Optimization of medium composition for microbial production of glutamic acid from Date fruit wastes using fractional factorial method

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Abstract

Date wastes, one of the abundantly available carbohydrate agricultural wastes in the South and East of the Mediterranean, were served as a novel substrate for the production of glutamic acid, the precursor of monosodium glutamate. A fractional factorial design was used to investigate the effects of variables, namely biotin content, urea content and agitation speed on the response glutamic acid production. A second order polynomial model was used to predict the response. Agitation speed was found to significantly influence the glutamic acid production. The highest glutamic acid production was recorded at agitation speed ranging 240-250 rpm and urea concentration of 2.8-3 g/l. The study showed that the wastes from dates could serve as a low-cost substrate for glutamic acid production.

Keywords: Glutamic acid, Date wastes, Submerge, Agitation speed, Fractional factorial design, Biotin

1- Introduction

L-amino acids have a wide spectrum of commercial uses as food additive, feed supplements, infusion compounds, therapeutic agents and precursors for the synthesis of peptides or agrochemicals [1]. In the food sector, more than 1,000,000 tons of L-glutamate is used per year as a flavor enhancer of different spices in food like sauces and in ready-to-eat meals, typically in concentrations between 0.1 and 0.4 %. Monosodium glutamate (MSG), the sodium salt of glutamic acid is widely used as a long established seasoning or flavor enhancer to improve the palatability of foods [2].

Owing to the importance of the particular industrial fermentation, much effort is still going on to improve the glutamic acid fermentation process especially from the stand point of saving in production cost [1, 3-5].

Dates production regions are semi-dry and sandy in the south and east of the Mediterranean. According to FAO's indications, the level of production in 2001 was 5.4 million tons. The largest producer was Egypt, with a volume slightly above 1 million tons, followed by Iran [6].

The fruits of the date palm contain a high percentage of carbohydrate (total sugars, 44-88%), fat (0.2-0.5%), 15 salts and minerals, protein, vitamins and a high

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percentage of dietary fiber [7].

The processing of dates for the large-scale production of products based on dates (including date paste, date honey and date syrup) results in fibrous and non-fibrous wastes. The non-fibrous slurry contains around 50-60% of reduced sugars. Date wastes which is generally discarded to the environment without any treatment, causes serious concern to environmental pollution in areas where the date processing factories are located.

The rich organic nature of the wastes makes it suitable as a good substrate for microorganisms for the production of valuable products like amino acids.

Principally for a novel substrate like date fruit wastes, reducing the costs of amino acid production by optimizing the fermentation medium and process is the basic goal of research in industrial applications. In general optimization by the traditional "one-factor at-a-time" technique was used. This method is determined by varying one factor while keeping the other factors at a constant level. This method, although simple, often requires a considerable amount of work and time. Recently, different statistical designs including fractional factorial design for optimization have been successfully employed in the fermentation industry [8-9]. These statistical methods have proved to be powerful and useful tools.

The aim of this work is to evaluate the glutamic acid production of *Corynebacterium glutamicum* CECT690 grown on date fruit wastes as a substrate in submerged fermentation. The effect of variables (urea, biotin concentration and agitation speed) on the production of glutamic acid was studied by two-level fractional factorial design.

2- Materials and Methods

2- 1- Microorganism and Fermentation

Stock cultures of *Corynebacterium glutamicum* CECT690 were obtained from the Spanish culture collection. The fungi were grown in the 500 ml shake flasks contenting 100 ml at 37 °C with agitation at 150 rpm. The medium used had the following composition (in g/100 ml): Glucose 5, yeast extract 0.5, $MnSO_4.H_2O$ 0.001, $FeSO_4.7H_2O$ 0.001, KH_2PO_4 0.1, K_2HPO_4, 0.1, $MgSO_4.7H_2O$ 0.2 and biotin 20 pg/l (Merk, Gemany) [3]. The initial pH of the medium was adjusted to 7.0 with potassium hydroxide or hydrochloric acid. The culture was incubated for 18 h before transferring it to the production medium.

