, the determination coefficient value was ($R^2=0.884$) showed that 94% of the variability in the response could be explained by the model.

In addition, the adjusted determination coefficient (Adj $R^2=0.884$) value was too high which specifies the significance of the model. The Figures 2-4 showed the three dimensionsal response surface plots of effect of urea, biotin and mineral salt solution concentration on the glutamic acid concentration. The final glutamic acid crystals concentration obtained in the fermentor was 30.2g/L. FTIR of glutamic acid (Figure 5) shows peak wave number values of all functional groups 3061.49cm^{-1} for N-H, 1613.75cm^{-1} for C=O, 2742.03 cm^{-1} for C-H exhibiting a similar frequency to standard values of glutamic acid.

Table 1: Independent variables used in the experimental design

Variables	Coded levels				
	–1.682	–1	0	+1	+1.682
Urea (g/l), X_1	0.659	1	1.5	2	2.341
Biotin (µg/l), X_2	1.159	1.5	2	3.0	3.409
Salt solution concentration (%v/w), X_3	6.59	10	15	20	23.41

Table 2: CCD matrix for three independent variables of glutamic acid production

The CCD matrix employed for three independent variables			
Run No.	X_1	X_2	X_3
1	-1	-1	-1
2	-1	-1	+1
3	-1	+1	-1
4	-1	+1	+1
5	+1	-1	-1
6	+1	-1	+1
7	+1	+1	-1
8	+1	+1	+1
9	-1.682	0	0
10	1.682	0	0
11	0	-1.682	0
12	0	1.682	0
13	0	0	-1.682
14	0	0	1.682
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0
19	0	0	0
20	0	0	0

Table 3: CCD matrix having real values along with the experimental and predicted values of L-Glutamic acid concentration

Run No.	X_1	X_2	X_3	L-Glutamic acid concentration (g/L)	
				Experimental	Predicted
1	1	1.5	10	6.37	9.4622
2	1	1.5	20	8.41	8.0340
3	1	3.0	10	11.58	12.3180
4	1	3.0	20	13.41	15.3048
5	2	1.5	10	10.12	12.6818
6	2	1.5	20	5.29	8.3486
7	2	3.0	10	6.73	8.2326
8	2	3.0	20	6.83	5.6344
9	0.659	2	15	9.35	7.6028
10	2.341	2	15	4.56	2.7578
11	1.5	1.159	15	4.52	10.2552
12	1.5	2.841	15	11.83	10.2153
13	1.5	2	6.59	21.37	18.1352
14	1.5	2	23.41	18.53	17.0954
15	1.5	2	15	24.63	24.2652
16	1.5	2	15	24.73	24.2652
17	1.5	2	15	24.13	24.2652
18	1.5	2	15	24.10	24.2652
19	1.5	2	15	24.18	24.2652
20	1.5	2	15	24.15	24.2652

Figure 1: CCD matrix having real values along with the experimental and predicted values of L-Glutamic acid concentration

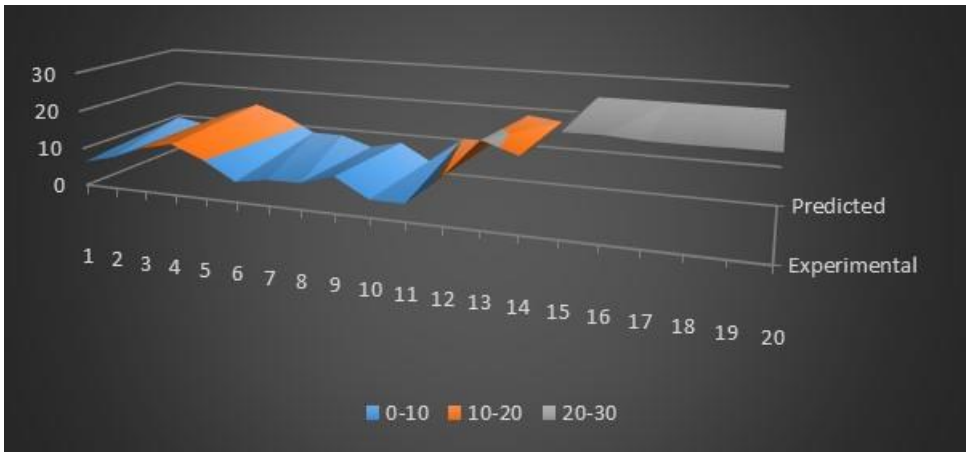


Table 4: Model coefficients estimated by multiple linear regression (significance of regression coefficients)

