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Production of glutamic acid by *Corynebacterium glutamicum* using dates syrup as carbon source

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The present study was an illustrative investigation on L-glutamic acid production by using *Corynebacterium glutamicum* employing a cheaply available dates syrup. Owing to the high sugar content of dates syrup (total sugar 80%) attempt was made to utilize the date syrup for the production of glutamic acid, in shaking culture. The acid treated dates syrup (ATDS) at concentration of 100g/L was the best sugar sources in the glutamic production. Penicillin addition at concentration of 4 U/ml after 12 h of incubation was superior in glutamic acid production. The selected temperature-sensitive mutants M5AJ2, showed 13.4% increase in glutamic production while, M5AJ4 and M7AJ6 showed 22.4 and 4.6% decrease respectively, in their glutamic acid production than their wild type bacterial strain. The specific production rate of glutamic acid in case of temperature shift-up from 31 to 39°C increased apparently 2 and 1.5 fold respectively on average from that under the constant temperature. 24g/L pure glutamic acid were precipitated in crystal at pH 3.2.

Key words: Glutamic acid, *Corynebacterium glutamicum,* dates syrup, fermentation, penicillin, mutation, UV, efflux.

INTRODUCTION

Glutamate is mainly used as a flavor agent , recently it is known as neurotransmitter, (Hawkins, 2009). It is widely used in food, pharmaceutical, medical, biochemical and analytical industries. *Corynebacterium glutamicum* and related organisms are used since 1957 for the production of glutamate (Kinoshita et al., 2004) to meet today an annual demand of approximately 1,500,000 tons. Over production of glutamate by *C. glutamicum* is induced by biotin limitation (Gutmann et al., 1992), or by adding specific detergents (Eggeling et al., 2001; Amin and Al-Talhi, 2007) or by adding sublethal amounts of penicillin in early exponential growth phase (Numheimer et al., 1970) or at last, by a temperature up-shift of the culture broth (Delaunay et al., 1999). Glucose, and other carbon source as, beet molasses (Yoshikiro et al., 1979) and cassava residues (Jyothi et al., 2005). Later on, an investigation carried out by other agriculture wastes which are the cheap carbon source such as *Muntingia calabura L.* are used by Vijayalakshmi and Sarvamangala (2011) for the production of glutamic acid. In addition to exploitation of wild type strains for amino acid biosynthesis some workers used advanced techniques for improvement of strains by mutagenesis, cloning and protoplasm fusion techniques. Atef et al. (2007) utilized mutant cells of *Brevibacterium flavum* produced by UV irradiation and by ethyl methane sulfonate (EMS) treatments, increased alanine productivity. Pasha et al. (2011) tested the UV and chemical mutation for *Corynebacterium glutamicum* for increasing glutamic productivity.

In the present study, an attempt was made to utilize the dates syrup as carbon source, penicillin or surfactant and UV mutation to produce glutamic acid by shaking fermen-

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tation.

MATERIALS AND METHODS

Microorganism

Corynebacterium glutamicum AJ1510 obtained from Ajinomoto company was used for this study.

Media

Minimal salt medium (MSM)

It containing : 1g; KH_2PO_4, 0.6 g; K_2 HPO_4, 1g; $(NH)_2$ SO_4, 0.5g; $MgSO_4$ .7 H_2O. in 1L distilled water. The medium pH was adjusted to pH 7-7.5. Maintenance medium(g/l): Meat extract, 10, peptone, 10; NaCl, 5, agar, 20. pH adjusted at 7.

Fermentation medium

For L-glutamic acid production by *C. glutamicum*, cells were cultured in basal salt (BS) medium per liter. Basal salt medium contained the following: 5 g, $(NH_4)_2SO_4$, 5 g, urea, 2 g, KH_2PO_4, 2 g, K_2HPO_4, 0.25 g , $MgSO_4 \cdot 7H_2O$, 0.01 g, $FeSO_4 \cdot 7H_2O$, 0.01 g, $MnSO_4 \cdot 5H_2O$, 0.01 g, $CaCl_2 \cdot 2H_2O$, 0.03 mg, $ZnSO_4 \cdot 7H_2O$, 0.1 mg, H_3BO_4, 0.07 mg, $CoCl_2 \cdot 6H_2O$, 0.03 mg, $CuCl_2 \cdot 2H_2O$, 0.01 mg, $NiCl_2$, 0.1 mg of $NaMo_2O_4 \cdot 2H_2O$, different concentration of dates syrup, and 200 µl of biotin (pH 7.0). Fifty milliliter (50 ml) of fermentation media were added to the flasks (250ml). To each flask, the 1 ml inoculums of a 24-h-old culture was added and incubated in a rotary flask shaker at 150 rpm at 31°C (Hoischen et al., 1990).