The composition of production media had the following composition (in g/100 ml): Date fruit wastes 10, KH_2PO_4 0.05, K_2HPO_4 0.05, $MnSO_4.H_2O$ 0.001, $FeSO_4.7H_2O$ 0.001 (Merk, Gemany). The different levels of urea concentration (1,2 and 3 g/l) and biotin concentration (0, 5 and 10 µg/l) were added in according to the used experimental design. Fifty milliliters of the medium was transferred to 500 ml flasks and pH was adjusted at 7.5. Beakers were autoclaved at 15 psi for 20 min. 10 ml of the inoculum from seed medium was added and incubated in a rotary flask shaker for 48 h. The *Corynebacterium glutamicum* was cultured on date wastes in the form of submerged fermentation. Incubation was carried out at 37 °C and fermentation period was 48 h. Three levels of agitation speed were used (150, 200 and 250 rpm).

After fermentation, each flask consisting of 100 ml broth was filtered using filter paper (Whatman No.1) under vacuum using a pump. Total filtrate of each flask was then centrifuged at 6000 rpm for 20 minutes to remove the cell particles and other wastes and glutamic acid in the broth was estimated.

2-2- Chemical Analysis

Sugar analysis of date fruit wastes was done using the DNS (dinitro salicylic acid) method. The absorbance readings were calibrated against glucose standards at 575 nm using a spectrophotometer (Scienco UV-2100, Korea) [10].

Some of the essential elements for bacteria growth such as phosphorus, potassium, manganese and magnesium were analysed in date wastes. Phosphorus was measured using a spectrophotometer. Manganese and magnesium were estimated using an atomic adsorption (Shimatzo, Japan). Potassium was

analysed using a flame photometer (Jenway, England) [11].

Thin layer chromatography (TLC) was used (silica gel G., solvent mixture phenol/water 80/20 V/V) for the qualitative detection of L-glutamic acid [12].

Quantitative measurement of glutamic acid was done using high-performance liquid chromatography (HPLC; Younglin instrument SDV 30 plus, Korea) with a AccQ-Tag column (Nova- pack ^{TM}C18) at a controlled temperature of 37 $^{\circ}$C with a mobile phase flow rate of 1.0 ml/min.

The column was an application-specific reversed-phase AccQ-Tag column placed in a column heater. The mobile phase system consisted of three eluents: eluent A (acetate-phosphate buffer), eluent B acetonitrile and Milli-Q water. The gradient was run at λ_{250} nm (excitation wavelength). Emission wavelength was λ_{390} nm [13].

2-3- Experimental Design

A two-level fractional design leading to eleven sets of experiments, performed in duplicate, was used to determine the most significant factor affecting the glutamic acid production. The variables were coded according to Eq.1:

(1) $x_i = (X_i - X_o)/\Delta X_i$

Where x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_o is the real value of an independent variable at the center point, and ΔX_i is the step change value.

The range and the levels of the variables investigated in this study are given in Table 1.

Table 1. Range of variables at different levels for the fractional factorial design

Independent variable	Units	Low level (-1)	High level (+1)	Center level (0)
Urea concentration	g/l	1	3	2
Biotin concentration	µg/l	0	10	5
Agitation speed	rpm	150	250	200

The center points were used due to no prior judgment can be made as to which factor causes the nonlinear behavior. The glutamic acid was taken as the dependent variable or response. Experimental fitting of the experimental data was by polynomial regression, based on analysis of variance (ANOVA).

In order to fit an experimental second-order polynomial model, a central two-level fractional factorial design with the coded levels was performed. The quadratic model for predicting the optimal point was expressed according to Eq. 2.

(2) $Y = b_0 + b_i X_i + b_{ii} X_i^2 + b_{ij} X_i X_j$

Where Y= predicted response, b_0= offset term, b_i= linear effect, b_{ii}= squared effect, and b_{ij}= interaction effect. Data were analyzed using the Sigmaplot software package (2000). Equation 2 enables evaluation of the effects of all main and two-factor interaction effects [14].

The statistical significance of the second-order model equation was determined by F-value and the proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R^2.

3- Results and Discussion

3-1- Nutrition Value of Date Fruit Wastes

The reducing sugar content in the wastes was about 50%. The composition of the date fruit wastes are given in Table 2. These results indicate that these wastes are a nutritive substrate for bacteria growth and production of glutamic acid.