	Coefficient value	Standard error Coefficients	t-Value	p-value
Coefficient constant	65.23	24.70	2.640	0.020
Urea concentration	-13.41	4.60	-2.916	0.012
Biotin concentration	7.56	3.26	2.318	0.040
Salt concentration	-0.66	0.29	-2.236	0.040
Urea x Biotin concentration	-0.66	0.24	-2.775	0.020
Urea x salt concentration	-0.33	0.19	-1.772	0.100
Biotin x salt concentration	-0.43	0.17	-2.568	0.020

Table 5: ANOVA for the entire quadratic model

Source of variation	Sum of squares (SS)	Degree of freedom (d.f.)	Mean squares (MS)	*F*-value	Probe>*F*
Model	1068.943	6	178.16	16.51	0.0000
Error	140.3	13	10.79		
Total	1209.25	19			
R= 0.94; R^2= 0.884 ; $P_{model} > F$ = 0.0000					

Figure 2: Response surface plot for the effect of urea and biotin concentration on glutamic acid yield

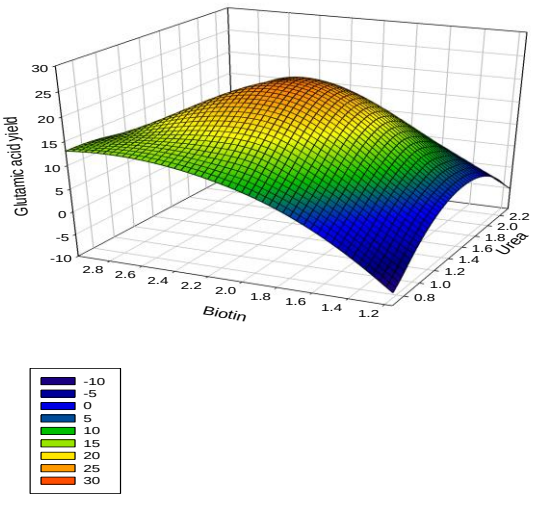
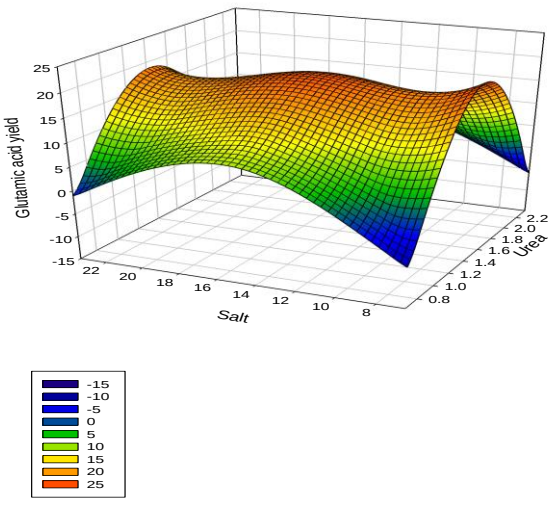


Figure 3: Response surface plot for the effect of biotin and salt concentration on glutamic acid yield