Mutation medium (MY)

It contained (g/L): 10, glucose; 3, yeast extract; 3, peptone extract; 5, malt extract; 15, agar.

Treatment of dates syrup

Alkaline treatment

The dates syrup (10 g) was diluted with distilled water (1 part ml), the pH of the mixture was adjusted to pH 9.5, and the mixture was shaking in water bath at 50°C for 10 min. Then it was kept undisturbed overnight, at room temperature. The precipitate solid material was separated from the supernatant by filtration. The supernatant was neutralized to pH 7 (Shah et al.. 2002).

Acid treatment

The dates syrup (10 g) was diluted with distilled water (1 part ml), 0.5 N oxalic acid were added and heated on a boiling water bath for 3 h. The supernatant solution was neutralized with barium carbonate then it was filtered and concentrated under vacuum to small volume, and it was added to the medium.

Mutagenesis

An aliquot of appropriate dilution of the *C. glutamicum* was grown in MY medium for 24 h. One milliliter (1 ml) of this culture from *C. glutamicum* cells were held in a Petri dish 20 cm and were exposed to UV lamp 254 nm at distance of 30 cm for 1, 2, 3, 4 and 5 min in a tightly closed wooden chamber. The mutated cells were plated into complex and minimal medium agar (MSM) plates and incubated for 48 h at 30°C. The numbers of survivals were calculated.

Analytical methods

Growth determination

The growths of cells were determined by measuring the absorbance at 600 nm spectrophotometrically (Shimadzu 24016), according to the methods of Hoischen et al. (1990). Absorbency was converted to dry weight by using a standard curve.

Determination of total carbohydrates

Total carbohydrates were determined by phenol sulphuric acid method according to the study of Dubois et al. (1956) .

Chromatography

Paper chromatography

Identification of the monosaccharides and oligosaccharides for the non treated, alkaline and acid treated dates syrup were proceeded by the paper chromatoghraphic technique using Whatman No. 1, and the solvent system n-butanol: acetic acid: water (60:15:25 v/v) for a period of 48 h. The chromatogram was dried, then dipped in alkaline -$AgNO_3$.

Estimation of glutamic by thin chromatography

The glutamic acid content in the broth after fermentation was estimated by thin chromatography using silica gel G and the solvent mixture of n-butanol, glacial acetic acid and water in the ratio 4:1:1 (v/v). Ninhydrine in ethanol was used to develop the colour of spots. For quantitative estimation of glutamic acid produced, the spots developed on plates were scraped and collected in microtubes which contained 5ml 75% ethanol (Lee, 1996). After shaking for 5 min the sample was centrifuged at 4000 rpm and its absorbance was recorded at 560 nm (Lee, 1996) on UV/VIS spectrophotometer.

Crystallization

Fermented broth (1.5 L) collected from culturing in shaking medium was centrifuged at 10,000 rpm for 10 min to get the supernatant, then the supernatant was partially evaporated in a rotary evaporator. The concentrating medium containing a high amount of glutamic acid was acidified to pH 3.2, the isoelectric point of glutamic acid with 1 N HCl and allowed to stand still in a refrigerator until the glutamic acid crystals were precipitated there from. The obtained clear crystals were identified subjected to HPLC. Glutamic acid after purification dissolved and 20 µ/l was applied to the HPLC, (Shimadzu, Shim-pack ISC-07/S 1504 Na, flow rate 0.3 ml/min, detector-Florescence D 6A). The samples were using methanol as a mobile phase at room temperature.

RESULTS

Dates syrup total sugars were determined to be about 80% according to the study of Dubois et al. (1956).

Identification of the sugar content of dates syrup before and after treatment

Different treatment were done for the dates syrup, alkali treatment was done by adjusting to pH 9.5 after shaking water bath at 50°C for 10 min, then it was kept overnight,

Table 1. Sugar content of dates surup before and after treatment.