Table 2. Sugar and some element contents in date fruit wastes

Element	Mg	Mn	K	P	Reducing sugar
Concentration (g/100 g of date fruit wastes)	0.05	0.7	0.5	0.232	49

3- 2- Optimization of Fermentation Medium

The TLC plate over laid with ninhydrin showed several spots which were identical with authentic samples and indicated that other than glutamic acid, some other amino acids were produced**.**

The three variables playing the most important role in the fermentation were chosen (Table 1) and the effect of each variable on the production of glutamic acid was investigated. The experimental data were noted in the various runs and the results of the two-level fractional factorial also shown in Table 3. Table also lists three additional runs 9-11 that were included to check reproducibility.

The glutamic acid concentration varied markedly with the tested conditions, in the range of 0-8.7 g/l. The lowest value of glutamic acid concentration was obtained when all variable levels were minimum (run3). Maximum glutamic acid concentration of 8.7 g/l was observed at agitation speed of 250 rpm, without added biotin and urea concentration of 3 g/l (Table 3).

Table 3. Factor levels and the response of the optimization runs

Run	Agitation speed (rpm) X_1	Biotin (µg/l) X_2	Urea (g/l) X_3	Glutamic acid (g/l)
1	150	0	1	0.45
2	150	0	3	1.35
3	150	10	1	0
4	150	10	3	0.39
5	250	0	1	1.86
6	250	0	3	8.7
7	250	10	1	1.5
8	250	10	3	4.5
9	200	5	2	1.95
10	200	5	2	2.25
11	200	5	2	2.4

ANOVA was employed for the determination of significant variables. By applying multiple regression analysis on the experimental data, the following second-order polynomial was obtained (Table 4).

Table 4. Regression coefficient and their P values for glutamic acid production

	Coefficient	P.Value
b_0	2.2	0.0124*
b_1	1.7962	0.0055*
b_2	-0.7462	0.0580
b_3	1.3912	0.0114*
b_{11}	0.1438	0.7829
b_{12}	-0.3937	0.2123
B_{13}	1.0687	0.0233*
B_{23}	-0.5437	0.1172

* Statistically significant at 95% of confidence level

$$Y=2.2+1.7962X_1-0.7462X_2+1.3912X_3+0.1438X_1^2-0.3937X_1X_2+1.0687X_1X_3-0.5437X_2X_3 \quad (3)$$

Except for the linear term agitation speed and the linear urea concentration ($P<0.05$) and interaction term between biotin and urea concentration ($P<0.05$), none of the other linear, quadratic and interaction terms were statistical significantly (Table 4). The agitation speed was found to have more significance than the other factors (Table 4).

The multiple coefficient correlation, R, for the model was 0.9875 which indicates a close agreement between experimental and predicted values of glutamic acid production. The multiple coefficient of determination, R^2, implies that the fitted model could explain 97.5% of the total variation (Table 5).

The F value is the ratio of mean square due to model to the mean square due to error and indicates the influence (significance) of each controlled factor on the tested model. The F value of 16.8255 is greater than F [3, 7] with in a rejection region having an α-level that is $P< 0.05$ (Table 5).

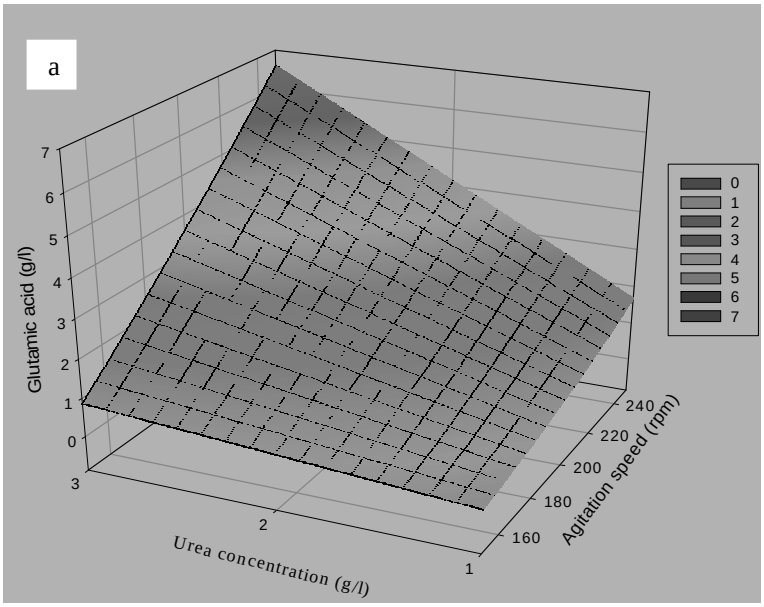
Table 5. ANOVA table for model (P< 0.05)