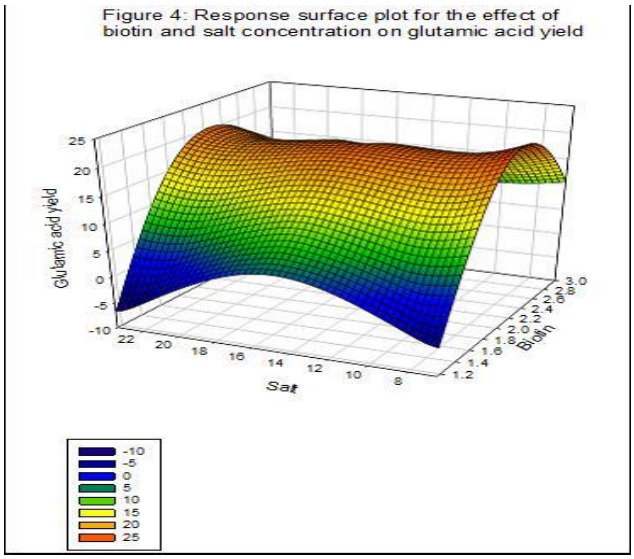
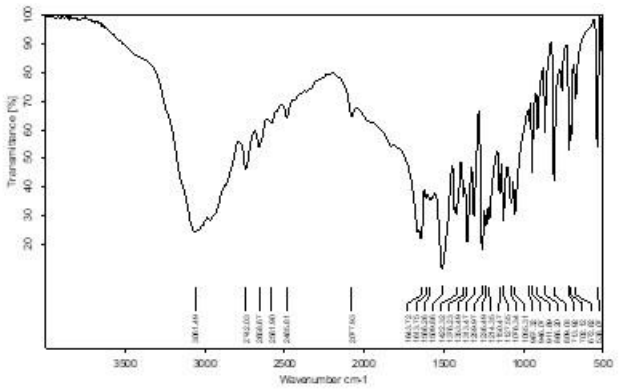


Figure 5: Fourier Transform Infrared Spectroscopy of glutamic acid



DISCUSSION

The present results revealed that high quantity of glutamic acid yield 30.2g/L was obtained from *M.elengi* using RSM methodology and implies significant increase in the glutamic acid yields during scale-up production. The yields obtained in the present study were significantly higher than reported earlier from the substrates like Date syrup (24g/L) by Ahmed et al. (2013)[12], corn pomace, cassava peel and pine apple waste (6.2g/L) by Lawal et al. (2011)[13], Date fruit waste (6g/L) by Davati et al. (2007) [14], Potato waste (5.6g/L) by Nasab et al. (2010)[15], sugarcane baggase (3.86g) by Jyothi et al. (2005) [16] , cassava starch hydrolysate (25g/L) by Nampoothiri and Pandey (1996) [17] , Mexican lime waste (13.75g/L) by Islas-Murguia et al. (1984) [18], but lesser from date waste juice (33.2g/L) by Mouffok Abdenacer et al. (2012). The Korean society for biotechnology and Bioengineering, 17, 795-803. (2012)[19], palm waste hydrolysate (88g/L) by Das et al. (1995)[20], Sugar cane baggase enriched with 10% glucose liberated glutamic acid 80mg/g of dry sugar cane baggase Nampoothiri and Pandey (1996)[17] and sugar beet molasses 100g/L Crueger and Crueger (2004) [21]. In view of the fact that, tons of these raw materials are being wasted or unutilized and containing a richest amount of sugars and other organic compounds conversion of these raw materials to a useful product like glutamic acid is a novel thought. Several findings from this study had proved that, the glutamic acid fermentation investigated in the present research was a cost-effective process and one of the best method for the production of opulent yield of glutamic acid was achieved by employing response surface methodology by using CCD matrix.

CONCLUSION

The present study used the methodology Response surface technique for the first time in production of glutamic acid using an inexpensive carbon source *Mimusops elengi* Linn. Relatively high yield of glutamic acid 30.2g/L was obtained in a fermenter from this carbon source which was higher than the yield obtained by the other studies. Fermentation conditions were optimized for glutamic acid production utilizing RSM technology to determine the effect of independent variables like urea, biotin and salt concentration on glutamic acid production. Since the raw materials are freely available in large quantities, it considerably reduces production cost. These studies highlight the possibility of producing glutamic acid at a commercial level.

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