Sugar	Date	Alkali treatment	Acid treatment
Mono sugar	Glucose, Fructose	Glucose, Fructose	Glucose, Fructose
Oligosugar	Sucrose	Sucrose	No oligosugars
	Other 3 oligo sugars	One oligosugars	

Table 2. Comparison between glucose , alkali or acid treated and non treated dates syrup in basal basal medium of *C. glutamicum.*

Carbon Source	pH		Cells dry weight (g/L)	Consumed Sugar (%)	Glutamic acid (g/L)
	Initial	Final			
Glucose	7.5	7.0	5.0	70	6.0
Non treated dates syrup	7.5	6.8	2.3	40	3.5
Acid treated dates syrup	7.5	7.8	4.35	80	8.7
Alkali treated dates syrup	7.5	8.1	5.23	74	7.0

the precipitate was removed by filtration and the supernatant was neutralized to pH 7. Acid treatments were done 0.5 N oxalic acid for 3 h, after neutralization with barium carbonate, the precipitate was removed by filtration. Both non treated and treated dates syrup with alkaline or acid supernatants were subject to paper chromategraphy. The data are shown in Table 1 that, the non treated, and alkali treated dates syrup contain glucose and fructose, sucrose and oligosaccharide. On the other hands, the acid treated dates syrup contained only glucoseandfructose,nosucroseoligosaccharidewasdetected.

A comparison between glucose, treated and non-treated dates syrup

In comparison between glucose, treated dates syrup by alkali or acid and non-treated dates syrup, 50 g/l of each carbon source was added to basal medium of *C. glutamicum*. The data in Table 2, indicated that acid treated was the best sugar source in glutamic production. Good cells growth was found in both alkali dates syrup and glucose, while weak cells growth was found in non treated dates syrup.

Effect of acid treated dates syrup (ATDS) concentration

Different concentration of (ATDS) (0,10, 20, 40 ,60, 80, 100 and 120) were added to basal medium of *C. glutamicum* as carbon source. The maximum glutamic acid was found at concentration of 100 g/l (ATDS) then the values of percentages decreased as the concentration of dates increased as shown in Table 3.

Effect of penicillin on L-glutamic acid

In culture media of *C. glutamicum* 100 g/l of (ATDS) replaced glucose, different units (0-8 units /ml) of penicillin were added to the media after 12 h of incubation time in rotary flask shaker at 150 rpm at 31°C, to study the optimum penicillin units for the secretion of glutamic acid.

The data obtained as shown in Table 4 indicated that supplementation of 4 units/ml penicillin was superior in glutamic acid production by *C. glutamicum* than other concentrations.

Induction of mutation by UV

C. glutamicum AJ1510 (AJ) strain was exposed to UV radiation at 254 nm for 1, 2, 3, 4 and 5 min at a distant of 30 cm. The results in Table 5 show a perfect negative relationship between UV- exposure time and survival percentages for bacterial strain

Isolation of temperature-sensitive mutants

Suitable dilution of each UV treatments were spread onto (MSM) medium and incubated at 40, 45 and 50°C. Colonies which showed weak growth at 40 and 45°C, were isolated. Among 26 tested colonies, 3 strains (AJ strain) were scored as temperature sensitive mutants which showed very good growth at 30°C.

The effect of mutation on glutamic acid production

The selected temperature-sensitive mutants *C. glutamicum* M5AJ2, M5AJ4 and M7AJ6 and their wild type bacterial strains (AJ) were cultured in basel medium, contained (ATDS) as carbon source, and incubated in shaking flask at 39°C to determine the effect of mutation on glutamic acid production (Table 6). Penicillin were added to culture medium after 12 h. The selected temperature-sensitive mutants M5AJ2, showed 13.4% increase in the glutamic production while, M5AJ4 and M7AJ6 showed 22.53 and 4.6% decrease respectively, in their glutamic acid production

Table 3. Effect of acid treated dates syrup concentration on glutamic production by *C. glutamicum.*

Dates Concentration (g/L)	Cells dry weight (g/L)	Consumed sugar (%)	Glutamic acid (g/L)
0	0.5	0.0	0.1
10	3.2	0.7	1.5
20	4.3	1.3	4.8
40	7.4	2.3	8.7
60	8.0	4.4	10.9
80	8.5	6.5	15.7
100	9.55	7.5	17.9
120	11.4	8.0	15.0

Table 4. Effect of penicillin addition on glutamic acid production by ***C.glutamicum AJ1510.***

Units	pH		Cells dry weight (g/L)	Consumed sugar (%)	Glutamic (g/L)
	Initial	Final			
0	7.0	5.6	6.75	8.15	6.7
2	7.0	6.8	7.12	7.7	8.7
4	7.0	7	5.34	7.73	20
6	7.0	7.1	3.38	8.17	12
8	7.0	7.5	2.12	5.0	9.6

Table 5. Effect of UV- exposure time on the survival bacteria.