Source	Free degree	Sum of squares	Mean square	F-Value	P-Value
Model	7	58.5404	8.3629	16.8255	0.0205
Error	3	1.4911	0.4970		
Total	10	60.0315	6.0031		

R^2=0.9752, R=0.9875

3-3- Effect of Urea Concentration and Agitation Speed

Fig.1 depicts the three dimensional plot of the two-level fractional factorial design experiment and contour plot of effects of urea concentration and agitation speed on glutamic acid concentration. The agitation speed and urea concentration demonstrated linear effects on glutamic acid concentration, however, the glutamic acid concentration increased as the agitation speed and the urea concentration increased. From contour plot shown in Fig.1, it is easily deduced that maximal glutamic acid production (6 g/l) could be obtained with a combination of urea 2.8-3 g/l and agitation speed from 240 to 250 rpm.



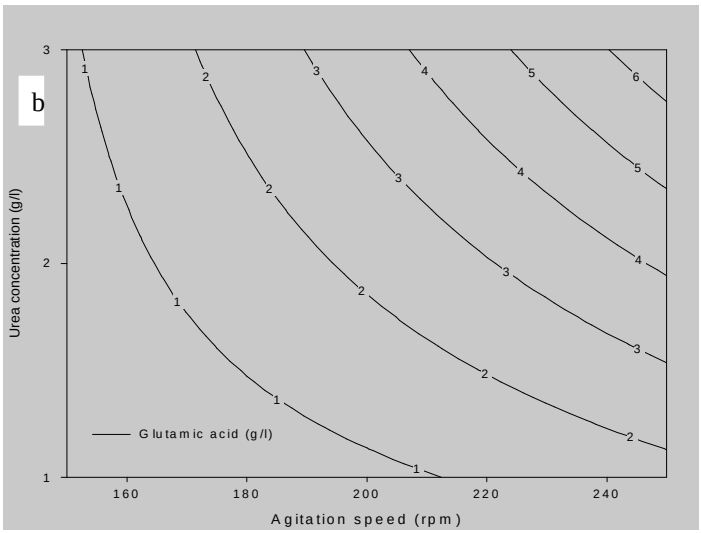


Fig 1. (a) Three dimensional plot of the fractional design and (b) contour plot of the effect of urea concentration and agitation speed on glutamic acid production

4- Conclusions

The possibility of using carbohydrate wastes like date fruit waste to produce glutamic acid using *corynebacterium glutamicum* CECT 690 for the first time was studied. An attempt to utilize the carbohydrates in the date processing factory wastes for value addition of these residues were found to serve as excellent raw material for glutamic acid production by submerged fermentation and the scaling up of this process is underway.

Although the concentration of glutamic acid produced in this study was low, it seems the optimization of the other conditions` such as phosphate concentration and aeration rate can increase the glutamic acid concentration.

5- References

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بهینه سازی ترکیبات محیط کشت برای تولید میکروبی گلوتامیک اسید از ضایعات خرما با استفاده از روش فاکتوریل جزئی

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چکیده

ضایعات خرما به عنوان یکی از ضایعات کشاورزی کربوهیدراتی که در دسترس و به مقدار فراوان در جنوب و غرب مدیترانه یافت می شود به عنوان سوبسترایی جدید برای تولید گلوتامیک اسید، پیش ساز گلوتامات منو سدیم، استفاده شد. طراحی فاکتوریل جزئی برای بررسی اثر متغیرهای مقدار بیوتین، مقدار اوره و سرعت همزدن بر روی مقدار گلوتامیک اسید تولید ی بکار گرفته شد. برای پیش بینی پاسخ از مدلی دو جمله ای استفاده شد. مشخص شد که سرعت همزدن تاثیر معنی داری بر روی تولید گلوتامیک اسید دارد. بیشترین مقدار گلوتامیک اسید در محدوده سرعت همزدن 240-250 rpm و غلظت اوره 2,8-3 g/l گزارش شد. مطالعه نشان داد که ضایعات خرما می توانند به عنوان سوبسترایی ارزان قیمت برای تولید گلوتامیک اسید استفاده شوند.

کلید واژگان: گلوتامیک اسید، ضایعات خرما، غوطه وری، سرعت همزدن، طراحی فاکتوریل جزئی، بیوتین

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