Expose time (min)	C. *glutamicum*-AJ1510 (AJ)	
	Cells / ml	Survival %
0	50×10^{10}	100.0
1	18×10^{10}	36.0
2	12×10^{8}	0.24
3	58×10^{7}	0.116
4	17×10^{6}	0.0034
5	13×10^{6}	0.0026

Table 6. Glutamic acid production by some selected temperature-sensitive mutants *C. glutamicum.*

Bacteria Strain	State	pH		Cells dry weight (g/l)	Residuals Sugars%	Glutamic acid %
		Initial	Final			
(AJ)	Wild type	7.5	7.0	6.90 4.1	0.329	19.6
M5 AJ2	Temperature	7.5	6.5	13.27	0.388	22.3
M5 AJ4	sensitive	7.5	7.4	1.80	0.336	15.2
M7AJ6	mutants	7.5	7.2		0.355	18.7

The fermentation process achieved by basal medium. The selected temperature-sensitive mutants after exposing the wild type bacterial strains to UV

than their wild type bacterial strain. As shown in Table 6, good cells growth was found in the case of M5AJ4 than other different mutants. On the other hand, residual sugar was approximately stable.

Effect of temperature shift-up on the efflux of glutamic acid

To study the effect of temperature shift-up on the efflux of

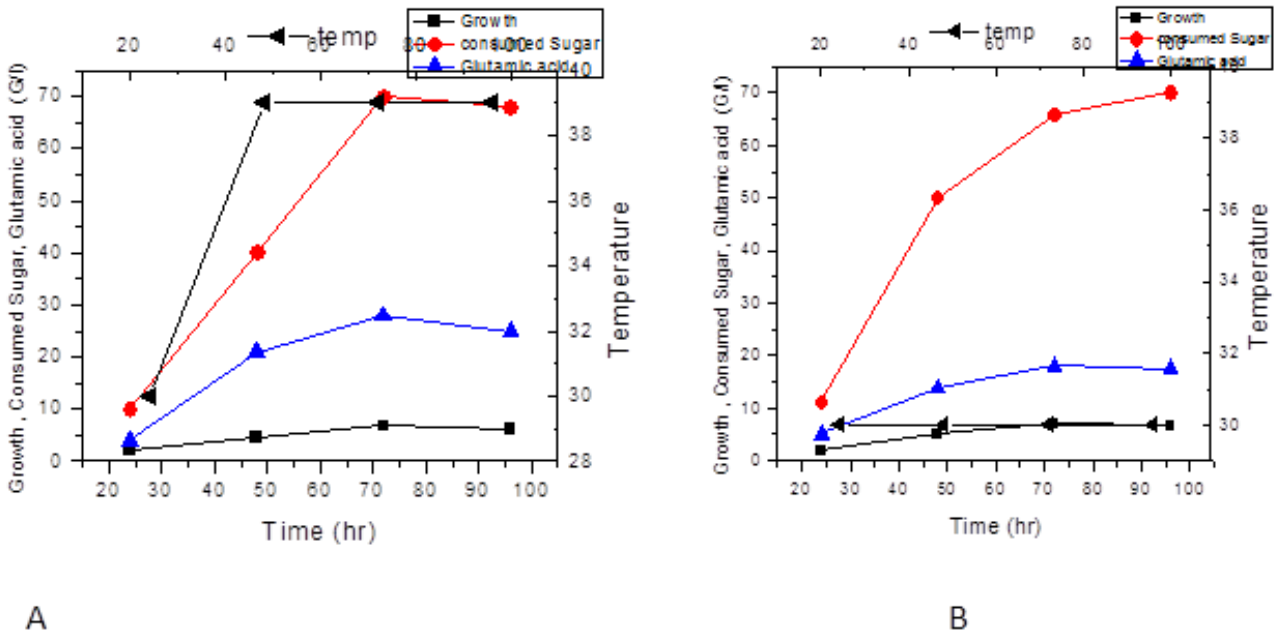

Figure 1. Effect of temperature shifting (30 to 39°C) on glutamic acid production by mutant strain; B: Effect constant temperature at (30°C) on glutamic acid production by mutant strain.

glutamic acid, the mutant strain *C. glutamicum* M5 AJ2 and wild strain (AJ) were cultured on basal medium with (ATDS) as carbon source and 4 units/ml of penicillin were added after 12 h. The fermentation temperature was changed from 30 to 39°C after 24 h of cultivation. Both strains wild AJ and mutant strain M5 AJ2 were compared with that under constant temperature for the entire period of cultivation. The results were shown in Figures 1A, B and 2A, B. Figures 1 and 2 A for mutant strain M5 AJ2 and wild strain shows that the specific production rate of glutamic acid increased apparently 2 and 1.5 fold respectively on average from that under the constant temperature.

The preliminary product identification was done qualitatively by thin layer chromatography and quantitatively by spectrophotometric analysis. The result of the former test was observed as a visible purple coloured spot having similar Rf value 0.26 similar to that of authentic standard sample. The concentration of glutamic acid was estimated by standard ninhydrin method according to the study of Lee (1996). Furthermore results of HPLC indicate the precipitated crystal at pH 3.2 is pure glutamic acid, which about 24g/l glutamic acid was produced at concentration of 100 g/l (ATDS).

DISCUSSION

The dates syrup is a very important cheap carbon source (total sugar 80%) which can be used for the production of different important product as glutamic acid. The non treated, treated with alkaline or acid supernatants were subject to paper chromatography, the non treated dates syrup, and alkali treated contain glucose and fructose, sucrose and oligosaccharide. While, acid treated contained glucose and fructose, no sucrose oligosaccharide was detected. Although, Mostafa and Ahamed (2006) referred that, Libyan date syrup contained glucose 48.70%, fructose-45.21%, and sucrose 6.09% were the major sugars. In comparison between glucose, treated dates syrup with alkali or acid and non treated dates syrup, the results indicated that acid treated was the best sugar source in glutamic production. The maximum glutamic acid and good cells growth were found at concentration of 100 g/l (ATDS). The same results was mentioned by Das et al. (1995) who referred that glutamic acid produced from palm waste hydrolysate by fermentation with *Brevibacterium lactofermentum* ATCC 13869 is produced with high yield than that produced from pure glucose as a carbon source. In agreement to our results, Roy and Chatterjee (1989) referred that glucose (8%) was the best than molasses in glutamic acid by *Arthrobacter globiformis*. In contrast to the results of Kyoichi et al. (1964) who found that the concentration of glutamic production by *Microbacterium ammoniaphilum* decrease as the concentration of glucose increased.

The data obtained as shown in Table 4 indicated that supplementation of 4 units/ml penicillin was superior in glutamic acid production than other concentrations used. Penicillin primarily inhibited cell wall synthesis, leaving the cell membrane unprotected, resulting in physical damage to the cell membrane, which increase the permeability of the cell wall, then increasing glutamic secretion (Shiio et al., 1962). The effect of penicillin was also investigated on glutamic acid production by *Micrococcus* that the addition of 4 U/ml increase the conversion of L-homo-

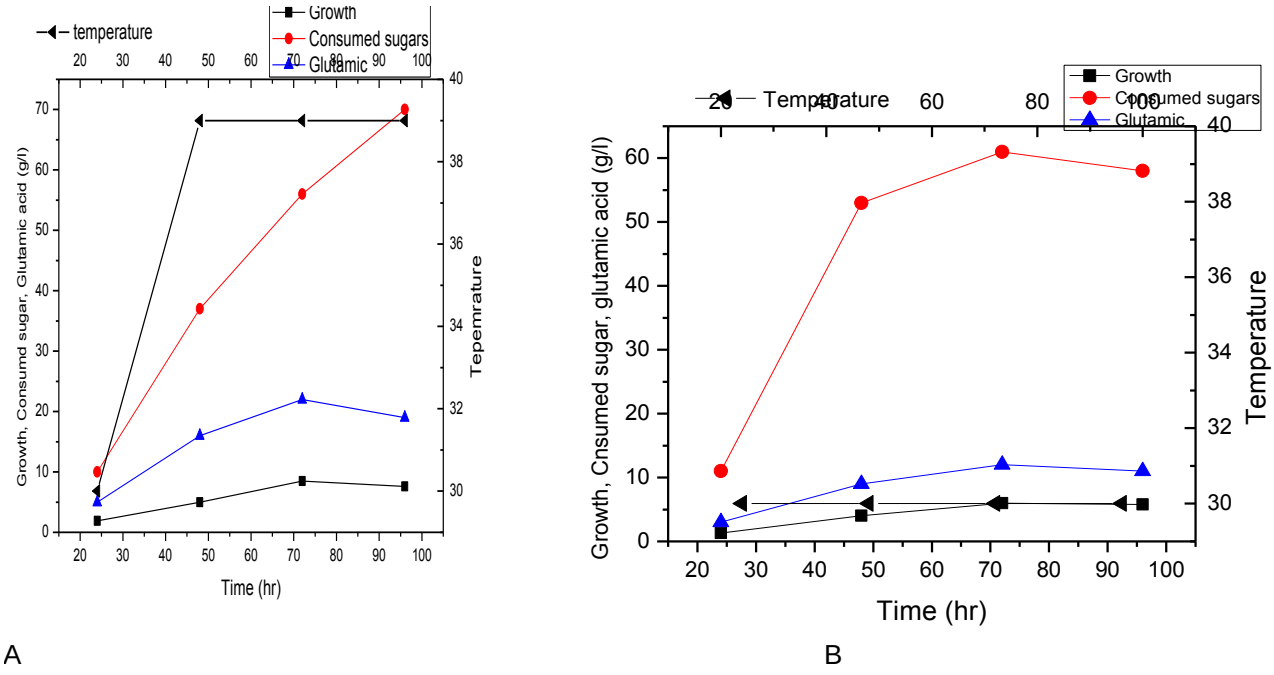

Figure 2. A Effect of temperature shifting (30 to 39°C) on glutamic acid production by wild strain; B: Effect constant temperature at (30°C) on glutamic acid production by wild strain.

homoserine to L-glutamic acid production. On the other hand, Vijayalakshmi and Sarvamangala (2011), found that addition 1 U/ml to the culture medium of *Arthrobacter globiformis* MTCC 4299 increase the glutamic production to about 87.5 g/l. As shown in Table 5, the survival cells decrease as the UV radiation time increase, 3 strains were scored as temperature sensitive mutants from wild strain AJ. The temperature sensitive mutants may causes higher producing ability of glutamic acid production than their wild type bacterial strain Uy et al. (2003) who, found that, at 39°C the glutamate was actively produced, while the activities of 2-oxoglutarate dehydrogenase complex (ODHC) and pyruvate dehydrogenase (PDH) were, respectively completely inhibited and 35% decreased in their activity. The selected temperature-sensitive mutants M5AJ2, showed 13.4% increase in the glutamic production while, M5AJ4 and M7AJ6 were showed 22.5 and 4.6% decrease respectively, in their glutamic acid production than their wild type bacterial strain. The same results of the increase in the production of glutamic acid cited by Hirasawa et al. (2001) indicated that a defect caused by a mutation is responsible for temperature-sensitive growth and L-glutamate overproduction by C. *glutamicum.* The glutamic acid production increased to about 43 g/l by immobilized UV mutant strains of *C. glutamicum* Pasha et al. (2011). The effect of temperature shift-up from 30 to 39°C through incubation were compared to that incubated at constant temperature, the results are shown in Figures 1and 2 A for mutant strain M5 AJ2 and wild strain shows that the specific production rate of glutamic acid increased apparently 2 and 1.5 fold respectively on average from that under the constant temperature (Figures 1 and 2B). The same results were done by Choi et al. (2004) who referred to, enhance glutamic acid production of *Brevibacterium* sp. with temperature shift-up cultivation from 30 to 38°C. The concentration of glutamic acid was estimated by standard ninhydrin method (Lee, 1996).

Furthermore results of HPLC, indicate the precipitated crystal at pH 3.2 is pure glutamic acid, which about 24 g/l glutamic acid was produced at concentration 100 g/l (ATDS).

CONCLUSION

Owing to the high sugar content of dates syrup (total sugars 80%), it can be used for the production of different important product as glutamic acid. By acid treated dates syrup (ATDS) only glucose and fructose were reveled by paper chromatography. In comparison between glucose, treated dates syrup with alkali or acid and non treated dates syrup, the results were indicated that acid treated was the beast sugar source in glutamic production. The optimum glutamic acid and good cells growth were found at concentration of 100 g/l of (ATDS). Penicillin addition at concentration 4 U/ml after 12 h of incubation was superior in glutamic acid production. 3 mutants were scored as temperature sensitive from AJ strain. The selected temperature-sensitive mutants M5AJ2, showed

13.4% increase in the glutamic production while, M5AJ4 and M7AJ6 showed 22.5 and 4.6% decrease respectively, in their glutamic acid production than their wild type bacterial strain. The specific production rate of glutamic acid in case of temperature shift-up from 30 to 39°C increased apparently 2 and 1.5 fold respectively on average from that under the constant temperature. 24g/l pure glutamic acid were precipitated in crystal at pH 3.2.

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